energetics and mechanisms of **phosphate transport** in *Acinetobacter johnsonii*

h.w. van veen

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in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
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van **Hendrik W. van Veen**

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Our understanding of the world is built up of innumerable layers. Each layer is worth exploring, as long as we do not forget that it is one of many. Knowing all there is to know about one layer, a most unlikely event, would not teach us much about the rest.

Erwin Chargaff, *Heraclitean Fire*

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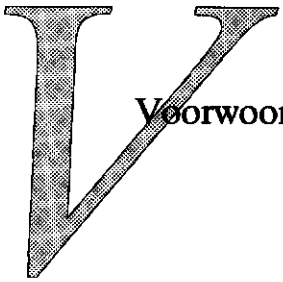

Voorwoord

Het leven als onderzoeker in opleiding is een ervaring die ik voor geen goud had willen missen. Het moment van euforie bij een succesvol experiment, de hoogte- en dieptepunten waardoor je de grenzen van jezelf en van anderen leert kennen. Talloze mensen zijn betrokken geweest bij de totstandkoming van dit proefschrift. Op deze eerste pagina's wil ik hen bedanken voor hun hulp en advies.

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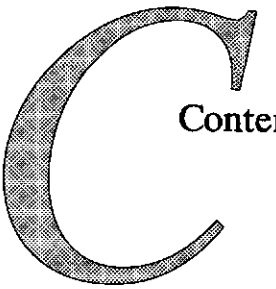
Ook bedank ik alle promovendi en andere medewerkers van Microbiologie die op enigerlei wijze hebben bijgedragen aan de totstandkoming van dit proefschrift of hebben bijgedragen aan de leuke sfeer op de vakgroep.

Tenslotte wil ik ook mijn nieuwe collega's in Groningen bedanken voor hun steun tijdens het afronden van dit proefschrift.

Voor het voltooien van een promotieonderzoek is het ook heel belangrijk om gesteund te worden door mensen die buiten het werk staan. Daar heeft het mij niet aan ontbroken. Mijn ouders verdienen hier een plaats. Zij hebben mij de mogelijkheid geboden om te gaan studeren. Bovendien is hun voortdurende belangstelling een goede stimulans geweest. Elvira (mijn coach), jij hebt deze promotietijd wel een hele speciale dimensie gegeven. In deze periode hebben wij besloten om samen door het leven te gaan. Dat besluit heb jij door dik en dun ten uitvoer gebracht. Bedankt voor je vertrouwen, steun, geduld en hulp met de lay-out van dit proefschrift. Bedankt voor alles.



Contents

General introduction 1

Part of this chapter will be published in: Van Veen HW, Abee T, Korststee GJJ, Konings WN and Zehnder AJB (1994) Phosphate inorganic transport system in *Escherichia coli* and *Acinetobacter johnsonii*. *In* Torriani-Gorini AM, Silver S and Yagil E (eds.), Phosphate in microorganisms: cellular and molecular biology. American Society for Microbiology, Washington, DC

Preview

This thesis describes the results of a research project entitled: "P_i transport in *Acinetobacter* in relation to the polyphosphate and energy metabolism". This study started in 1989 and was financed for four and a half years by the Netherlands Organization for Scientific Research (NWO) and the Dutch Technology Foundation (STW). The aim of the project was to examine the mechanism and energetics of P_i uptake and efflux in the polyphosphate-accumulating *Acinetobacter johnsonii* strain 210A. The project arose from previous studies on the nature, localization and metabolism of polyphosphates in *A. johnsonii* 210A. Insight into P_i transport processes in *Acinetobacter* is important for two reasons. Firstly, polyphosphate-accumulating acinetobacters may play a major role in the biological removal of P_i from waste water. Fundamental knowledge about the parameters which influence the uptake and release of P_i in these organisms may help to improve the design and development of phosphorus-removal systems. Secondly, insight into P_i transport processes in polyphosphate-accumulating acinetobacters may further unravel the metabolic functions of polyphosphate, and may contribute to our knowledge of the mechanisms and bioenergetics of solute translocation across biomembranes. In the following, two topics will be discussed: (i) the application and physiology of poly-phosphate-accumulating acinetobacters, and (ii) our current knowledge of P_i transport processes in prokaryotes. This introduction will be concluded by an outline of the thesis.

1. Application and physiology of polyphosphate-accumulating *Acinetobacter* spp.

1.1 Biological phosphorus removal

The removal of phosphorus from waste water is an essential feature of sewage treatment facilities because of the threat of eutrophication. Although the addition of various agents, such as salts of calcium, iron or aluminium, to activated sludge can be used to remove P_i from the waste water by settling, the treatment causes the

accumulation of large quantities of chemical sludge. In addition, the initial costs of the materials required for chemical treatment have stimulated a search for alternative biological methods for phosphate removal (11, 12, 39, 52, 53, 75, 119, 171, 186, 193, 194). Any practical application of biological removal of dissolved phosphate compounds from sewage effluent depends on the ability of sludge organisms to take up these substances in quantities exceeding those required for normal metabolic activities (81, 106). Polyphosphate, the biopolymer in which excess P_i is frequently stored, has been detected in a wide variety of pro- and eukaryotic microorganisms (50, 84, 112, 113). Effective removal of phosphates from sewage due to enrichment of activated sludge with polyphosphate-accumulating bacteria requires alternating aerobic and anaerobic cycles (11, 53, 75, 150, 187). Analysis of the population structure of activated sludge have focussed attention on *Acinetobacter* as being one of the important genera in the process of enhanced biological phosphorus removal (15, 20, 43, 52, 75, 121, 145, 186), with *A. johnsonii* as the major and *A. lwoffi*, *A. calcoaceticus* and *A. junii* as the minor constituents of the *Acinetobacter* population (21, 31, 60, 104, 123). Similar to the behaviour of activated sludge, these *Acinetobacter* species accumulate P_i as polyphosphate under aerobic conditions. Polyphosphate is subsequently broken down and released as P_i in response to the anaerobic treatment.

1.2 The genus *Acinetobacter*

Members of the genus *Acinetobacter* are strictly aerobic, non-motile, non-fastidious gram-negative bacteria which are ubiquitously present in soil, water and sewage. Studies on its biochemistry and physiology have indicated that *Acinetobacter* is typical of other gram-negative bacteria with distinctive metabolic features which are well-adapted to its lifestyle and support its nutritional versatility (197).

General physiology

Metabolism in *Acinetobacter* spp. centres on a Krebs tricarboxylic acid cycle which is fed by catabolic pathways for the degradation of a wide variety of carbon sources

including sugars, fatty acids, aliphatic alcohols, dicarboxylic acids, amino acids, unbranched hydrocarbons and many aromatic and alicyclic compounds (100, 197). The ability of *Acinetobacter* to form acid from D-glucose (and other sugars) has been used as a taxonomic criterion within the genus (34) and depends on the presence of a functional, cytoplasmic membrane-bound quinoprotein aldose dehydrogenase that catalyzes the oxidation of glucose to gluconate (56, 79, 204, 205). *Acinetobacter* species able to grow on gluconate, use the Entner-Doudoroff pathway for its degradation. However, most *Acinetobacter* strains are unable to use gluconate as a carbon and energy source and release this compound into the growth medium during the oxidation of glucose.

The composition of the electron transfer chain in acinetobacters has not been completely elucidated. NADH dehydrogenase can catalyse the input of electrons from NADH into the quinone pool, which consists of ubiquinones, but not of menaquinones (13, 14, 32, 66). The electrons can be transferred from the level of ubiquinone into two branches. In general, under conditions of high aeration a cytochrome *o*-containing oxidase and a cytochrome b_{554} are the predominant species, whereas under oxygen-limitation a cytochrome *d*-containing oxidase is predominant (57, 66, 67, 68, 83). *Acinetobacter* does not contain a cytochrome *c* oxidase, and is therefore classified as oxidase-negative. Cytochrome b_{562} is involved in linking glucose dehydrogenase to the electron transport chain (58, 76).

Many *Acinetobacter* strains accumulate polyphosphate (30, 31, 70, 168, 169), poly-ß-hydroxybutyrate (30, 31, 70, 168, 169, 207) or wax esters (69, 70) as reserve polymers. Poly-ß-hydroxybutyrate and wax esters can fulfill a role as energy reserves in these organisms. Poly-ß-hydroxybutyrate has been postulated to play an important role in the polyphosphate metabolism of acinetobacters isolated from sewage (52, 75, 94, 122, 124). However, the exact nature of the linkage between poly-ß-hydroxubutyrate and polyphosphate metabolism is presently unclear.

Polyphosphate metabolism

Pure cultures of *Acinetobacter* spp. isolated from activated sludge have been used to investigate the metabolism and metabolic functions of polyphosphate. *A. johnsonii*

210A, one of the most extensively studied strains (for review, see 110 and 223), grows well within the pH range of 6 to 9. In the presence of excess energy and substrates, it is able to accumulate phosphate up to 300 mg P_i per gram (dry weight) at all pH values. Although this accumulation is independent of the growth phase in batch cultures, it is stimulated at decreasing growth rates in carbon or sulphur-limited chemostat cultures (203). Concomitantly with P_i, Mg^{2+} and K^+ are taken up by *A. johnsonii* 210A. Mg^{2+} can be replaced by Ca^{2+}. Both divalent cations can act as counterion of polyphosphate in polyphosphate granules in the cytoplasm of the organism (27, 200, 202). K^+ is essential for P_i uptake. In its absence P_i uptake is strongly reduced (202).

When incubated under anaerobic conditions, *A. johnsonii* 210A degrades the polyphosphate which has been accumulated under aerobic conditions. Other manipulations which interfere with oxidative phosphorylation in this organism induce the release of P_i as well. Thus, besides by anaerobiosis (lack of electron acceptor), polyphosphate degradation is also triggered by (i) the absence of a carbon and energy source (lack of electron donor), or the presence of (ii) the respiratory chain inhibitor CN^-, (iii) the H^+-ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide, or (iv) the uncoupler α-dinitrophenol (198). P_i release is stoichiometrically paralleled by the excretion of Mg^{2+} (202).

A. johnsonii 210A contains two polyphosphate degrading enzymes: polyphosphatase and polyphosphate:AMP phosphotransferase (199-201). Polyphosphatase hydrolyzes polyphosphate to P_i (Eqn. 1). Polyphosphate:AMP phosphotransferase phosphorylates AMP to ADP with polyphosphate as phosphoryl donor (Eqn. 2).

$$PP_n \text{ (polyphosphate)} + H_2O \rightarrow PP_{n-1} + P_i \text{ (Eqn. 1)}$$
$$PP_n + AMP \rightarrow PP_{n-1} + ADP \text{ (Eqn. 2)}$$

The enzymes have been partially purified and characterized (26, 28, 29). They both show high affinity for highly polymeric polyphosphates, as encountered in the cytoplasm of the organism, with an apparent K_m of 5.9 μM polyphosphate (average chain length of 64 residues) for polyphosphatase and an apparent K_m of 0.8 μM polyphosphate (average chain length of 35 residues) for polyphosphate:AMP

phosphotransferase. The enzymes degrade polyphosphate via a processive mechanism and are stimulated by Mg^{2+}, but inhibited by small polyphosphates such as pyro- or triphosphate (28, 29). Pyro- and triphosphate (in complex with divalent cations) are further hydrolysed to P_i by a pyrophosphatase (26).

Polyphosphate:AMP phosphotransferase allows *A. johnsonii* 210A to conserve metabolic energy released during the degradation of polyphosphate. The combined action of this enzyme with adenylate kinase can produce ATP in cell-free extracts from the degradation of polyphosphate (201). This energy conserving mechanism may also be relevant for other strains of *Acinetobacter* spp. in which significant levels of polyphosphate:AMP phosphotransferase and adenylate kinase are detected (15, 104, 199). In *A. johnsonii* 210A polyphosphate meets the three criteria which are used to define energy reserves (215): (i) it accumulates under conditions of carbon and energy source excess when growth is limited by another nutrient, (ii) it is degraded during carbon and energy source starvation, and (iii) its degradation via polyphosphate:AMP phosphotransferase yields metabolic energy. In addition to its role as an energy reserve, polyphosphate can also act as a P_i and/or Mg^{2+} reserve when these nutrients are limiting in the growth medium. These compounds can be released from polyphosphate by the action of polyphosphatase (200, 202).

The observations on P_i uptake and release in *A. johnsonii* 210A raised questions about the P_i transport mechanism(s) in this organism. In the following sections, the current knowledge of P_i transport processes in prokaryotes will be summarized.

2. P_i transport in prokaryotes

Phosphorus is an integral part of the cellular metabolism of bacteria since it is indispensible for energy supply and for DNA, RNA and phospholipid biosynthesis. In order to grow, microorganisms must therefore take up P_i and/or phosphorus-containing nutrients from the environment. These compounds have to pass the cell envelope which encloses the cytoplasm.

In gram-negative bacteria, this envelope consists of two membranes, the cytoplasmic (or inner) membrane and the outer membrane, which are separated by

the peptidoglycan containing periplasm (Fig. 1) (24, 33, 151). Together with the underlying peptidoglycan, the outer membrane gives the cell its rigidity and shape. It contains proteinaceous pores that allow the diffusion of most small molecules such as P_i, amino acids, and sugars. The cytoplasmic membrane is quite different from the outer membrane in that it allows nutrients to be concentrated within the cytoplasm. All cell membranes allow the free diffusion of water but have proteins that form permeases for active transport of other hydrophilic solutes. In contrast to gram-negative bacteria, gram-positive bacteria are not enclosed by an outer membrane or periplasm. Instead, their cytoplasmic membrane is directly surrounded by a thick and rigid cell wall (Fig. 1) (24, 151).

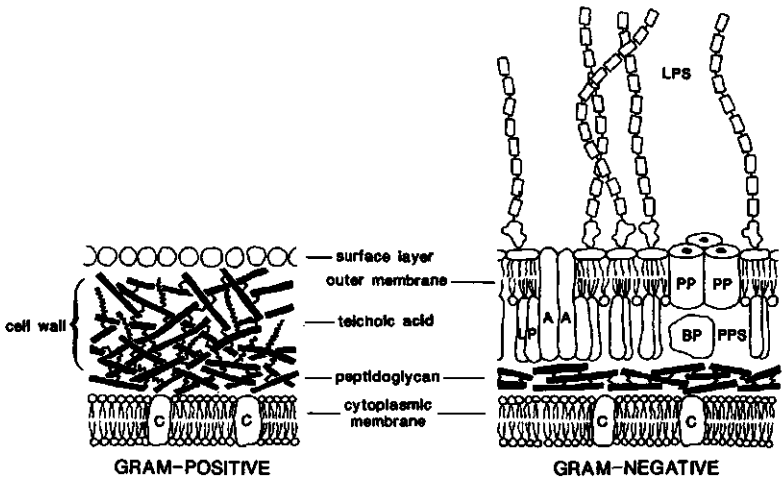


Figure 1. Schematic presentation of the cell envelope of gram-positive and gram-negative bacteria. PP, protein porine; C, cytoplasmic membrane embedded protein (*e.g.*, carrier); BP, binding protein; PPS, periplasmic space; A, outer membrane protein; LP, lipoprotein; LPS, lipopolysaccharide.

P_i transport has been documented in a number of microorganisms, including *Escherichia coli* (173), *Pseudomonas aeruginosa* (114, 157), *Micrococcus lysodeikticus* (1, 71, 72), *Enterococcus faecalis* (87-90), *Lactococcus lactis* (132, 159, 161),

Staphylococcus aureus (161), and several other bacterial species (10, 40, 92, 170, 176, 177, 224). The assimilation of P_i and phosphate-containing nutrients has been most intensively studied in *E. coli*. One of its P_i transport systems is part of a regulatory complex concerning phosphorus supply, and has been studied since the early 1960s. In addition, its molecular mechanisms for the extraction of P_i from the environment seem to represent the most common solutions to the problem of P_i transport in bacteria. The P_i transport systems of *E. coli* will therefore form the main portion of the second part of this introduction, but relevant information on other organisms has also been included.

2.1 P_i uptake through the outer membrane and the periplasm

While P_i is the preferred P source, *E. coli* also uses organo-phosphates that occur in nature. P_i esters like *sn*-glycerol-3-P, glucose-6-P or mannose-6-P can enter the cell intact. However, a wide range of organic phosphate compounds cannot be metabolized unless they are degraded to P_i. Such P_i esters cross first the outer membrane, and are then hydrolysed to release P_i in the periplasm. This P_i is then transported across the cytoplasmic membrane into the cytoplasm.

The outer membrane functions as a molecular sieve. It contains water-filled protein channels through which hydrophilic solutes with a molecular mass of less than 600 to 1000 Da can pass in a diffusion-like process. When grown at excess P_i, *E. coli* produces two general pore forming proteins, OmpF and OmpC (126). The synthesis of a third pore forming protein, PhoE, is induced when cells are grown under P_i limitation (153). The OmpF and OmpC pores have a preference for cations, whereas PhoE pores are more efficient for anions, *e.g.* P_i and phosphate-containing nutrients (17, 18, 23, 108, 109). Site-specific mutation analysis and determination of the three-dimensional structure of the PhoE pore at 3 Å resolution have indicated that the anion selectivity is provided by a positively charged lysyl group which protrudes into the channel (18, 44). PhoE-like pores have also been found in *Pseudomonas aeruginosa* (82, 158), *Enterobacter cloacae* (206), *Klebsiella aerogenes* (184), *Klebsiella pneumoniae* (158) and *Salmonella typhimurium* (16). Thus, diffusion through these anion-selective pores seems to provide gram-negative

bacteria with an unspecific mechanism for the transport of P_i and P_i-containing nutrients through the outer membrane.

The periplasm of *E. coli* contains a number of proteins, including phosphatases and solute-binding proteins (167). Among the phosphatases, the *phoA*-encoded nonspecific phosphomonoesterase with alkaline pH optimum has attracted much attention since its discovery (195, 196). Its increased synthesis during P_i limitation allows *E. coli* to use many non-transportable P_i-esters as sole P sources. Other phosphatases in the periplasm of *E. coli* include acid phosphatase (optimum pH 2.5), which is able to degrade short-chain polyphosphates (47), 2',3'-cyclic phosphodiesterase (19), and glycero-phosphoryl-diesterase (115). PhoA-like periplasmic phosphatases have also been found in other gram-negative bacteria, such as *Salmonella typhimurium* (212), *Pseudomonas fluorescens* (73), *Pseudomonas aeruginosa* (42, 192), and *Acinetobacter lwoffi* (222).

Solute-binding proteins are part of permeases which transport solutes with high-affinity across the cytoplasmic membrane. These permeases are inactivated when the binding protein is released into the medium as a result of disruption of the periplasmic space, *e.g.* by osmotic shock or during the formation of spheroplasts (147). Several functions have been assigned to solute-binding proteins. Two of the most common suggestions are that the binding proteins increase the effective concentration of the solute in the periplasm or that they enhance the affinity of otherwise binding protein-independent transport systems (9, 49, 162). Both suggestions can be persuasively excluded (93). Binding proteins may facilitate the movement of the solute through the periplasm by restricting diffusion to two rather than three dimensions (36, 95). Thus, the lateral diffusion of periplasmic binding proteins may resemble that of the binding proteins in gram-positive bacteria which, in the absence of a periplasm, are anchored to the cytoplasmic membrane by a lipid group (59, 80, 155). Finally, binding proteins may impose directionality on transport via the uptake system with which it is associated (95). One of the P_i permeases of *E. coli*, the phosphate specific transport system (Pst), acts in conjunction with a periplasmic P_i-binding protein (see below).

2.2 P_i transport across the cytoplasmic membrane

The cytoplasmic membrane of bacteria forms a permeability barrier for hydrophilic solutes (41). The translocation of these compounds is mediated by specific transport proteins which are embedded in the membrane. The mechanisms of solute transport can be divided into two classes: (i) primary transport, and (ii) secondary transport (86). By definition, primary transport systems mediate the vectorial movement of the solute across the cytoplasmic membrane coupled to a chemical reaction. These transport systems comprise, amongst others, ion translocating ATPases (*e.g.* F_0F_1 H^+-ATPase) and ATP-driven, binding protein-dependent permeases for solutes. Secondary transport systems (carriers) catalyze the translocation of a solute across the membrane in uniport, or in symport or antiport with other solutes without being associated with a chemical reaction. The driving force for transport is supplied by electrochemical solute gradients. Carriers mediate facilitated diffusion, but may perform osmotic work by coupling the flux of one solute to that of another, such as protons or sodium ions. In these latter cases, the transport systems are indicated as proton and sodium motive force-driven, respectively. Thus, while primary transport systems are *intrinsically unidirectional*, secondary transport systems mediate solute fluxes in uptake or efflux direction in accordance with the prevailing electrochemical gradients (86, 160).

E. coli possesses four P_i transport systems which can be classified on the basis of substrate specificity, and bioenergetic and structural criteria (Fig. 2). The phosphate specific transport system (Pst) and phosphate inorganic transport (Pit) system are specific for P_i and designed for net P_i movement. The remaining two P_i transporters mediate an anion exchange reaction in which P_i is accepted as an analog of an organo-phosphate, such as *sn*-glycerol-3-P (GlpT) or glucose-6-P (UhpT) (116, 132, 173). Pit, GlpT and UhpT are secondary transport systems. Pit is probably a nH^+/P_i symport system (173). GlpT and UhpT are antiport systems that couple the accumulation of sugar phosphates to the downhill release of P_i under physiological conditions (2, 3, 181, 182). Pit, GlpT and UhpT do not use a periplasmic binding protein and apparently consist of a single transmembrane protein (61, 64, 74, 98, 103, 120). The Pst system operates as a primary transport

mechanism. Its structure is far more complex, involving several membrane proteins in addition to the periplasmic P_i-binding protein (188). In the following sections, the mechanism and regulation of the P_i transport systems of *E. coli* are described in more detail.

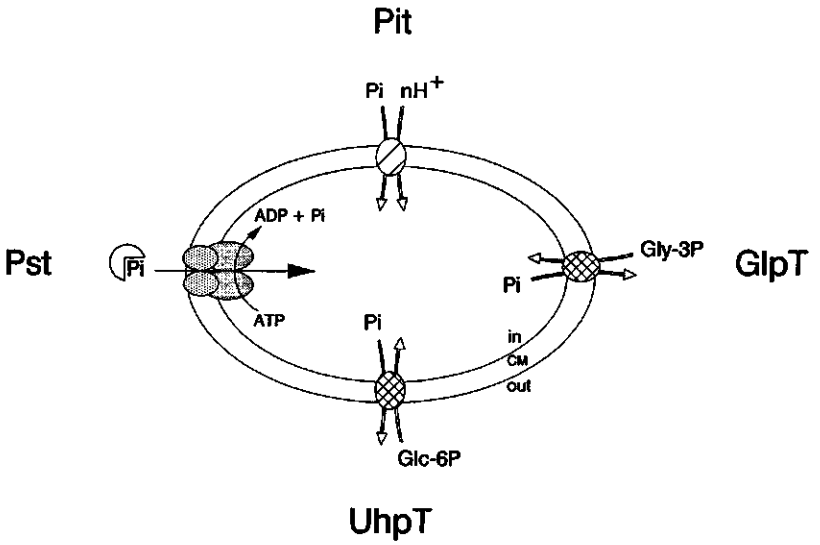


Figure 2. P_i transport systems in *Escherichia coli*. Pst, phosphate specific transport system; Pit, phosphate inorganic transport system; UhpT and GlpT, P_i-linked antiport systems for the transport of glucose-6-P and *sn*-glycerol-3-P, respectively.

Pst system

The Pst system of *E. coli* is typical of a class of periplasmic permeases which are composed of one periplasmic substrate-binding protein and three membrane-bound components (96). This class of bacterial uptake systems belongs to an expanding family that includes several important eukaryotic proteins, *e.g.* the multidrug resistance glycoprotein of tumor cells (65) and the cystic fibrosis transmembrane conductance regulator (172), and has been given the name "Traffic ATPases" (7, 9, 55) or "ABC transporters" (95, 96). The latter designation refers to the highly

conserved ATP-binding cassette which is the most characteristic feature of this superfamily.

There are four genes (*pstS*, *pstA* (formerly *phoT*), *pstC* (*phoW*), and *pstB*), encoding proteins required for P_i transport through the Pst system (190). The *pstS*-encoded P_i-binding protein binds P_i in the periplasm and then apparently shepherds this solute to the transporter in the cytoplasmic membrane (78, 137, 138, 189, 217). The P_i-binding protein has been purified to homogeneity from crude shock fluid. Like other periplasmic binding proteins, the P_i-binding protein consists of a single polypeptide with one tight solute-binding site with a dissociation constant (K_D) of 0.8 μM P_i (77, 111, 138, 191). This K_D value is comparable to the K_t of the Pst system for P_i transport (K_t between 0.3 and 0.7 μM P_i) (139, 174, 218). DNA sequence determinations of the *pstS* gene indicate a molecular weight of 34,400 for 321 amino acid residues (127, 189). A high-resolution (1.7 Å) X-ray crystal structure is available for the P_i-binding protein (111, 125), from which information about the selectivity at the atomic level is deduced. The P_i-binding protein is ellipsoidal with an axial ratio of 2 : 1, consisting of two similar, globular domains with a cleft in between which forms the solute binding site. Anhydrous monovalent and divalent P_i ($H_2PO_4^-$ and HPO_4^{2-}) are bound with coordination via 12 hydrogen bonds to amino acid residues, while sulfate is excluded (125). A conformational change in the binding protein, induced by binding of the solute, facilitates the interaction with the transmembrane transport proteins (135, 148, 149, 152, 163).

The transmembrane portion of the Pst system is formed by the hydrophobic PstA and PstC proteins. The *pstA* and *pstC* genes encode integral membrane proteins with six membrane spanning helices (45, 46, 211). The *pstB* gene encodes a hydrophilic protein containing an ATP-binding sequence motif. It interacts with PstA and/or PstC on the cytoplasmic side of the membrane (45). PstB shares extensive homology around the nucleotide-binding with HisP, OppD and MalK proteins of the similarly organized, binding protein-dependent histidine, oligopeptide and maltose permeases, respectively (96). Recently, the direct involvement of ATP hydrolysis was demonstrated in the histidine and maltose transport systems reconstituted in membrane vesicles and proteoliposomes (8, 25, 48, 49, 51, 99, 162). By analogy with other Traffic ATPases the PstB protein probably functions as a dimer

(209). Mutational analysis of PstA and PstC suggest that P_i is translocated through PstA and PstC via a "P_i relay" of three arginine/glutamate (or aspartate) salt bridges. The necessary movement of the participating PstA and PstC helices, to open or close the "phosphate channel", may be achieved by cis-trans isomerisation of two pairs of proline residues, energized by ATP hydrolysis via PstB (35, 211).

Together with the *phoU* gene, encoding a regulator protein (144, 146, 183, 185), the *pst* genes form a *pstSCAB-phoU* operon (190), the expression of which is highly regulated. The *pstS* promoter is expressed at a low, basal level when P_i is present in excess. It shows a 100-fold derepression during P_i-limited growth (141). The *pstSCAB-phoU* operon is part of the phosphate (Pho) regulon which consists of several phosphate-starvation-induced (*psi*) genes, the products of which act primarily in the assimilation of environmental phosphorus (209). Besides the Pst system, these products comprize the outer membrane porin PhoE, the periplasmic alkaline phosphatase PhoA, a periplasmic binding protein-dependent uptake system for *sn*-glycerol-3-P together with a periplasmic glycero-phosphoryl-diesterase (encoded by the *ugp* operon) (37, 38, 105, 115, 154), and proteins involved in the transport and metabolism of phosphite and phosphonates (encoded by the *phn* operon) (141). In addition, *psi* genes are involved in the regulation of the synthesis of high molecular-weight linear polyphosphates (165).

P_i control over the synthesis of Pho regulon proteins uses a transmembrane signal transduction mechanism in which the extracellular P_i level is sensed (166). A model for signal transduction is shown in Fig. 3. Signal transduction requires the Pst system, PhoU and the partner proteins PhoR and PhoB which make up a two-component gene regulatory system for P_i control (185, 209). PhoR is an integral membrane sensor protein which autophosphorylates itself (from ATP) and acts as a histidine kinase that phosphorylates the response regulator PhoB when cells are under P_i limitation (128, 131, 221). PhoB is a soluble DNA-binding effector protein that functions only when it is phosphorylated. Phospho-PhoB activates transcription by binding to the consensus "Pho box" sequences upstream the Pho regulon promoters (129, 130). The regulation of (de)phosphorylation of PhoR and PhoB is a complex process, which is not fully understood. The model in Fig. 3 depicts three events important in P_i repression. The first step involves the saturation of PstS by

and Rosenberg (107) who studied the effect of ionophores on P_i transport in membrane vesicles from *E. coli* energized by the oxidation of D-lactate or ascorbate/phenazine methosulphate. Although in these experiments, P_i transport was biased towards the transmembrane pH gradient (ΔpH) component of the proton motive force, the transport process appeared to be electrogenic. This latter property seems to be restricted to Pit since GlpT and UhpT mediate an electroneutral P_i transport mechanism (132). The absence of proton motive force-driven uptake of P_i in membrane vesicles prepared from the Pit-deficient *E. coli* strain K-10 is consistent with this notion (107).

Like other secondary transport systems, *e.g.* LacY (101), Pit is probably composed of a single polypeptide. All known *pit* mutations map within the same locus (77 min) of the *E. coli* chromosome and are complemented by transformation with a plasmid carrying a 2.2 kb chromosomal *SalI-AvaI* fragment. This fragment was sequenced and an open reading frame comprising 1287 bp was found. The deduced polypeptide contains 429 amino acids corresponding with a molecular mass of 46.2 kDa (62, 64). The actual sequence data have not yet been published.

UhpT and GlpT system

Although earlier classifications considered the P_i transporters GlpT and UhpT of *E. coli* to be further examples of nH^+/anion symport (85, 117), we now know that these systems belong in the category of P_i-linked antiport (2, 61, 63, 91, 116, 132, 133, 156, 181, 220). Besides from studies on the *E. coli* systems, most of our current knowledge about P_i-linked antiport also comes from the characterization of the sugar-phosphate transporters in *Lactococcus lactis* and *Staphylococcus aureus* (for review, see 132 and 159).

The P_i-linked anion exchangers in these three organisms appear to share the following properties. First, they mediate the homologous P_i:P_i and organo-phosphate:organo-phosphate exchange and the heterologous exchange of P_i and organo-phosphate (2, 4, 134, 143, 181, 182). Arsenate freely substitutes for P_i during both homologous and heterologous exchange (134). Second, the antiporters strongly favor $H_2PO_4^-$ above HPO_4^{2-}, but select randomly among the available mono- and

divalent organo-phosphates. The affinity of the exchangers for the organo-phosphate species is at least 10-fold higher than for P_i (or arsenate). Thus, the UhpT system has a K_t of about 1.2 mM for P_i, but a K_t of 20 μM for glucose-6-P (3, 132-134, 143). Third, the antiporters maintain electroneutrality during the homologous and heterologous exchange reactions by translocating P_i and organo-phosphates with a pH-dependent variable exchange stoichiometry. A carrier protein can be envisaged in which a bifunctional active site accepts either two monovalent anions, or a single divalent species. During heterologous exchange at pH values at which the divalent organo-phosphate is prevailing in aqueous solution, the antiporters will predominantly mediate the exchange of two molecules of $H_2PO_4^-$ against one molecule of divalent organo-phosphate. Concomitantly with decreasing pH (7 to 5), the heterologous exchange ratio will fall from 2:1 down to 2:2 (= 1:1) as pairs of mono anions move against each other (3, 132, 133). Besides via heterologous exchange, the P_i-linked antiporters can mediate the net accumulation of organo-phosphate via a pH gradient (interior alkaline)-promoted asymmetrical, homologous exchange of one molecule of divalent organo-phosphate (on the inner surface of the membrane) against two molecules of the monovalent species (on the outer surface of the membrane) (3).

Production of the UhpT and GlpT transporters is not P_i regulated, but induced specifically by extracellular glucose-6-P and 2-deoxyglucose-6-P (54), or *sn*-glycerol-3-P (116), respectively. The expression of the *uhpT* gene is dependent on the function of three regulatory genes, *uphABC* (213, 214). UhpA and UhpB are members of two-component regulatory proteins, such as PhoB/PhoR and others (185). Sequence homology suggests that the membrane protein UhpB may be a histidine-protein kinase and, in the presence of exogenous glucose-6-P and the membrane protein UhpC, may phosphorylate and activate UhpA, a transcriptional activator for the *uhpT* gene (98, 140, 214). In addition, the *uhpT* gene expression is subject to catabolite repression and is reduced 2- to 3-fold when cells are grown in the presence of both glucose-6-P and glucose. This decrease is reversed by cyclic AMP, and it has been found that the *uhpT* promoter contains a typical binding site for the cyclic AMP receptor protein (CAP) (140). This catabolite repressibility of Uhp synthesis may be necessary to prevent excessive flux of metabolites through

the glycolytic pathway, which can lead to growth inhibition or killing (102).

3. Outline of this thesis

Some acinetobacters have the ability to accumulate more P_i than is required for growth. Together with metal ions, this P_i is stored as a metal polyphosphate chelate in granules in the cytoplasm of the cell. Though acinetobacters are strictly aerobic organisms, polyphosphate-accumulating strains are enriched in wastewater treatment plants running in anaerobic/aerobic cycles. There, they accumulate polyphosphate during the aerobic phase, and subsequently degrade this polymer during anaerobiosis. The polyphosphate metabolism of one of these strains, *A. johnsonii* 210A, is relatively well studied (Fig. 4) and there is evidence that polyphosphate plays a role as an energy reserve. In contrast, virtually nothing is known about the transport processes which are involved in the uptake or release of P_i in *Acinetobacter*. Therefore, the aim of this thesis was to study the mechanisms of P_i transport in *A. johnsonii* 210A in relation to the synthesis and degradation of polyphosphate. Special attention was given to the possibility of recycling of metabolic energy during the excretion of P_i. This mechanism would enable the organism to regain the energy which was invested in the uptake of P_i in a similar way as was proposed by Michels et al. (142) in their "energy recycling model".

The investigations on P_i transport in *A. johnsonii* 210A started with the identification of the P_i transport systems in whole cells. Chapter 2 describes the presence of a primary, periplasmic binding protein-dependent P_i transport system and a secondary P_i transport system in *A. johnsonii* 210A. These permeases show analogy to the Pst and Pit system of *E. coli*. The energetics and mechanism of the secondary P_i transport system of *A. johnsonii* 210A were studied in detail in membrane vesicles and in proteoliposomes in which the transport protein was functionally reconstituted. Strikingly, the secondary P_i transport system shows specificity for a soluble, neutral metal phosphate ($MeHPO_4$) complex rather than P_i. Several modes of carrier-mediated transport of $MeHPO_4$ have been analysed as a function of pH and proton motive force. The data have been incorporated into a kinetic model of the transport cycle of $H^+/MeHPO_4$ cotransport which is presented

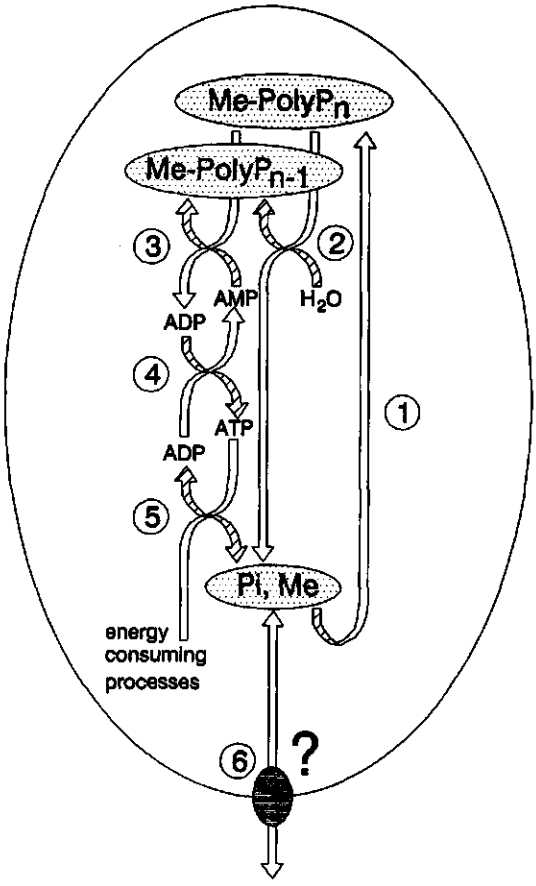


Figure 4. Polyphosphate (PolyP) metabolism in *A. johnsonii* 210A: 1, polyphosphate synthesis; polyphosphate degradation via 2, polyphosphatase and 3, polyphosphate: AMP phosphotransferase; 4, adenylate kinase; 5, ATP consumption by energy-requiring processes; 6, P_i and cation (Me) transport.

in Chapter 3. The properties of the Pit system of *E. coli* were studied for comparison. In Chapter 4, the Pit system is identified as a $MeHPO_4$ permease operating via a similar mechanism as the secondary $MeHPO_4$ transport system of *A. johnsonii* 210A. Both $MeHPO_4$ transport systems may represent a new class of bacterial porters. Chapter 5 reports on the relation between the substrate specificity of the

two P_i transport systems of *A. johnsonii* 210A, the availability of P_i species in its aquatic environment, and the metabolism of metal polyphosphate granules. In view of the limited insight into the bioenergetics of the transport of amino acids in *Acinetobacter*, and the possible energetic role of a $MeHPO_4$ efflux-induced proton motive force in the accumulation of these solutes, several secondary amino acid transport systems were characterized in *A. johnsonii* 210A. A description of the energetics of three such systems is presented in Chapter 6. Evidence for energy recycling by $MeHPO_4/H^+$ excretion via the secondary $MeHPO_4$ transport system in *A. johnsonii* 210A is summarized in Chapter 7. In this chapter it is demonstrated that a $MeHPO_4/H^+$ efflux-induced proton motive force can drive various energy consuming processes, such as the synthesis of ATP and the retention of amino acids, when oxidative phosphorylation is impaired. Finally, important implications of the results presented in the preceding chapters are discussed in Chapter 8.

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2

Characterization of two phosphate transport systems in *Acinetobacter johnsonii* 210A

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Summary

The transport of P_i was characterized in *Acinetobacter johnsonii* 210A, which is able to accumulate an excessive amount of phosphate as polyphosphate under aerobic conditions. P_i is taken up against a concentration gradient by energy-dependent, carrier-mediated processes. *A. johnsonii* 210A, grown under P_i limitation, contains two uptake systems with K_t values of 0.7 ± 0.2 μM and 9 ± 1 μM. P_i uptake via the high-affinity component is drastically reduced by *N,N'*-dicyclohexylcarbodiimide, an inhibitor of the H^+-ATPase, and by osmotic shock. Together with the presence of P_i-binding activity in concentrated periplasmic protein fractions, these results suggest that the high-affinity transport system belongs to the group of ATP-driven, binding-protein-dependent transport systems. Induction of this transport system upon transfer of cells grown in the presence of excess P_i to P_i-free medium results in a 6- to 10-fold stimulation of the P_i uptake rate. The constitutive low-affinity uptake system for P_i is inhibited by uncouplers and can mediate counterflow of P_i, indicating its reversible, secondary nature. The presence of an inducible high-affinity uptake system for P_i and the ability to decrease the free internal P_i pool by forming polyphosphate enable *A. johnsonii* 210A to reduce the P_i concentration in the aerobic environment to micromolar levels. Under anaerobic conditions, polyphosphate is degraded again and P_i is released via the low-affinity secondary transport system.

Introduction

Enhanced biological phosphorus removal from domestic wastewaters in full-scale activated sludge plants is currently perceived to hinge on the provision of alternate stages in which the activated sludge is subjected to anaerobic and aerobic conditions (40). A characteristic feature of such plants is that P_i, after being released from the biomass in an anaerobic stage, is reincorporated in the biomass during aeration, together with part or all of the influent P_i (16). Significant numbers of polyphosphate-accumulating bacteria, especially from the gram-negative genus *Acinetobacter*, have been isolated from activated sludge in which biological phosphorus removal

has been observed (14, 29). Similar to activated sludge, polyphosphate-accumulating *Acinetobacter* spp. take up P_i under aerobic conditions and release it anaerobically (10). One of these strains, *Acinetobacter johnsonii* 210A, is able to accumulate up to 300 mg of P_i per g (dry weight). The extent of P_i accumulation depends on growth rate, carbon and energy source, limiting nutrients and temperature (46). When oxidative phosphorylation is impaired (*e.g.*, in the absence of oxygen or an electron donor), polyphosphate is degraded and P_i is released into the medium (43). Two enzymes are involved in the degradation of polyphosphate: (i) polyphosphatase and (ii) polyphosphate:AMP phosphotransferase (44). The latter enzyme has been characterized recently (8). In combination with adenylate kinase, this enzyme enables the organism to conserve the energy from the phosphate bonds in polyphosphate and to use the accumulated polymer as a source of ATP when energy cannot be obtained otherwise (45).

The possible role of *Acinetobacter* spp. in the enhanced biological phosphorus removal from domestic wastewaters raised questions about the involvement of P_i transport systems in the uptake and release of P_i in *A. johnsonii* 210A. The nature and properties of P_i transport systems have been investigated in other bacterial strains, including *Escherichia coli* (36, 37, 49), *Pseudomonas aeruginosa* (24, 30), *Lactococcus lactis* (31) and *Micrococcus lysodeikticus* (12). In *E. coli* and *P. aeruginosa*, two major P_i transport systems with low and high affinity for P_i are present (36). In the work reported here, the presence of two P_i transport systems in *A. johnsonii* 210A is demonstrated. One system is an inducible, ATP-dependent, binding protein-dependent permease enabling the organism to reduce the P_i concentration in the aerobic environment to micromolar concentrations. The other system is a constitutive, reversible secondary transport system which mediates the efflux of P_i under anaerobic conditions.

Materials and methods

Organism and culture conditions

A. johnsonii 210A was grown aerobically at 30 °C in a buffered medium (pH 7.2)

containing 20 mM Na-butyrate, 20 mM NH_4Cl, 5 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM $CaCl_2 \cdot 2H_2O$, 10 mM KCl, 2 ml trace element solution per liter, and 50 mM Tris-HCl. The composition of the trace element solution has been described by Van Groenestijn et al. (45). Sterile sodium phosphate, pH 7.2, was added to a final concentration of 5 mM (high-P_i medium) or 20 μM (low-P_i medium) for cultivation of high-P_i-grown and low-P_i-grown cells, respectively. *E. coli* K-12 was grown under P_i limitation in a minimal medium with glucose and 0.1 % (wt/vol) yeast extract as phosphorus source (17). For growth in medium with excess P_i, 5 mM sodium phosphate buffer was added. Cells at the logarithmic phase were harvested by centrifugation (7,000 x *g*, 10 min). The pellet was washed and resuspended as indicated below.

Transport assays

Cells, washed and resuspended in 20 mM potassium Pipes (pH 7.0), containing 10 mM $MgSO_4$ and 50 μg chloramphenicol per ml, were stored on ice and used within 2 h. Transport assays were performed at 30 °C. Cells were diluted in 100 μl of air-saturated buffer to about 0.5 mg of protein per ml. The suspension was kept aerobic by flushing with water-saturated air. Cells were preincubated for 3 min with 2 μM PQQ, after which 20 mM glucose was added. Two minutes later uptake was started by the addition of ^{32}P-labeled potassium phosphate (0.33 to 1.47 TBq/mol) or ^{14}C-labeled L-lysine at concentrations as specified in the figure legends. At given time intervals, 2 ml of ice-cold 0.1 M LiCl was added and the samples were filtered immediately through cellulose-nitrate filters. Filters were washed once with 2 ml of 0.1 M LiCl. The radioactivity on the filters was measured with a liquid scintillation counter. To remove contaminating P_i, the glassware used for $^{32}P_i$ transport assays was kept in chromic acid and rinsed 15 times with distilled water before use. $^{32}P_i$-labeled potassium phosphate was filtered through a 0.45-μm-pore-size cellulose nitrate filter prior to use, in order to remove $^{32}P_i$ adsorbed to particles (27).

EDTA treatment of intact cells

To permeabilize the outer membrane, cells were washed three times with 20 mM potassium Pipes (pH 7.0), containing 50 μg chloramphenicol per ml and suspended in this buffer at an A_{660} of about 30. After 3 min of preincubation of the cell suspension at 30 °C, 1 mM sodium EDTA (pH 7.0) was added. $MgSO_4$ was added 10 min later to a final concentration of 10 mM. Cells were washed once with 20 mM potassium Pipes (pH 7.0), containing 10 mM $MgSO_4$ and 50 μg chlorampenicol per ml, stored on ice and used within 2 h.

Determination of Δp_{H^+}

The $\Delta\psi$ (interior negative) was determined from the distribution of TPP^+, using a TPP^+-selective electrode (39). The standard assay was done at 30 °C with EDTA-treated cells in 1 ml of oxygen-saturated buffer [50 mM potassium Pipes, 10 mM $MgSO_4$, 50 μg chloramphenicol per ml, pH 7.0] in the presence of 4 μM TPP^+. Cells were supplied with metabolic energy by glucose oxidation as described in "Transport assays". Measurements were corrected for nonspecific probe binding to the cells (25). The pH-gradient across the membrane was calculated from the increase in $\Delta\psi$ upon the addition of nigericin, assuming a complete interconversion of the ΔpH into the $\Delta\psi$ (11).

Osmotic shock, preparation of periplasmic protein fractions and P_i-binding experiments

Cells were exposed to an osmotic shock procedure, essentially as described by Neu and Heppel (28). Cells were washed three times with 20 mM potassium Pipes (pH 7.5), containing 50 μg chloramphenicol per ml and suspended to an A_{660} of about 5.0 in 20 mM potassium Pipes (pH 7.5), containing 0.75 M sucrose, and 1 mM EDTA. This cell suspension was incubated for 10 min at 20 °C to induce plasmolysis. Cells were collected by centrifugation (7,000 x *g*, 20 min). Periplasmic proteins were released upon resuspension of the cell pellet in 50 volumes of 0.1

mM $MgSO_4$ at 20 °C. The osmotic shock treated cells were collected by centrifugation and used for transport assays. The supernatant (shock fluid) was concentrated 20-fold via ultrafiltration under N_2 pressure as described by Abee et al. (1) and dialyzed extensively at 4 °C against 20 mM potassium Pipes (pH 7.5). Binding of $^{32}P_i$ to concentrated shock fluid was measured according to Richarme and Kepes (35), by the addition of 10 μM $^{32}P_i$ (2.81 TBq/mol) to 0.5 ml of shock-fluid (0.3 mg of protein per ml). After incubation for 10 min at 20 °C, proteins were precipitated by the addition of 4 ml of an ice-cold saturated ammonium sulphate solution. The mixture was immediately passed through a nitrocellulose filter, which was washed twice with 2 ml ice-cold saturated ammonium sulphate. The radioactivity retained on the filter was measured by scintillation counting. In control experiments, binding of $^{32}P_i$ to the filter and to bovine serum albumin was determined.

Counterflow of ^{32}Pi and ^{14}C-lysine

Intact cells, grown in high-P_i medium, were deenergized by incubation for 12 h at 30 °C in 20 mM potassium Pipes (pH 7.0), containing 10 mM $MgSO_4$, 50 μg chloramphenicol per ml, and 2.5 mM DNP. Cells were washed twice with 20 mM potassium Pipes (pH 7.0) containing 30 mM sodium azide and 50 μg chloramphenicol per ml and suspended in this buffer to an A_{660} of about 30. After the addition of 1 mM sodium EDTA, the cell suspension was incubated at 30 °C for 10 min. Subsequently, the cells were washed and resuspended to a concentration of 0.5 mg of protein per ml in loading buffer (pH 7.5), containing 100 mM potassium Pipes, 10 mM $MgSO_4$, 30 mM sodium azide, 50 μg of chloramphenicol per ml, and 20 μM CCCP. Cells were loaded for 3 hours at 20 °C with 10 mM potassium phosphate or 3 mM L-lysine. Control cells were incubated in a buffer without potassium phosphate or L-lysine. Cells were concentrated to 20 mg protein per ml and diluted 200-fold in 400 μl loading buffer, containing a final concentration of 10 μM ^{32}P-labeled potassium phosphate (3.91 TBq/mol) or 16 μM L-[U-^{14}C]lysine (1.26 TBq/mol). The uptake of the radio-labeled substrates was monitored in time at 30 °C as described in "Transport assays".

Analytical procedures

Intracellular ATP concentrations were determined by the luciferin-luciferase method of Jetten et al. (22). The assay of alkaline phosphatase activity in concentrated shock fluid with *p*-nitrophenylphosphate as the substrate was carried out according to Yashphe et al. (50). The respiration rate was measured with a Clark-type oxygen-electrode. Protein was determined by the method of Lowry et al. (26) with bovine serum albumin as a standard. Soluble phosphates were extracted from cells by incubation in cold 5 % (wt/vol) TCA for 20 min according to the method of Hellingwerf et al. (19). P_i in the cells was assayed according to Avron (5) as a phosphomolybdate complex extracted into an organic phase of isobutanol-benzene.

Materials

Cellulose-nitrate filters (0.45 μm pore-size) were supplied by Millipore, Etten-Leur, the Netherlands, and Schleicher und Schuell, Dassel, Germany. ^{32}Pi (carrier-free) and L-[U-^{14}C]lysine (11.5 TBq/mol) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, UK. The ATP bioluminescence constant light signal kit was from Boehringer Mannheim. Other chemicals were reagent grade and obtained from commercial sources.

Results

Active P_i transport

(i) Energy-dependent uptake of P_i. Washed cells of *A. johnsonii* 210A, grown in high-P_i medium with butyrate as the carbon and energy source, took up P_i in the absence of an exogenous energy supply (Fig. 1). Initial experiments revealed the presence of a membrane-bound glucose dehydrogenase in *A. johnsonii* 210A, like in *Acinotobacter lwoffi* (47), which requires PQQ for activity (48). Although *A. johnsonii* 210A cannot grow on glucose with or without PQQ, or on gluconate, the addition to washed cells of 20 mM glucose plus 2 μM PQQ resulted in an increase

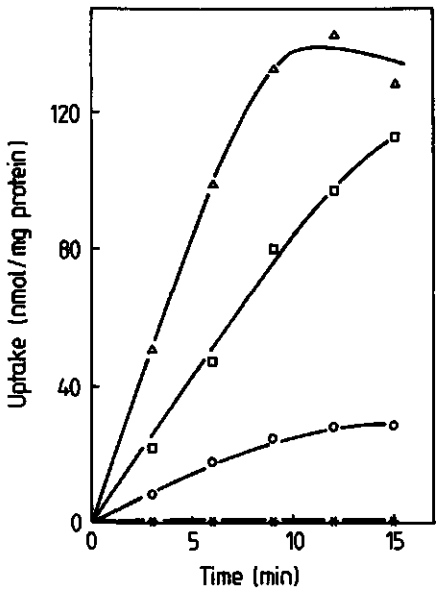

Figure 1. Uptake of 100 μM P_i by washed, high-P_i-grown cells of *A. johnsonii* 210A in the absence (O) and presence of 20 mM butyrate (□) or 20 mM glucose-2 μM PQQ (Δ), or by cells treated with 1% chloroform (*).

of the respiration rate from 41 to 145 nmol O_2 per min per mg protein and a stimulation of the Δp_H+ from -90 to -152 mV (inside negative and alkaline). In the presence of 20 mM butyrate, the oxidation rate was 83 nmol of O_2 per min per mg of protein and the Δp_H+ -115 mV (inside negative and alkaline). The rate of Pi uptake was stimulated in proportion with the respiration rate and the Δp_H+ (Fig. 1).

The uptake of P_i was severely impaired by preincubation of the cells with the respiratory chain inhibitor CN^-, the uncouplers DNP or CCCP, or the H^+-ATPase inhibitor DCCD (Table 1). A total collapse of the Δp_H+ in EDTA-treated cells after the addition of a combination of the ionophores nigericin and valinomycin in the presence of glucose-PQQ resulted in a complete abolition of the uptake of P_i (Table 1). The EDTA treatment itself had no effect on the uptake of P_i and was given to facilitate the incorporation of the ionophores into the cytoplasmic membrane of this bacterium. When the cell membranes were permeabilized by a treatment with chlo-

Table 1. Effect of inhibitors on P_i uptake in *A. johnsonii* 210A[a]

Inhibitors[b] (mM)		Uptake (%)
None (control)		100
CN^-	0.5	8
	1.0	5
DNP	0.5	54
	1.0	24
CCCP	0.02	32
DCCD	0.005	30
Valinomycin + nigericin[c]		2

[a] Cells grown in high-P_i medium were washed, treated with 1 mM EDTA, and diluted to a protein concentration of 0.3 mg/ml, as described in "Materials and methods". After addition of 20 mM glucose and 2 μM PQQ, the initial rate of P_i uptake over the first 60 s was determined at a P_i concentration of 100 μM. Cells incubated without inhibitors (control) took up P_i at a rate of 18 nmol/min/mg of protein (100%).

[b] Inhibitors were added 5 min prior to the uptake experiments, except for DCCD, which was added 45 min prior to the uptake experiment.

[c] Valinomycin and nigericin were added to final concentrations of 1.5 and 0.15 μM, respectively.

roform, no P_i was taken up (Fig. 1). These results indicate that the uptake of P_i in *A. johnsonii* 210A is an energy-dependent process. Because of the strong stimulation of the P_i uptake rate in the presence of glucose-PQQ, this substrate was used for energization of cells in further experiments.

(ii) Fate of intracellular P_i. Soluble phosphates were extracted from high-P_i-grown cells of *A. johnsonii* 210A as described in "Materials and methods". The total amount of P_i entering energized cells increased approximately linearly in time for at least 10 min. Most of this P_i was incorporated rapidly into cold TCA-soluble organic phosphates and into TCA-insoluble phosphates. A minor fraction remained in the cells as P_i. Assuming an internal volume of these cells of 3 μl/mg protein (7), an internal P_i concentration of 3 mM can be calculated, implying a 40-fold

accumulation of P_i under these conditions.

Effect of P_i deprivation on P_i uptake and accumulation

The rate of uptake of P_i in cells grown in high-P_i medium was 15 to 25 nmol of P_i per min per mg of protein. After transfer of these cells into a P_i-free medium, the rate of P_i uptake gradually increased to as much as 100 to 150 nmol of P_i per min per mg of protein during incubation for 4 h. This stimulation was inhibited in the presence of chloramphenicol. Since chloramphenicol had no effect on the P_i uptake rate itself, it is concluded that the stimulation reflects de novo protein synthesis. Washed cells grown in low-P_i medium took up P_i at a rate of 100 to 150 nmol of P_i per min per mg of protein. This rate was hardly stimulated by the addition of an exogenous source of energy. The high endogenous respiration rate of these cells (358 nmol of O_2 per min per mg of protein) indicates the presence of an internal energy reserve. Recently it has been reported that *A. johnsonii* 210A cells form poly-β-hydroxybutyrate when grown at a low P_i concentration (9). Most likely, the oxidation of this polymer supplies the energy for the uptake of P_i. An intensive accumulation of polyphosphate, known as the overplus phenomenon (18) or as polyphosphate supersynthesis (23), has been observed in several microorganisms after the addition of P_i to phosphorus-starved cells.

The fate of internalized P_i under these conditions has not yet been investigated. Low-P_i-grown cells of *A. johnsonii*, which took up P_i at a rate of 120 nmol/min/mg of protein, maintained an internal concentration of free Pi of 1.5 mM, comparable to internal concentrations found in high-P_i-grown cells. However, within 2 min of uptake a considerable amount of P_i (more than 85%, versus 15% in high-P_i-grown cells) was found in the TCA-insoluble fraction (data not shown). In *M. lysodeikticus* this fraction was shown to be composed mainly of polyphosphate (12, 13). Although most of the P_i taken up by the low-P_i-grown cells was subsequently metabolized, a maximal concentration gradient of 150 was obtained at an external Pi concentration of 10 μM, showing that P_i was taken up against a concentration gradient.

Table 2. Kinetic parameters of P_i uptake in *A. johnsonii* 210A[a]

	Low-affinity uptake		High-affinity uptake	
	K_t (μM)	V_{max} (nmol/min/mg of protein)	K_t (μM)	V_{max} (nmol/min/mg of protein)
High-P_i-grown cells	41 ± 7	15 ± 3	4 ± 1	12 ± 4
Osmotic shock	31 ± 6	10 ± 3	-[b]	-
DCCD	34 ± 7	5 ± 2	-	-
Low-P_i-grown cells	9 ± 1	15 ± 5	0.7 ± 0.2	80 - 120[c]

[a] The kinetics of P_i uptake was analysed in energized high-P_i-grown control cells, shocked cells, cells treated with 15 nmol DCCD per mg of protein, and in low-P_i-grown cells, using Lineweaver-Burk and Eadie-Hofstee plots. Initial velocities in high- and low-P_i-grown cells were determined over the first 20 and 8 s, respectively, at a P_i concentration between 0.025 and 500 μM. Values are means from four separate experiments.

[b] -, high-affinity P_i uptake system could not be detected under these conditions.

[c] V_{max} was dependent on growth conditions.

Kinetic parameters of P_i uptake

The uptake of P_i in high- and low-P_i-grown cells, energized by glucose-PQQ, was linear for at least 60 and 8 s, respectively, in the range of 0.025 to 500 μM P_i. The kinetic parameters of this uptake, K_t and V_{max}, were determined via linear regression analysis of Lineweaver-Burk and Eadie-Hofstee plots. The results show the presence of two transport systems in high- and low-P_i-grown cells (Table 2).

Presence of a binding protein-dependent P_i transport system

(i) Effect of DCCD. In order to examine the presence of an ATP-driven P_i transport system, the effect of the H^+-ATPase inhibitor DCCD on the internal ATP concentra-

tion, the Δp_H+, and P_i uptake was studied in EDTA-treated, high-P_i-grown cells. In a control experiment, the internal ATP concentration increased from 0.5 to 2.8 *mM* within 2 min after glucose-PQQ addition to washed cells. In a parallel experiment in which the cells were preincubated with 15 nmol of DCCD per mg protein, a decrease in the ATP concentration was observed from 2.8 to 0.4 mM. The -$Z\Delta$pH remained constant at -10 mV (inside alkaline) in DCCD-treated cells, whereas the $\Delta\psi$ was stimulated from -139 to -180 mV (inside negative). Since the oxidation of glucose-PQQ results primarily in the generation of a Δp_H+, which is partly used for ATP synthesis by the H^+-ATPase, the higher Δp_H+ and the lower internal ATP concentration in DCCD-treated cells are indications for the inhibition of the H^+-ATPase. Uptake of L-lysine, which is mediated by a secondary transport system in this organism (48), was not affected by DCCD up to 30 nmol per mg protein. P_i uptake was strongly inhibited by 15 nmol DCCD per mg of protein (Table 1). Only one component, resembling the low-affinity transport system kinetically, could be demonstrated in DCCD-treated cells (Table 2). This result indicates that phosphate bond energy is required for the energization of the high-affinity P_i uptake system.

(ii) Effect of osmotic shock and binding of P_i to concentrated shock fluid. ATP plays a role in the energization of periplasmic binding protein-dependent transport systems in gram-negative bacteria (2). These transport systems are called osmotic shock sensitive because of the loss of the periplasmic binding proteins into the medium by osmotic shock (15). Pi uptake in cells of *A. johnsonii* 210A grown in high-P_i (Fig. 2A) and in low-P_i medium (Fig. 2B) was inhibited by an osmotic shock by approximately 45 and 80%, respectively.Shocked cells maintained an intact cytoplasmic membrane and a constant internal pH as was shown in high-P_i-grown cells in which the respiration rate, the $\Delta\psi$ or the -$Z\Delta$pH remained constant at 108 nmol of O_2 per min per mg of protein, -100 mV (inside negative) and -12 mV (inside alkaline), respectively, before and after osmotic shock. This conclusion is consistent with the observed insensitivity of the uptake of L-lysine to the shock procedure in high-P_i-grown cells (Fig. 2C). Kinetic analysis of P_i uptake in shocked cells strongly suggests a specific inhibition of the high-affinity P_i uptake system by osmotic shock (Table 2).

To investigate whether a P_i binding protein was released by osmotic shock, P_i

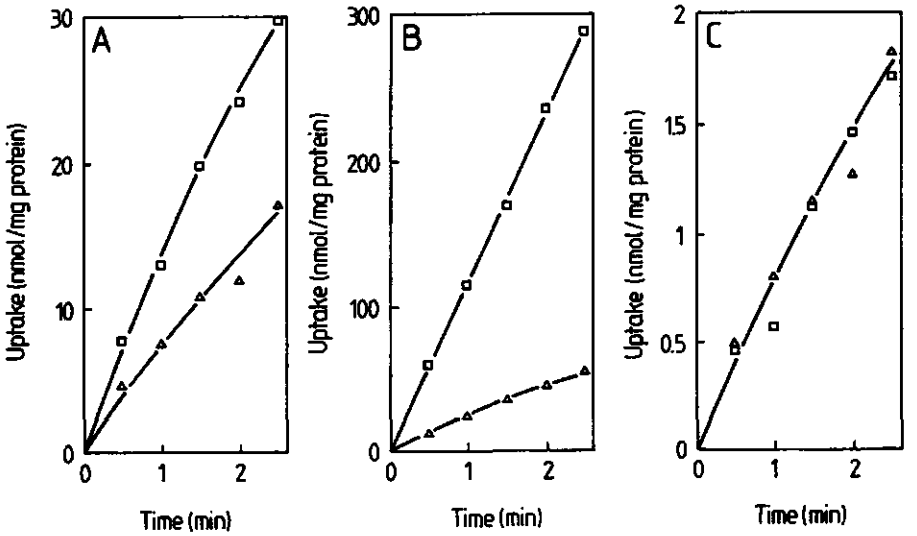

Figure 2. Effect of an osmotic shock on the uptake of P_i and L-lysine in *A. johnsonii* 210A. The uptake of P_i and L-lysine was determined in control cells (□) and shocked cells (△) at concentrations of 100 μM P_i and 1.6 μM L-lysine. (A) P_i uptake in high-P_i-grown cells; (B) P_i uptake in low-P_i-grown cells; (C) uptake of L-lysine in high-P_i-grown cells.

binding experiments were performed with concentrated periplasmic protein fractions of high-P_i-grown and low-P_i-grown cells. Alkaline phosphatase, which is known to be present in the periplasm of gram-negative organisms (30, 42), served as a control for the presence of periplasmic proteins in the concentrated shock fluids. As an additional control, concentrated shock fluid was prepared from *E. coli* in which the presence of a binding protein-dependent Pi transport system has been reported (36). The results are very similar for the two organisms (Fig. 3). P_i-binding activity was detectable in concentrated shock fluids of high-P_i-grown cells of *A. johnsonii* 210A. However, along with the activity of alkaline phosphatase, the P_i-binding activity was appreciably higher in concentrated shock fluid of low-P_i-grown cells. This result is consistent with the observation that P_i uptake in low-P_i-grown cells was more sensitive to osmotic shock than P_i uptake in high-P_i-grown cells (Fig. 2) and points to the presence of an inducible binding protein-dependent transport system for P_i in *A. johnsonii* 210A.

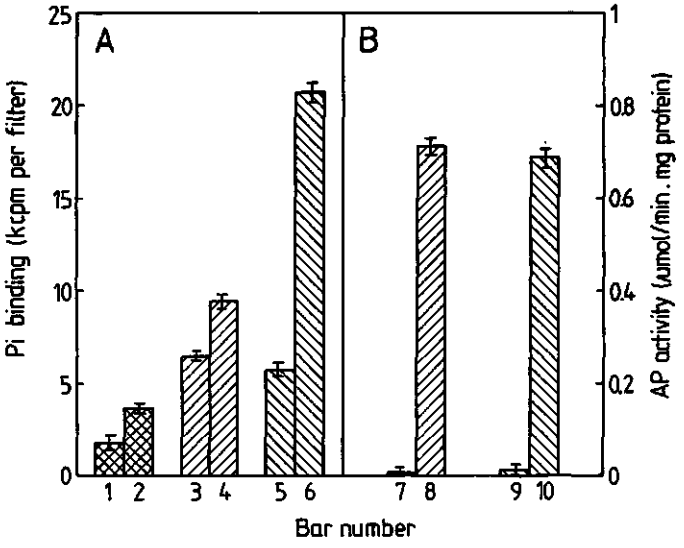

Figure 3. P_i-binding and alkaline phosphatase (AP) activity in concentrated shock fluid of *E. coli* and *A. johnsonii* 210A. (A) Binding of 10 μM P_i to the filter (bar 1), to bovine serum albumin (bar 2), and to concentrated shock fluid of high- and low-P_i-grown cells of *A. johnsonii* 210A (bar 3 and 4) and high- and low-P_i-grown cells of *E. coli* (bar 5 and 6); (B) alkaline phosphatase activity in concentrated shock fluid of high- and low-P_i-grown cells of *A. johnsonii* 210A (bar 7 and 8) and of high- and low-P_i-grown cells of *E. coli* (bar 9 and 10). Each value is the mean of two separate determinations.

Presence of a secondary P_i transport system

ATP-dependent uptake systems for solutes are in general unidirectional and should not allow counterflow of substrates. On the other hand, secondary transport systems are reversible and should allow an easy exchange of P_i inside and outside the cell in a deenergized state (1, 31, 32). In order to obtain more evidence for reversible secondary P_i and L-lysine transport, counterflow of P_i and L-lysine was studied in high-P_i-grown cells of *A. johnsonii* 210A which were depleted of endogenous energy reserves by an aerobic incubation in the presence of 2.5 mM DNP for 12 h at 30 °C. Within 60 min of incubation with DNP, the internal ATP concentration was reduced from 1.5 mM to below 0.01 mM. Endogenous respiration and Pi uptake

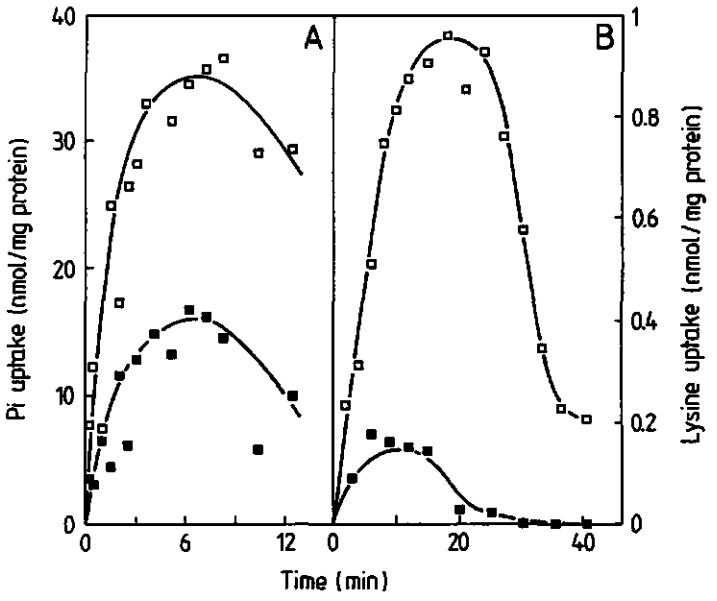

Figure 4. Uptake of 10 μM P_i (A) and 16 μM L-lysine (B) via counterflow in deenergized cells of *A. johnsonii* 210A, which were unloaded (■), or loaded with 10 mM P_i or 3 mM L-lysine (□).

decreased to very low rates after 12 h of incubation. Upon addition of glucose-PQQ to these starved and washed cells, the respiration rate and P_i uptake rate were restored to levels comparable to those in energized cells, indicating that (i) DNP was removed effectively to allow an energization of the starved cells and (ii) the starved cells had retained the transport system in an active form.

The amounts of P_i and L-lysine taken up via counterflow in deenergized cells of *A. johnsonii* 210A loaded with 10 mM P_i or 3 mM L-lysine and diluted into media containing a final concentration of 10 μM $^{32}P_i$ and 16 μM L-[U-^{14}C]lysine, respectively, were significantly higher than in unloaded cells (Fig. 4). Counterflow activity of P_i and L-lysine clearly indicates the presence of reversible secondary transport systems for these substrates.

Discussion

The uptake of P_i by *A. johnsonii* 210A is an active process. P_i uptake occurs against a concentration gradient, depends on the presence of an oxidizable energy source, and is inhibited by respiratory chain inhibitors and uncouplers of the oxidative phosphorylation. Kinetic analysis reveales the presence of two P_i uptake systems with low and high affinity for P_i. The K_t values observed in low-P_i-grown cells (0.7 ± 0.2 μM and 9 ± 1 μM) are very similar to those reported for *E. coli* (0.7 μM and 9.2 μM) (27) and *P. aeruginosa* (1.1 μM and 10 μM) (24), but are sixfold lower than those observed in high-P_i-grown cells of *A. johnsonii* 210A (4 ± 1 μM and 41 ± 7 μM). High-P_i-grown cells contain polyphosphate granules, which are absent in low-P_i-grown cells. Whether polyphosphate granules and/or the slow degradation of polyphosphates in washed cell suspensions of high-P_i-grown cells (43) affects the kinetics of P_i transport is unclear. However, since the high- and the low-affinity systems in both low- and high-P_i-grown cells are similarly affected by several treatments of the cells, it is concluded that the uptake of P_i in cells grown in low P_i as well as in high P_i medium is mediated by the same two transport systems. Comparison of the maximal uptake rates of the transport systems in low- and high-P_i-grown cells suggests the presence of a constitutive low-affinity system and an inducible high-affinity system. The maximal level of induction after transferring high-P_i-grown cells to P_i-free medium is obtained within 4 h or approximately two cell divisions.

Strong evidence is obtained for the presence of a periplasmic binding-protein-dependent high-affinity P_i uptake system. The uptake of P_i in cells is inhibited by an osmotic shock, as is observed for all binding-protein-dependent systems but not for secondary transport systems (15). Kinetic experiments show the inactivation of the high-affinity transport system by osmotic shock. Further evidence comes from the demonstration of P_i binding activity in concentrated periplasmic fractions of shocked cells. The observed induction of this activity under P_i limitation is consistent with its involvement in the high-affinity P_i uptake system. Binding-protein-dependent transport systems are energized by a high-energy phosphate bond (2-4, 20, 21). P_i transport in *A. johnsonii* 210A can be energized by the oxidation of

glucose via a membrane bound, PQQ-dependent glucose dehydrogenase. Since *A. johnsonii* 210A is unable to grow on glucose ± PQQ or on gluconate as the sole carbon source, glucose oxidation can result only in ATP synthesis coupled to the Δp_H+ by a membrane bound H^+-ATPase. Inhibition of the H^+-ATPase by DCCD results in a stimulation of the Δp_H+, a sevenfold decrease of the internal ATP concentration, and a drastic reduction in the uptake of P_i via the high-affinity transport system. These effects of DCCD point to the involvement of ATP, or a related compound, in the energization of P_i uptake via the binding-protein-dependent transport system.

The low-affinity uptake of P_i is hardly affected by osmotic shock or by DCCD, but dissipation of the Δp_H+ by valinomycin/nigericin decreases the activity strongly, suggesting that it is mediated by a Δp_H+-dependent secondary transport system. In contrast to ATP-dependent, binding-protein-dependent uptake systems which are usually unidirectional, secondary transport systems mediate reversible transport (1, 31, 32). Counterflow of P_i in deenergized cells loaded with 10 mM P_i confirmed the presence of a reversibel (secondary) transport system for P_i.

The P_i uptake systems in *A. johnsonii* 210A resemble the two major transport systems of *E. coli*: the high-affinity P_i-specific transport system and the constitutive low-affinity P_i transport system (37, 49). In both organisms, the two systems are present in cells grown in high P_i medium. In low P_i-medium, the rate of P_i uptake via the high-affinity system is increased by a factor of 6 to 10, just as the activity of alkaline phosphatase. This phenomenon is well documented for *E. coli*, where the phosphate regulon is an interlocking assembly of genes, transport systems, and enzymes dedicated to the singular purpose of ensuring that the cell obtains an adequate supply of P_i for growth under adverse conditions (33, 36, 41). The enhanced activity of the binding-protein-dependent transport system and of alkaline phosphatase in cells of *A. johnsonii* 210A under P_i limitation suggests the presence of a similar regulatory mechanism to scavenge the last traces of P_i and phosphorus-containing nutrients from the surrounding medium.

In *A. johnsonii* 210A regulation of expression of the high-affinity P_i uptake apparently takes place via a mechanism of (de)repression of protein synthesis as described in *E. coli*. The ability of *A. johnsonii* 210A to accumulate polyphosphate

during logarithmic growth may result in P_i uptake characteristics which differ from those of other microorganisms. In *E. coli* (27) and *Bacillus cereus* (38), the uptake of P_i in time is biphasic: an initial high rate is followed by a lower one. This biphasic kinetics was explained by the presence of two transport systems, of which the primary transport system is subjected to inhibition when the primary pool of P_i within the cells is filled up. Since phosphate bond-driven transport systems are essentially unidirectional and can catalyze the uptake of solutes to much higher accumulation levels than secondary transport systems, *trans*-inhibition acts as a regulatory device to prevent solute accumulation to unacceptably high internal levels. This type of regulation of transport activity, usually not found for secondary transport systems, has been described for the major potassium transport system of *Enterococcus faecalis* (6), the potassium transport systems TrkA and Kup of *E. coli* (34), and the P_i uptake system of *L. lactis* (31). In contrast to the biphasic uptake of P_i in *E. coli* and *B. cereus*, monophasic uptake of P_i is maintained in *A. johnsonii* 210A. P_i uptake is linear in time until a maximal level is reached. However, the bulk of P_i that is taken up is rapidly metabolized and most likely incorporated into polyphosphate in low- and high-P_i-grown cells. As a result of this incorporation into a polymer, a low intracellular P_i concentration can be maintained. It is very likely that the free-P_i concentration is too low to result in *trans*-inhibition of the ATP-dependent high-affinity uptake system, allowing the organism to efficiently take up large amounts of P_i.

The results in this paper allow the following conclusions to be drawn: (i) *A. johnsonii* 210A is able to reduce the P_i concentration in its environment to micromolar levels (or lower) because of the presence of an inducible high-affinity P_i uptake system in combination with its ability to synthesize and accumulate polyphosphate, and (ii) P_i efflux is mediated by a low-affinity secondary transport system. The secondary transport system could be involved in the anaerobic energy metabolism of *A. johnsonii* 210A. Besides a conservation of metabolic energy liberated from the cleavage of polyphosphate via a direct enzymatic synthesis of ATP, metabolic energy could additionally be conserved by the generation of an electrochemical ion gradient across the cytoplasmic membrane, when P_i is excreted together with ions.

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3

Mechanism and energetics of the secondary phosphate transport system in *Acinetobacter johnsonii* 210A

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Summary

The mechanism and energetics of the secondary P_i transport system of *A. johnsonii* were studied in membrane vesicles and proteoliposomes in which the transport protein was functionally reconstituted. P_i uptake is strictly dependent on the presence of divalent cations, like Mg^{2+}, Ca^{2+}, Mn^{2+}, or Co^{2+}. These cations form a $MeHPO_4$ complex with up to 87% of the P_i present in the incubation mixture, suggesting that divalent cations and P_i are co-transported via a metal phosphate chelate. Metal phosphate uptake is driven by the proton motive force (interior negative and alkaline). The metal phosphate/proton stoichiometry was close to unity. The transport system mediates efflux and homologous exchange of metal phosphate, but not heterologous exchange of metal phosphate and glycerol-3-P or glucose-6-P. Exchange and counterflow were essentially pH-independent while efflux and uptake increased with increasing pH. Efflux was inhibited by the proton motive force, whereas exchange was inhibited by the membrane potential only. These observations are consistent with an ordered mechanism for binding and dissociation of metal phosphate and proton to and from the carrier protein and point to the recycling of a positively charged, protonated carrier protein during exchange.

Introduction

Acinetobacter johnsonii 210A is able to accumulate an excessive amount of phosphate as polyphosphate under aerobic conditions. When proton motive force (Δp_H+)-driven ATP synthesis is impaired (*e.g.*, in the absence of oxygen or an electron donor), polyphosphate is degraded and P_i is released into the medium. The organism possesses a high-affinity P_i transport system which is ATP- and binding protein-dependent. Derepression of its activity in response to starvation for P_i enables the organism to reduce the P_i concentration in the aerobic environment to nanomolar concentrations. A constitutive low-affinity secondary transport system mediates the efflux of P_i under anaerobic conditions (38). Recently, the presence of two P_i uptake systems was also demonstrated in *Acinetobacter lwoffi* (41). The P_i transport systems in *A. johnsonii* and *A. lwoffi* show analogy with the two major P_i

transport systems in *Escherichia coli*, designated Pst (for phosphate-specific transport) and Pit (for P_i transport) (26, 40).

E. coli contains a set of at least four transport systems, each of which carries P_i into the cell. The Pst and Pit systems are highly specific for P_i, while the remaining two accept P_i as a low-affinity analog of glycerol-3-P (GlpT) or glucose-6-P (UhpT). The Pst system is known to be an inducible solute ATPase that acts in conjunction with specific periplasmic binding proteins to accumulate P_i at the expense of ATP (5, 10, 27). The other three transport systems are chemiosmotic carriers. The GlpT and UhpT systems are induced upon addition of the phosphorylated substrate to the growth medium and mediate exchange of P_i, the phosphorylated substrate, or both. P_i-linked exchange carriers of *E. coli*, *Lactococcus lactis* and *Staphylococcus aureus* have been well characterized in cells, membrane vesicles and in reconstituted systems (1, 3, 9, 21, 24, 31, 32). On the other hand, the Pit system of *E. coli* has not been studied extensively. P_i uptake studies in Pst-deficient strains and in membrane vesicles of wild-type cells revealed the absolute requirement of the Δp_H+ for the energization of this constitutive system (18, 27).

In view of the current descriptions of secondary P_i transport systems and the apparent similarities between P_i uptake in *E. coli* and *Acinetobacter*, it was of interest to study the energetics and mechanism of the secondary P_i transport system of *A. johnsonii* 210A. Experiments in natural and artificial membranes indicate an electrogenic symport mechanism of a metal phosphate chelate and a proton. On the basis of effects of pH and membrane potential on the different modes of facilitated diffusion processes, a kinetic scheme of the translocation cycle of metal phosphate/metal phosphate exchange and metal phosphate/proton symport is proposed.

Materials and methods

Preparation of membrane vesicles

The method for the preparation of membrane vesicles was obtained after modification of protocols, originally developed for *Pseudomonas aeruginosa* (11, 33) and *Acinetobacter calcoaceticus* (37). *A. johnsonii* 210A was grown at 30 °C in a

synthetic Tris-buffered medium (pH 7.0) containing 5 mM sodium phosphate and 20 mM sodium butyrate as sole carbon and energy source (38). Cells were harvested in midexponential phase by centrifugation at 20 °C and immediately suspended at 20 °C to an A_{660} of about 10 in 10 mM potassium Pipes (pH 7.0), 0.75 M sucrose, 10 mM $MgSO_4$, 2.5% (wt/vol) lithium-chloride, and 50 μg of chloramphenicol/ml. After addition of 1 mg of lysozyme/ml, the cell suspension was chilled for 15 min to 2 °C in an ice-bath, then warmed up for 5 min to 30 °C in a water bath of 40 °C, and subsequently incubated at 30 °C for 30 min with gentle shaking. This treatment turned cells into spheroplasts, which were collected by centrifugation (3,500 x *g* for 15 min) at 20 °C and lysed at 30 °C by dilution of the cell pellet into a 50-fold volume of (prewarmed) 10 mM potassium Pipes (pH 7.0) containing 1 mM $MgSO_4$, 1 mM dithiothreitol, 10 μg of deoxyribonuclease/ml, and 10 μg of ribonuclease/ml. The suspension was incubated at 30 °C for 30 min and then centrifuged (10,000 x *g* for 60 min) at 4 °C. The pellet was resuspended in ice-cold 50 mM potassium Pipes (pH 7.0) containing 10 mM $MgSO_4$. Intact cells and cell debris were removed by centrifugation at 4,500 x *g* for 6 min. The supernatant was carefully decanted, after which this centrifugation step was repeated. The supernatant fluid was centrifuged (30,000 x *g* for 40 min) at 4 °C. Membrane vesicles were suspended at a final concentration of about 10 mg of membrane protein/ml in 50 mM potassium Pipes (pH 7.0) containing 10 mM $MgSO_4$, and rapidly frozen and stored under nitrogen.

Solubilization and reconstitution

Thawed membrane vesicles (10 mg of membrane protein) were suspended for solubilization in 50 mM potassium Pipes (pH 7.0) to which subsequently was added 20% (vol/vol) glycerol (2), 1 mM dithiothreitol, 100 mg of acetone-ether-washed phospholipids [a mixture of *E. coli* phosphatidylethanolamine and egg phosphatidylcholine (3:1, wt/wt) in 50 mM potassium Pipes (pH 7.0) containing 4% (wt/vol) *n*-octyl-ß-glucopyranoside (12)], and additional octylglucoside (25) to 1.60% (wt/vol) in a final volume of 10 ml. After incubation for 30 min on ice, the suspension was clarified by centrifugation (110,000 x *g* for 3 h) at 4 °C. The detergent

extract was rapidly frozen and stored under nitrogen. For reconstitution, a sample of the clarified extract containing 1 mg of protein was diluted to an octyl glucoside concentration of 1.25% (wt/vol) in 4.5 ml (final volume) of 50 mM potassium Pipes (pH 7.0) containing 20% (vol/vol) glycerol and 100 mg of phospholipids (in 4% octyl glucoside). Proteoliposomes were formed by a 40-fold dilution into 20 mM potassium Pipes (pH 7.0) containing 100 mM potassium acetate. Proteoliposomes were pelleted by centrifugation (110,000 x *g* for 3 h) at 4 °C, resuspended to about 1.5 mg of protein/ml in 20 mM potassium Pipes (pH 7.0) containing 100 mM potassium acetate, and rapidly frozen and stored under nitrogen.

Transport assays

Δp_H+-driven uptake. P_i uptake in membrane vesicles, driven by a Δp_H+ generated by the oxidation of glucose via the membrane-bound glucose dehydrogenase, was assayed at 30 °C. Membrane vesicles were diluted to a final protein concentration of about 0.1-0.5 mg protein/ml in air-saturated 50 mM potassium Pipes (pH 7.0) containing 10 mM $MgSO_4$ or in 20 mM potassium Mes, 20 mM potassium Pipes, 20 mM potassium Hepes (pH 5.0-8.0) containing 10 mM $MgSO_4$ (MPH buffer). Membrane vesicles were preincubated for 3 min with 2 μM PQQ after which 20 mM glucose was added. The incubation mixture was kept under continuous aeration. Transport was initiated upon addition of ^{32}P-labeled potassium phosphate (1.7 TBq/mol) at concentrations as indicated in the legends to the figures. At given time intervals, samples were withdrawn, diluted with 2 ml of ice-cold 0.1 M lithium-chloride, filtered immediately through cellulose nitrate filters (0.45 μm pore size), and washed once with 2 ml of 0.1 M lithium chloride. Radioactivity was measured by liquid scintillation spectrometry. To remove contaminating P_i, the glassware used for $^{32}P_i$ transport assays was kept in chromic acid and rinsed 15 times with distilled water before use. ^{32}P-labeled potassium phosphate was filtered through a cellulose nitrate filter prior to use in order to remove $^{32}P_i$ adsorbed to particles (22). For calculations, a specific internal volume of 3 μl/mg of protein was used (13, 31). Transport data were corrected for binding of P_i to the nitrocellulose filters.

Imposed Δp_H+-driven uptake. For P_i uptake driven by artificially imposed diffusion gradients, proteoliposomes in 20 mM potassium Pipes (pH 7.0) containing 100 mM potassium acetate were diluted 100-fold into the same buffer (no gradient), into 20 mM potassium Pipes (pH 7.0) containing 100 mM potassium Mes to generate a ΔpH (interior alkaline) or into 20 mM sodium Pipes (pH 7.0) containing 100 mM sodium acetate to generate a transmembrane chemical Na^+ gradient. To generate a $\Delta\psi$ (interior negative), proteoliposomes were incubated in the presence of 1 nmol valinomycin/mg of protein, and diluted 100-fold into 20 mM *N*-methyl-*o*-glucamine-Pipes (pH 7.0) containing 100 mM *N*-methyl-*o*-glucamine acetate. For generation of a Δp_H+ (interior negative and alkaline) in the absence or presence of a chemical Na^+ gradient, the properly oriented potassium, sodium and acetate-diffusion gradients were combined. In all cases, the dilution media were supplemented with ^{32}P-labeled potassium phosphate (1.7 TBq/mol) and cations as specified in the text and in the legends to the figures. Uptake was assayed at 30 °C by filtration as described above.

Counterflow. Membrane vesicles in MPH (pH 6.0, 6.8 and 7.7) containing 20 μM CCCP, were equilibrated for 3 h at 20 °C in the presence of 5 mM potassium phosphate. Control membrane vesicles were incubated in buffer without potassium phosphate. Samples of 4 μl were diluted 100-fold to a final protein concentration of about 0.25 mg/ml in MPH buffer of the indicated pH containing 20 μM CCCP and ^{32}P-labeled potassium phosphate at a final concentration of 50 μM (0.8 TBq/mol). The uptake of radiolabeled P_i was followed in time at 30 °C and assayed by filtration as described under Δp_H+-driven uptake.

Efflux and exchange. Membrane vesicles in MPH (pH 6.0, 6.8 and 7.7) containing 20 μM CCCP were equilibrated for 3 h at 20 °C in the presence of 600 μM ^{32}P-labeled potassium phosphate (1.7 TBq/mol) and diluted 100-fold into buffer without (efflux) or with 600 μM potassium phosphate (equilibrium exchange). For efflux and exchange in the presence of artificially imposed diffusion gradients, proteoliposomes in 20 mM potassium Pipes (pH 7.2) containing 100 mM potassium acetate were equilibrated for 3 h at 20 °C in the presence of 500 μM ^{32}P-labeled potassium phosphate (1.7 TBq/mol) and 10 mM $MgSO_4$. Subsequently, loaded proteoliposomes were diluted 100-fold into buffers (pH 7.2) containing 10 mM

$MgSO_4$ without or with 500 μM sodium phosphate, as described under "Imposed Δp_H+-driven uptake". The release of radiolabeled P_i was followed in time at 30 °C and assayed by filtration as described under "Δp_H+-driven uptake".

Determination of Δp_H+

The $\Delta\psi$ (interior negative) in membrane vesicles and proteoliposomes was determined from the distribution of the lipophilic cation TPP^+, using a TPP^+-selective electrode (29). The $\Delta\psi$ was calculated from the steady state level of TPP^+ accumulation and was corrected for nonspecific probe binding to the membranes (19). Membrane vesicles were supplied with metabolic energy by glucose oxidation as described under "Δp_H+-driven uptake". The ΔpH in membrane vesicles was calculated from the increase in $\Delta\psi$ upon the addition of nigericin, assuming a complete interconversion of the ΔpH into the $\Delta\psi$ (8).

Miscellaneous

The method of Lowry et al. (20) was used for the determination of the amount of protein in membrane vesicles. The protein concentration in detergent extracts and proteoliposomes was determined in a bicinchoninic acid protein assay (30) in the presence of 0.2% (wt/vol) SDS. Bovine serum albumin was used as the standard. *E. coli* phosphatidylethanolamine was aceton/ether-washed as described (16, 39).

Chemicals

Cellulose nitrate filters (0.45 μm pore-size) were supplied by Schleicher und Schuell, Dassel, Germany. Radiolabeled phosphate (carrier-free) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, UK. Crude *E. coli* phosphatidylethanolamine and a Bicinchoninic Acid Protein Assay Kit were obtained from Sigma Chemicals Co., St. Louis, MO, USA. Other chemicals were reagent grade and obtained from commercial sources.

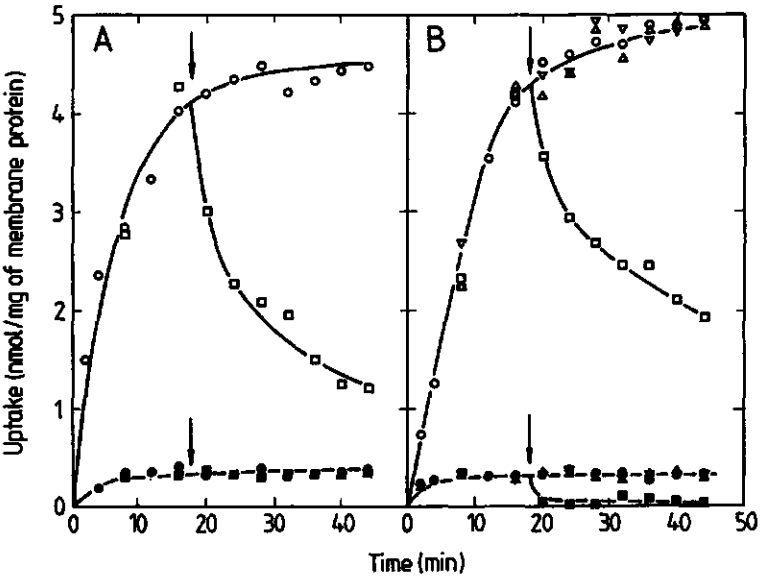

Figure 1. P_i efflux and exchange in membrane vesicles. Uptake of 50 μM $^{32}P_i$ in membrane vesicles in the absence (●) and presence (○) of glucose/PQQ. A, P_i efflux, 20 μM CCCP was added at the *arrow* (■,□). B, P_i exchange: 10 mM unlabeled P_i (■,□), glycerol-3-P (▲,△), or glucose-6-P (▼,▽) was added at the *arrow*.

Results

P_i transport in membrane vesicles

Membrane vesicles from *A. johnsonii* 210A were prepared by osmotic lysis of cells exposed to lithium chloride, a high concentration of lysozyme and a temperature shock. The membrane vesicles contain a PQQ-dependent glucose dehydrogenase which is functionally linked to the respiratory chain (38). In the presence of PQQ and 10 mM Mg^{2+}, the oxidation of glucose at pH 7.0 resulted in the generation of a $\Delta\psi$, inside negative, of -98 mV and a $-Z\Delta$pH, inside alkaline, of -12 mV. This Δp_H+ can drive the uptake of P_i. A steady-state level of P_i accumulation ($[P_i]_{in}/[P_i]_{out}$) of about 30 was reached in 40 min. Dissipation of the Δp_H+ by protonophore CCCP resulted in a rapid efflux of previously accumulated P_i (Fig. 1A).

Efflux of P_i was also observed upon addition of excess unlabeled P_i to membrane vesicles which had accumulated $^{32}P_i$ via Δp_H+-driven uptake (Fig. 1B). This homologous P_i:P_i exchange was also detected in intact cells of *A. johnsonii* 210A in counterflow experiments (38). In contrast, addition of excess unlabeled glycerol-3-P or glucose-6-P did not result in efflux of previously accumulated $^{32}P_i$, indicating the lack of heterologous P_i:glycerol-3-P or P_i:glucose-6-P exchange.

Kinetic analysis of P_i transport

The rate of Δp_H+-driven uptake of P_i in membrane vesicles was linear for at least 60 s in the range of 1 to 300 μM P_i (not shown). At pH 7.0, the apparent K_t of this uptake was 10.4 μM and the V_{max} reached 0.43 nmol/min/mg of membrane protein. The apparent K_t corresponds well with that of the secondary P_i transport system in cells of *A. johnsonii* 210A (38). When the pH was lowered from 8.0 to 5.5 the K_t increased almost 3-fold whereas V_{max} fell at least 10-fold (Fig. 2). The Δp_H+ remained nearly constant which indicates the influence of other parameters on P_i uptake. Kinetic analysis of facilitated diffusion of P_i in the absence of a Δp_H+ at pH 7.0, yielded a K_t of 8.1 μM, which is nearly identical to the K_t value found for Δp_H+-driven transport. The V_{max} was significantly lower under these conditions (0.08 nmol/min/mg of membrane protein).

Dependency on divalent cations

Divalent cations have been shown to be required for the binding of PQQ to *apo*-glucose dehydrogenase in *A. lwoffi* and other bacteria (36) and may influence the magnitude or composition of the Δp_H+ generated by glucose oxidation in membrane vesicles. Therefore, the effect of metal ions on P_i transport was studied in proteoliposomes in which the P_i carrier of *A. johnsonii* 210A was functionally reconstituted. The magnitude and stability of an artificially imposed Δp_H+ of -240 mV is not affected by the presence of 2 mM of Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, or 0.5 mM of EDTA as was concluded from direct measurements of the $\Delta\psi$ (not shown). The addition of 0.5 mM EDTA reduced imposed Δp_H+-driven uptake of P_i in proteo-

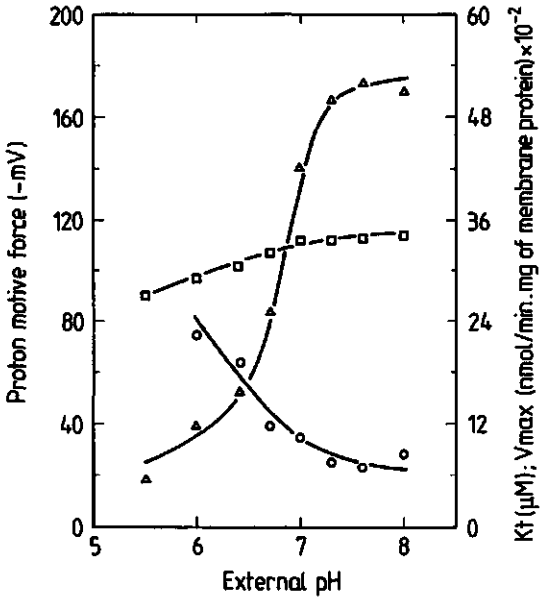

Figure 2. Effect of pH on the Δp_H+ and the kinetic parameters of Δp_H+-driven uptake of P_i in membrane vesicles. Initial velocities were determined over the first 60 s, at a P_i concentration between 1 and 300 μM. The kinetic parameters of P_i uptake, K_t (○) and V_{max} (△), were determined via linear regression analysis of Lineweaver-Burk plots. The Δp_H+ (□) was measured under conditions similar to those in P_i uptake experiments.

liposomes to equilibration levels, whereas P_i uptake was strongly stimulated in the presence of 2 mM of various divalent cations (Fig. 3). These divalent cations form a stable, soluble, electroneutral metal phosphate complex ($MeHPO_4$) with P_i. From the stability constants (28) it can be calculated that at pH 7.0, 31% (Ca^{2+}) to 87% (Mn^{2+}) of the P_i in the uptake assay is in the metal phosphate form. The extensive complexation of P_i into metal phosphate chelates together with the strict metal dependency of P_i uptake suggest that a metal phosphate complex is transported via the secondary transport system rather than P_i. Unless indicated otherwise, all further P_i transport measurements in proteoliposomes and membrane vesicles were done with 10 mM Mg^{2+} in the incubation mixture.

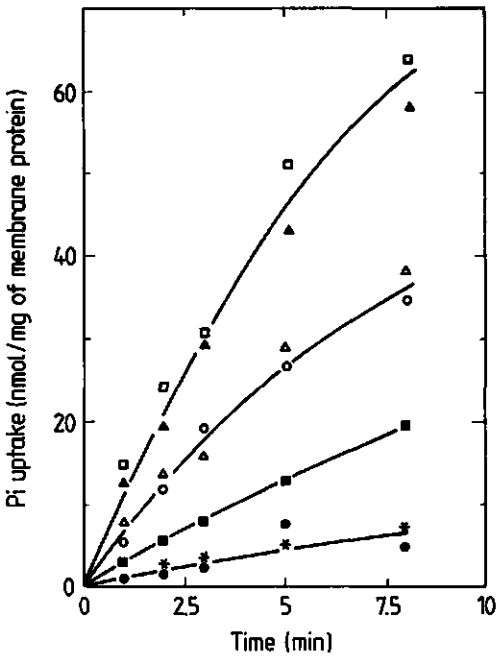

Figure 3. P_i transport in proteoliposomes is dependent on the presence of divalent cations. Δp_H+-driven uptake of 50 μM $^{32}P_i$ was measured in proteoliposomes in the presence of 2 mM of Mg^{2+} (○), Ca^{2+} (△), Co^{2+} (▲), Mn^{2+} (□), 0.5 mM of EDTA (●), or in the absence of added divalent cations (■) or a Δp_H+ (✻).

Mechanism of energy coupling

The relation between the magnitude and composition of the Δp_H+, and the uptake of metal phosphate was studied. The selective dissipation of the $\Delta\psi$ and ΔpH by valinomycin and nigericin, respectively, resulted in a significant inhibition of the P_i uptake rate and P_i accumulation level in membrane vesicles. In the presence of both ionophores, nonenergized P_i uptake rates were observed (data not shown). To verify that both components of the Δp_H+ function as the driving force for metal phosphate uptake, proteoliposomes were subjected to artificial gradients of protons, potassium ions, sodium ions, or combinations thereof.

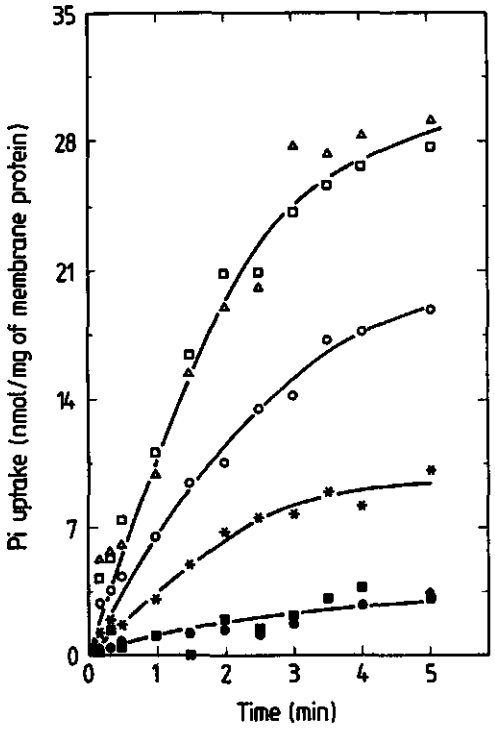

Figure 4. Effect of artificially imposed ion gradients on P_i transport in proteoliposomes. The uptake of 50 μM $^{32}P_i$ was measured in the absence (●) and in the presence of a $\Delta\psi$ (interior negative) (*), ΔpH (interior alkaline) (○), chemical Na^+ gradient (■), Δp_H+ (△), and a Δp_H+ and chemical Na^+ gradient (□).

Figure 4 shows the effects of these established gradients on P_i uptake. Accumulation of P_i could be detected upon imposition of a $\Delta\psi$ of -120 mV or a -$Z\Delta$pH of -120 mV. In the presence of both gradients, the highest initial rates of P_i uptake and the highest P_i accumulation levels were obtained. The effects of $\Delta\psi$ and ΔpH were additive. The presence of a chemical Na^+ gradient (ΔpNa of -120 mV) on top of a Δp_H+ (-240 mV) did not affect the uptake of P_i. In the absence of any gradient or in the presence of a ΔpNa, no P_i uptake was observed.

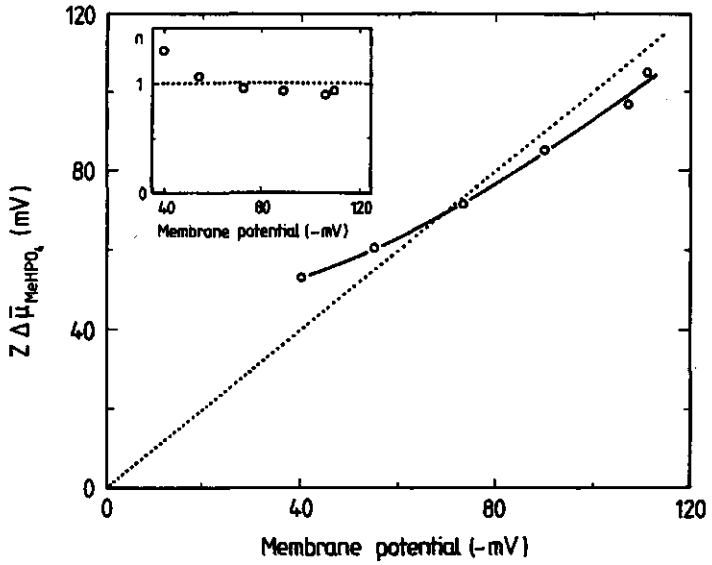

Figure 5. Metal phosphate/proton stoichiometry in membrane vesicles. Upon addition of 50 μM $^{32}P_i$, accumulation levels of $\Delta\psi$-driven metal phosphate uptake were determined after 35 min of incubation (pH 7.8). The $\Delta\psi$ was generated by glucose oxidation in the presence of 0.1 nmol of nigericin/mg of membrane protein and was varied by a titration with 0.01 to 0.08 nmol valinomycin/mg of membrane protein. Calculated values for n (number of protons symported with a metal phosphate) are presented in the *inset*. The *dashed line* represents $n = 1$.

Metal phosphate/proton stoichiometry

The metal phosphate/proton stoichiometry of the secondary transport system was determined in membrane vesicles at pH 7.8 from the steady state accumulation level of P_i in the presence of a $\Delta\psi$ only, by performing the studies in the presence of 0.1 nmol nigericin/mg of membrane protein ($\Delta p_H{}^+ = \Delta\psi$). Under the experimental conditions, 85% of the P_i was present in a magnesium-phosphate complex. The $\Delta\psi$ was varied by titration with valinomycin and was measured simultaneously with P_i uptake. If the metal phosphate complex is translocated with $n\mathrm{H}^+$ at thermodynamic equilibrium, $Z\Delta\bar{u}_{MeHPO4}$ equals $-n\Delta\psi + nZ\Delta\mathrm{pH}$, in which $Z\Delta\bar{u}_{MeHPO4}$ represents the

metal phosphate complex concentration gradient (in mV) and n the number of protons translocated in symport with metal phosphate. In the absence of a ΔpH, n equals $-Z\Delta\bar{u}_{MeHPO4}/\Delta\psi$. Figure 5 shows the existence of a linear relationship between the steady state accumulation level of metal phosphate and the $\Delta\psi$. This relationship indicates that the metal phosphate/proton stoichiometry is 1 and that metal phosphate is symported with 1 H^+ (inset to Fig. 5).

pH Dependency of facilitated diffusion of metal phosphate

The effects of pH on metal phosphate efflux, equilibrium exchange and counterflow were examined in membrane vesicles under conditions in which Δp was 0 (short-circuited by 20 μM concentration of the protonophore CCCP). Membrane vesicles were equilibrated with 600 μM $^{32}P_i$, a concentration considerably higher than the K_t for influx, and then diluted rapidly 100-fold into a medium free of P_i (efflux) or with 600 μM $^{31}P_i$ (exchange). P_i efflux and exchange occurred with pseudo-first order kinetics (Fig. 6). At pH values below 7.7, exchange rates were faster than efflux rates. The P_i efflux rates increased with increasing pH while the rate of P_i exchange exhibited virtually no pH dependence. These results were confirmed in counterflow experiments in which membrane vesicles were equilibrated with 5 mM unlabeled P_i and subsequently diluted 100-fold into a buffer containing a final concentration of 50 μM $^{32}P_i$. By this means an outwardly directed P_i gradient was imposed and rapid exchange and efflux occurred in which the initial phase caused a transient accumulation of $^{32}P_i$ (Fig. 7). The initial rate of $^{32}P_i$ uptake was essentially independent of pH, whereas the rate of decay after reaching the maximal uptake level displayed a pH dependency similar to that observed for P_i efflux. The results imply that for efflux of P_i the release of a H^+ at the outer surface of the membrane is rate-determining whereas in the exchange process no release of H^+ is needed.

Effect of the proton motive force on efflux and exchange of metal phosphate

In order to impose a membrane potential and/or a ΔpH during P_i efflux and exchange, proteoliposomes loaded with 500 μM $^{32}P_i$ were diluted rapidly 100-fold into

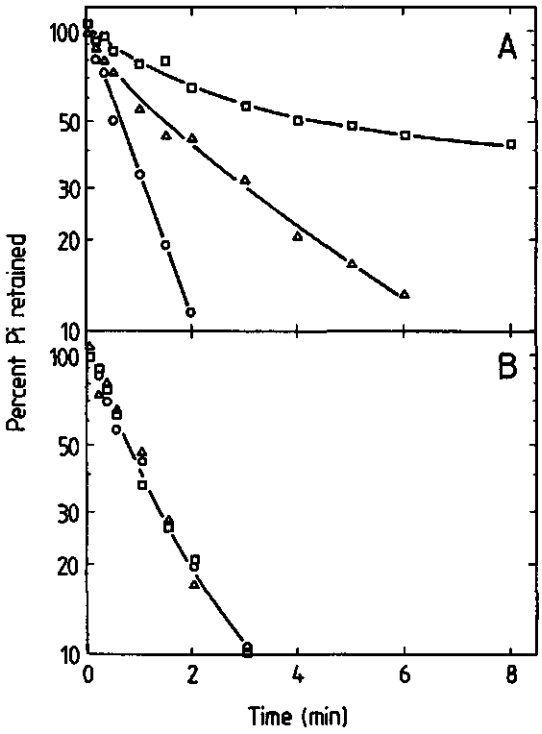

Figure 6. Effect of pH on P_i efflux (*panel A*) and exchange (*panel B*) in membrane vesicles. Membrane vesicles were loaded at pH 6.0 (□), pH 6.8 (△), or pH 7.7 (○) with 600 μM of $^{32}P_i$ and subsequently diluted 100-fold into buffer without (efflux) or with 600 μM P_i (exchange).

the appropriate buffers without P_i (efflux) or with 500 μM unlabeled P_i (exchange). P_i efflux was retarded by a ΔpH and a membrane potential as would be expected for an electrogenic H^+-symport mechanism (Fig. 8, *panel A*). Moreover, when both a $\Delta\psi$ (interior negative) and a ΔpH (interior alkaline) are imposed concurrently, leading to the generation of a Δp_H+, the rate of P_i efflux was diminished even further indicating that the effects of $\Delta\psi$ and ΔpH are additive. P_i efflux was stimulated by the addition of the protonophore CCCP. P_i exchange was not affected by the imposition of a ΔpH (interior alkaline) or by the addition of CCCP, but was retarded in the presence of a $\Delta\psi$ (interior negative) (Fig. 8, *panel B*). These results

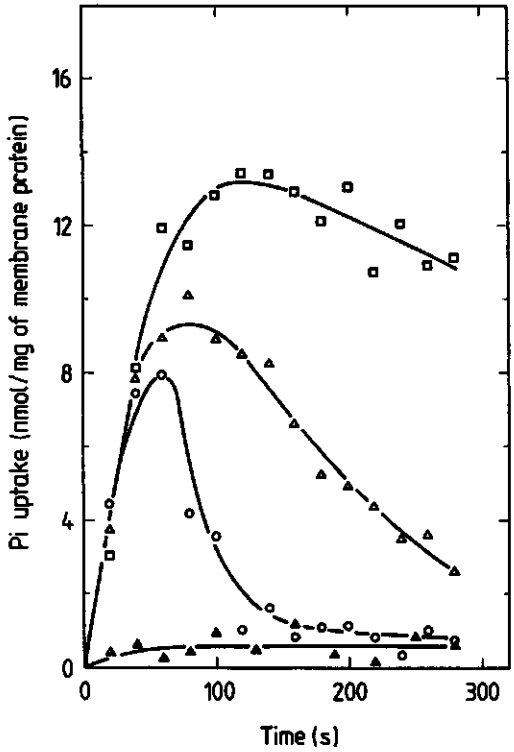

Figure 7. Effect of pH on P_i counterflow in membrane vesicles. The uptake of 50 μM $^{32}P_i$ via counterflow was monitored in membrane vesicles equilibrated with 5 mM P_i at pH 6.0 (□), pH 6.8 (△), or pH 7.7 (○). Control uptake in unloaded membrane vesicles at pH 6.0 and 7.7 was similar to that at pH 7.0 (▲).

are consistent with a translocation cycle for metal phosphate efflux in which positive charge moves to the outside during reorientation of the ternary carrier/proton/metal phosphate complex.

Discussion

The kinetic mechanism of the secondary P_i transport system of *A. johnsonii* 210A was studied. In membrane vesicles the uptake of P_i at pH 7.0 in the presence or absence of a Δp_{H^+} exhibited an apparent K_t of 10.4 μM and 8.1 μM P_i, respectively.

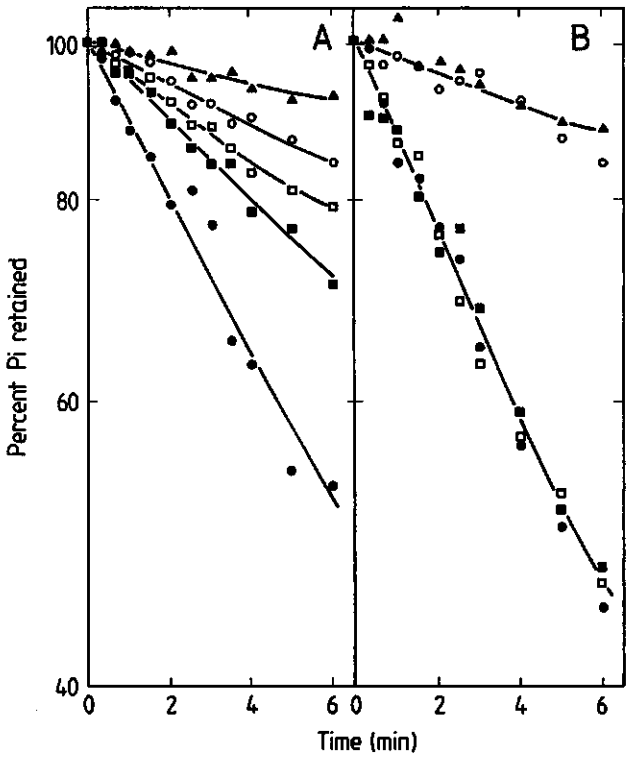

Figure 8. Effect of Δp_H+ and CCCP on P_i efflux (*panel A*) and exchange (*panel B*) in proteoliposomes. Proteoliposomes containing 500 μM of $^{32}P_i$ were diluted 100-fold into buffers (pH 7.2) without (efflux) or with 500 μM P_i (exchange). Efflux and exchange were studied in the presence of a $\Delta\psi$ (interior negative) (○), a ΔpH (interior alkaline) (□), a Δp_H+ (▲) and in the absence of imposed diffusion gradients with (●) or without (■) 20 μM of CCCP.

This K_t corresponds well with a K_t of 9 μM previously determined for the secondary P_i transport system in low-P_i-grown cells (38). These membrane vesicles contain a PQQ-dependent glucose dehydrogenase. In the presence of PQQ and Mg^{2+}, the oxidation of glucose resulted in the generation of a Δp_H+ in the presence of which the apparent V_{max} of P_i uptake was increased more than 5-fold.

The strict dependency of P_i uptake on divalent cations and the extensive com-

plexation of P_i to divalent metal ions point to the translocation of a metal phosphate complex rather than P_i via the secondary transport system of *A. johnsonii* 210A. The transport of citrate in membrane vesicles of *B. subtilis* is similarly affected by the addition of divalent cations or EDTA. These results were explained by the translocation of a metal citrate chelate which is formed upon addition of divalent cations to citrate (4). The substrate specificity of the secondary phosphate transport system of *A. johnsonii* 210A offers an explanation for the apparent pH dependency of the K_t for P_i. Under the experimental conditions employed in the kinetic experiments, 30% of P_i is present as a neutral $MgHPO_4$ complex at pH 6.0, 74% at pH 7.0, and 86% at pH 8.0 (28). A re-evaluation of the kinetic data reveals a corrected K_t for $MgHPO_4$ of 7.9 μM which is independent of pH. The higher P_i uptake rate in the presence of Mn^{2+} compared to that in the presence of Mg^{2+} at pH 7.0 (Fig. 3) can be explained by the difference in the metal phosphate concentration during uptake. Co-transport of P_i and divalent metal ions in *A. johnsonii* is consistent with (i) the accumulation of higher amounts of Mg^{2+} in cells when P_i is present in the medium (35) and (ii) the variable metal composition of polyphosphate granules which is related to relative concentration of Mg^{2+} and Ca^{2+} in the growth medium (6).

Selective manipulation of the components of the proton motive force in membrane vesicles and artificial imposition of diffusion gradients in proteoliposomes revealed that both a membrane potential and a pH gradient can drive the uptake of P_i. Determination of the metal phosphate/proton stoichiometry suggests the translocation of a (neutral) metal phosphate together with one proton via an electrogenic mechanism. Together with the observations discussed above and the absence of heterologous exchange of (metal)phosphate and glycerol-3-P or glucose-6-P, these results exclude an electroneutral anion exchange mechanism similar to that mediated by the GlpT or UhpT system of *E. coli*.

Dissipation of the Δp_H+ by CCCP resulted in the efflux of previously accumulated P_i from membrane vesicles. The stimulation of P_i efflux by the addition of an uncoupler has also been observed in cells of *A. johnsonii* 210A (34). For efflux of metal phosphate down a concentration gradient, a proton and a metal phosphate have to be bound by the carrier protein on the inside, and both have to be released

on the outside. P_i efflux was measured at a saturating metal phosphate concentration on the inside of the membrane. The retardation of P_i efflux by a ΔpH (interior alkaline) and/or a $\Delta\psi$ (interior negative) is consistent with an electrogenic H^+-symport mechanism. Under conditions that the internal and external pH are equal (in the presence of CCCP), the rate of P_i efflux increased with increasing pH. A similar pH effect has been observed on P_i efflux in cells of *A. johnsonii* 210A[1]. When saturating amounts of metal phosphate are present both on the inside and the outside (*e.g.* exchange and counterflow), rates of transport are essentially independent of pH and faster than the rates of efflux. The results indicate that the P_i efflux rate is limited by deprotonation of the carrier protein on the outer surface of the membrane and that the carrier recycles in a protonated form during exchange and the initial events of counterflow. The reactions involved in the translocation are schematically represented (Fig. 9).

During efflux, the ternary carrier/proton/metal phosphate complex is formed through the sequential binding of a proton and a metal phosphate to the unloaded carrier on the inner surface of the membrane. The ternary complex reorients its binding sites to the outer surface after which metal phosphate is released first from the carrier, followed by the loss of a proton. The unloaded carrier reorients its binding sites to the inner surface of the membrane in order to bind another proton and metal phosphate. During metal phosphate exchange the carrier recycles via the ternary carrier/proton/metal phosphate complex without being deprotonated. The inhibition of metal phosphate exchange by the membrane potential (inside negative) is in accordance with the recycling of a protonated, positively charged carrier protein during exchange and counterflow.

Deprotonation of the carrier protein on the outer surface of the membrane is one of the rate-limiting steps in the efflux of metal phosphate. Under conditions of uptake, the release of metal phosphate and a proton occurs at the inner surface of the membrane. Similar to effects of external pH on metal phosphate efflux, internal pH effects on metal phosphate uptake may be anticipated. The decrease of the V_{max} of metal phosphate uptake at low pH can be explained by the drop in the internal

[1]Van Veen HW and Kortstsee GJJ, unpublished observation.

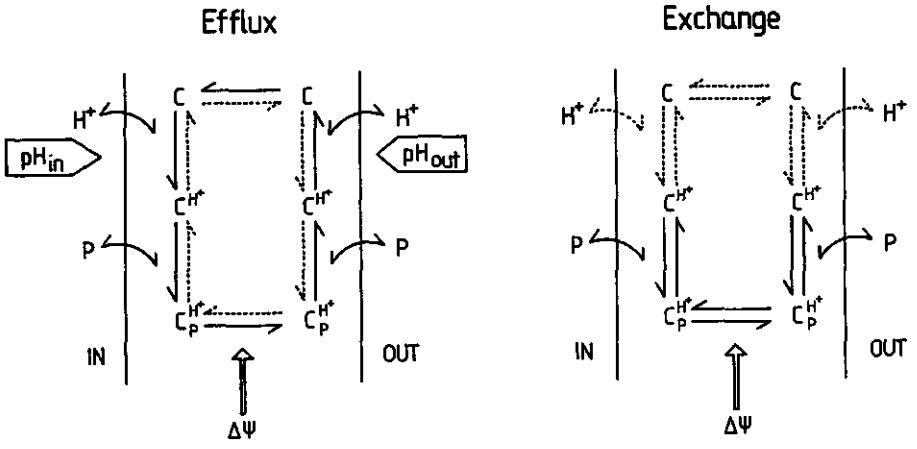

Figure 9. Schematic representation of reactions involved in metal phosphate efflux and exchange. The model consists of a single transport loop linking six discrete states of the $MeHPO_4$ carrier. The state transitions include one transmembrane charge-transport step, and one step each for binding of $MeHPO_4$ and proton at each side of the membrane. In the model an ordered mechanism for binding and dissociation of metal phosphate and proton to and from the carrier protein is suggested. The carrier protein is indicated by *C*, $MeHPO_4$ by *P*, and protons by H^+. *Solid* and *dashed arrows* indicate the major and minor steps (in terms of rates), respectively, involved in the efflux and exchange reactions. The internal and external pH have influence upon the (de)protonation of the carrier protein. The reorientation of the binding sites of the positively charged ternary carrier/H^+/P complex is affected by the $\Delta\psi$.

pH from 8.0 to 6.1 when the external pH was lowered from 8.0 to 5.5.

The pH dependence of metal phosphate transport via the secondary P_i transport system of *A. johnsonii* 210A shows analogies with the pH dependence of transport of lactose via the LacY system of *E. coli* (14, 15) and of L-leucine in *S. cremoris* (7). Like the secondary P_i transport system, these carriers catalyse symport of substrate and a proton via an electrogenic mechanism. However, the exchange mediated by these carriers is unaffected by membrane potential (interior negative), whereas metal phosphate exchange is inhibited under these conditions. In the case of the lactose and L-leucine carriers a kinetic scheme was put forward in which the membrane potential (interior negative) drives the reorientation of a negatively

charged unloaded carrier from the inner to the outer membrane surface during $\Delta\psi$-driven uptake of substrate. In metal phosphate transport, the $\Delta\psi$ most likely drives the inward movement of a positively charged carrier/proton/metal phosphate complex.

The question arises whether the Pit system of *E. coli* operates in a similar way as the secondary transport system discussed in this work. Although conflicting results and conclusions have been reported in literature, Konings and Rosenberg (18) have observed a stimulation of Δp_H+-driven uptake of P_i via Pit in the presence of Mg^{2+}. Our preliminary results suggest that metal phosphates are indeed translocated via the Pit system of *E. coli* (see Chapter 4 for detailed information).

In conclusion, the secondary P_i transport system of *A. johnsonii* 210A catalyzes the electrogenic uptake and efflux of a metal phosphate chelate and a proton. During uptake, internal metal phosphate is polymerized into metal polyphosphate which is stored in granules present in the cytoplasm. Under anaerobic conditions, these polyphosphates are degraded, resulting in the efflux of metal phosphate. Our results indicate that during this efflux the components of the proton motive force will be formed resulting in the conservation of metabolic energy from polyphosphate degradation, in a similar way as has been proposed in the energy recycling model (17, 23).

Acknowledgements

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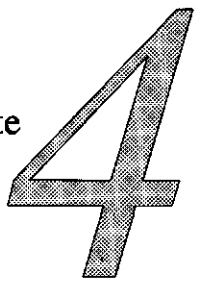
Translocation of metal phosphate via the phosphate inorganic transport system of *Escherichia coli*



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Summary

P_i transport via the phosphate inorganic transport system (Pit) of *Escherichia coli* was studied in natural and artificial membranes. P_i uptake via Pit is dependent on the presence of divalent cations, like Mg^{2+}, Ca^{2+}, Co^{2+}, or Mn^{2+}, which form a soluble, neutral metal phosphate ($MeHPO_4$) complex. P_i-dependent uptake of Mg^{2+} and Ca^{2+}, equimolar cotransport of P_i and Ca^{2+}, and inhibition by Mg^{2+} of Ca^{2+} uptake in the presence of P_i, but not of P_i uptake in the presence of Ca^{2+}, indicate that a metal phosphate complex is the transported solute. Metal phosphate is transported in symport with H^+ with a mechanistic stoichiometry of 1. Pit mediates efflux and homologous exchange of metal phosphate, but not heterologous metal phosphate exchange with P_i, glycerol-3-P or glucose-6-P. The metal phosphate efflux rate increased with pH, whereas the rate of metal phosphate exchange was essentially pH independent. Metal phosphate uptake was inhibited at low internal pH. Efflux was inhibited by a proton motive force (interior negative and alkaline), whereas exchange was inhibited by the membrane potential only. These results have been evaluated in terms of ordered binding and dissociation of metal phosphate and proton on the outer and inner surface of the cytoplasmic membrane.

Introduction

Escherichia coli possesses four systems via which P_i can enter the cell (27). The two major systems, the phosphate specific transport system (Pst) and the phosphate inorganic transport system (Pit) are highly specific for P_i and were originally described by Medveczky and Rosenberg (23) and by Willsky et al. (42). In addition, two transport systems, designated GlpT and UhpT, accept P_i as a low affinity analog of glycerol-3-P (11) and glucose-6-P (24, 44), respectively. The Pst system is an inducible, periplasmic binding protein-dependent solute ATPase that accumulates $H_2PO_4^-$ and HPO_4^{2-} at the expense of ATP (5, 10, 20, 29). Pit, GlpT and UhpT are chemiosmotic carriers. GlpT and UhpT belong to a family of P_i-linked antiporters which are induced in the presence of the phosphorylated solute and which mediate electroneutral exchange of $H_2PO_4^-$, organic phosphate anions, or both. P_i-linked exchange

carriers of *E. coli*, *Lactococcus lactis* and *Staphylococcus aureus* have been well characterized in cells, membrane vesicles, and reconstituted systems (for review, see 22). Pit has received much less attention over the past 20 years. Studies in the 1970s and early 1980s with wild-type cells (23, 43), Pst-deficient mutants (28, 29) and membrane vesicles (18) of *E. coli* indicated that Pit is a constitutive system which probably catalyzes an electrogenic nH^+/P_i symport. Proton motive force (Δp_H+)-driven uptake of P_i was not observed in membrane vesicles prepared from the Pit-deficient *E. coli* strain K-10 (18).

Recent studies on P_i transport in *Acinetobacter johnsonii* 210A revealed the presence of two transport systems which show a strong analogy with the Pst and Pit system of *E. coli* (39). Experiments aimed to clarify the mechanism of the secondary P_i transport system of *A. johnsonii* 210A point to an electrogenic symport of a proton and a neutral metal phosphate ($MeHPO_4$) chelate which is formed by complexation of divalent metal ions and P_i (40). In view of the apparent similarities between Pi transport in *A. johnsonii* 210A and *E. coli*, these results have led us to reevaluate and reexamine P_i transport via Pit. The substrate specificity and mechanism of Pit were characterized in membrane vesicles and proteoliposomes in which the transport protein was successfully reconstituted. In this paper, evidence will be presented for an electrogenic metal phosphate/proton symport mechanism. The effects of pH and Δp_H+ on the different modes of metal phosphate transport via Pit are consistent with the ordered binding model which was recently put forward for the secondary phosphate transport system of *A. johnsonii* 210A (40).

Materials and methods

Membrane vesicles and proteoliposomes.

Cells of *Escherichia coli* K-12 strain PC 1012[1] (*pit*$^+$ *pst*$^+$) were grown aerobically at 37 °C to an A_{660} of 0.6 in minimal glucose medium (9) supplemented with 5 mM

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sodium phosphate to repress the synthesis of the Pst system. Membrane vesicles were prepared as described (12) in 10 mM potassium Pipes (pH 7.0) containing 0.1 mM $MgSO_4$ and finally suspended in 50 mM potassium Pipes (pH 7.0) supplemented with 10 mM $MgSO_4$. Solubilization of membrane vesicles with *n*-octyl-β-glucopyranoside and reconstitution of membrane proteins by detergent dilution were performed by the procedures described (40, 41).

Transport assays

Δp_H+-driven uptake of $^{32}P_i$ in membrane vesicles and the determination of the membrane potential ($\Delta\psi$) using a tetraphenylphosphonium ion-selective electrode (35) were performed as described (39, 40) in 50 mM potassium Pipes (pH 7.0) or 20 mM potassium Mes-Pipes-Hepes (pH 6.0 - 8.0) (MPH buffer), supplemented with 10 mM $MgSO_4$. The procedure for uptake of $^{32}P_i$ or $^{45}Ca^{2+}$ in proteoliposomes driven by an artificial $\Delta\psi$ and/or pH gradient (ΔpH) was essentially as described previously (40) using sodium Pipes-based buffers, pH 7.0, for dilution. For metal phosphate efflux and exchange, proteoliposomes in 20 mM potassium Pipes (pH 7.0) containing 100 mM potassium acetate (PPA buffer) or MPH containing 100 mM potassium acetate (MPHA buffer) were preloaded for 3 h at 20 °C with $^{32}P_i$ and divalent cations as specified in the legends to figures. Loaded proteoliposomes were subsequently diluted 100-fold into buffer [PPA plus 20 μM carbonyl cyanide (3-chlorophenyl)hydrazone (CCCP), MPHA plus 20 μM CCCP, or the appropriate buffers to impose artificial diffusion gradients] without Pi (efflux), with an equimolar concentration nonlabeled potassium phosphate (homologous exchange), or with organic phosphate anions (heterologous exchange). Dilution buffers were supplemented with divalent cations as described in the legends to figures. Transport of $^{32}P_i$ (1.7 TBq/mol) and $^{45}Ca^{2+}$ (2.1 TBq/mol) was assayed at 30 °C by the filtration method (13). $^{32}P_i$ (carrier-free) and $^{45}CaCl_2$ (14.8 TBq/mol) were purchased from Amersham, United Kingdom. Uptake of Mg^{2+} was monitored at 30 °C in proteoliposomes loaded with 5 mM Mag-Quin-2 by freeze-thaw sonication (6). The fluorescence intensity at 490 nm was measured at an excitation of 335 nm with slit widths of 4 and 7.5 nm, respectively. Mag-Quin-2 was obtained from Molecular

Probes, Inc., USA.

Results

Kinetic analysis of P_i uptake

P_i was accumulated about 30-fold in membrane vesicles from *E. coli* suspended in Pipes buffer when a Δp_H+ of -109 mV was established by oxidation of glucose via the membrane-bound, pyrrolo quinoline quinone (PQQ)-dependent glucose dehydrogenase. Initial rates of Δp_H+-driven uptake were determined from time points taken during the first 60 s of linear uptake of P_i between 1 μM and 300 μM. Kinetic analysis using Lineweaver-Burk plots revealed the presence of one P_i transport system with an apparent K_t of 11.9 μM and a V_{max} of 0.74 nmol/min/mg of protein (data not shown). This K_t corresponds well with the reported K_t of 9.2 μM for the Pit system in cells (23).

Substrate specificity

Divalent cations are required for binding of apo-glucose dehydrogenase to its prosthetic group PQQ and may influence the magnitude or composition of the Δp_H+ generated by glucose oxidation (34). Such an influence of divalent cations on the stability and magnitude of an artificially imposed Δp_H+ in proteoliposomes was not observed (40). However, uptake of 50 μM P_i driven by an imposed Δp_H+ in proteoliposomes in which Pit protein was reconstituted, was inhibited to equilibration levels in the presence of 0.5 mM EDTA. Furthermore, the rate of P_i uptake was stimulated from 1.4 nmol/min/mg of protein in the absence of added cations to 3.7, 4.0, 6.9 and 7.5 nmol/min/mg of protein in the presence of 2 mM Mg^{2+}, Ca^{2+}, Co^{2+}, or Mn^{2+}, respectively. These cations form a soluble, electroneutral metal phosphate complex ($MeHPO_4$) with 31% (Ca^{2+}), 36% (Mg^{2+}), 70% (Co^{2+}), and 87% (Mn^{2+}) of the P_i present in the incubation (36). This complexation could be detected with the Mg^{2+} indicator Mag-Quin-2 which undergoes a fluorescence excitation intensity enhancement upon binding of Mg^{2+}. Addition of 100 mM P_i to a solution of 520 μM

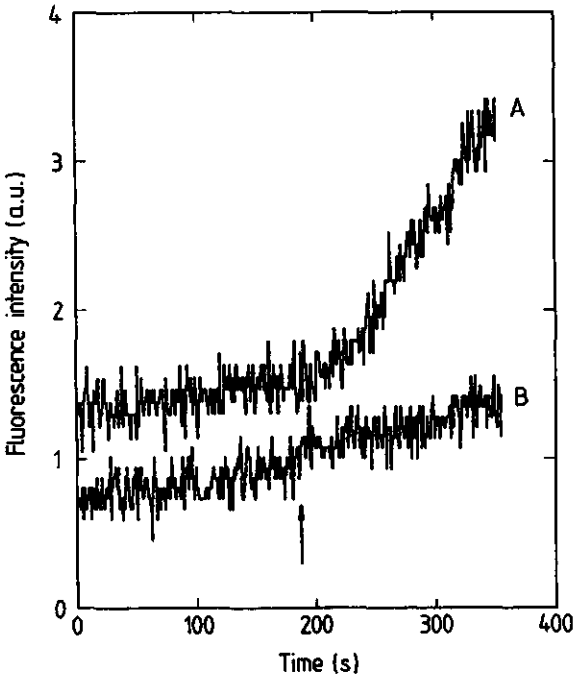

Figure 1. Time course of the fluorescence emission of Mag-Quin-2-loaded proteoliposomes representing the uptake of 200 μM Mg^{2+} in the presence (A) and absence (B) of 5.4 mM P_i. At the arrow, 1 nmol valinomycin/mg of protein was added to impose a potassium diffusion potential (interior negative) of -138 mV.

probe and 400 μM Mg^{2+} at pH 7.0 resulted in a 4-fold decrease of the fluorescence intensity, corresponding to a decrease of the Mg^{2+}/probe complex concentration from 130 to 18 μM, due to the formation of about 350 μM $MgHPO_4$ (data not shown). In view of the extensive complexation of P_i and Me^{2+}, the strict metal dependency of P_i uptake may be interpreted as the translocation of metal phosphate rather than P_i. This was investigated by measuring the uptake of Mg^{2+} and Ca^{2+} in the presence and absence of P_i. Mg^{2+} transport was monitored in proteoliposomes containing Mag-Quin-2. A significant increase in fluorescence intensity due to uptake of Mg^{2+} was only observed in the presence of a $\Delta\psi$ (inside negative) when P_i was present in the incubation mixture (Fig. 1).

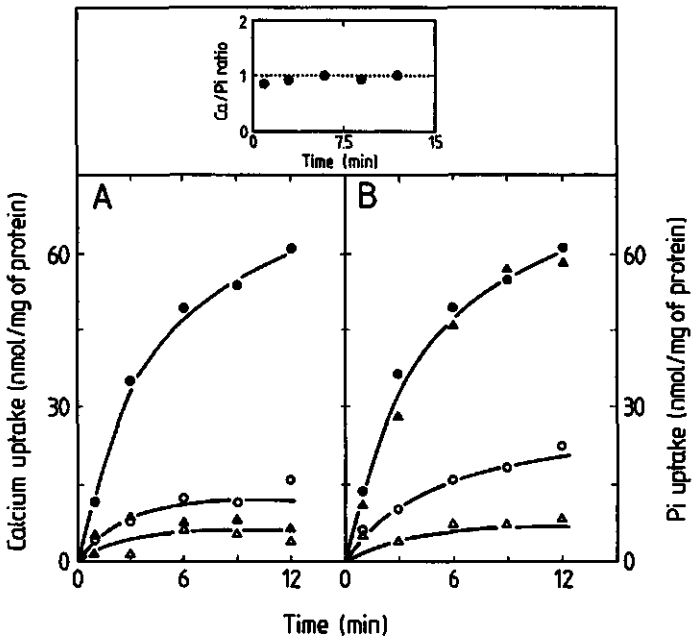

Figure 2. Δp_H+-driven uptake of Ca^{2+}, P_i and $CaHPO_4$ in proteoliposomes. Uptake of 100 μM $^{45}Ca^{2+}$ (A) or 100 μM $^{32}P_i$ (B) (○); uptake of 100 μM $^{45}Ca^{2+}$ plus 100 μM $^{45}CaHPO_4$ (A) or 100 μM $^{32}P_i$ plus 100 μM $CaH^{32}PO_4$ (B) in the absence (●) and presence of 10 mM Mg^{2+} (▲) or 0.5 mM EDTA (△). The Ca/P_i ratio for uptake of $CaHPO_4$ (●, *panel* A and B) is presented in the *inset*.

Similar results were obtained for Ca^{2+} uptake. The rate of Δp_H+-driven uptake of $^{45}Ca^{2+}$ in proteoliposomes was low in P_i-free buffer (Fig. 2). In the presence of P_i a considerably higher rate of Ca^{2+} uptake was observed. $^{32}P_i$ uptake was measured under identical conditions. The same initial rate and steady-state level of accumulation of P_i as Ca^{2+} was found, suggesting a Ca^{2+}/P_i ratio of 1. Ca^{2+} uptake but not P_i uptake was strongly inhibited by excess Mg^{2+}. These uptake experiments provide strong evidence for the transport of Ca^{2+} or Mg^{2+} via a metal phosphate complex. The substrate specificity of Pit was further studied in efflux and exchange experiments under conditions in which no Δp_H+ was imposed. At pH 7.0, the efflux of ^{32}P-labeled magnesium-phosphate from proteoliposomes was stimulated by the

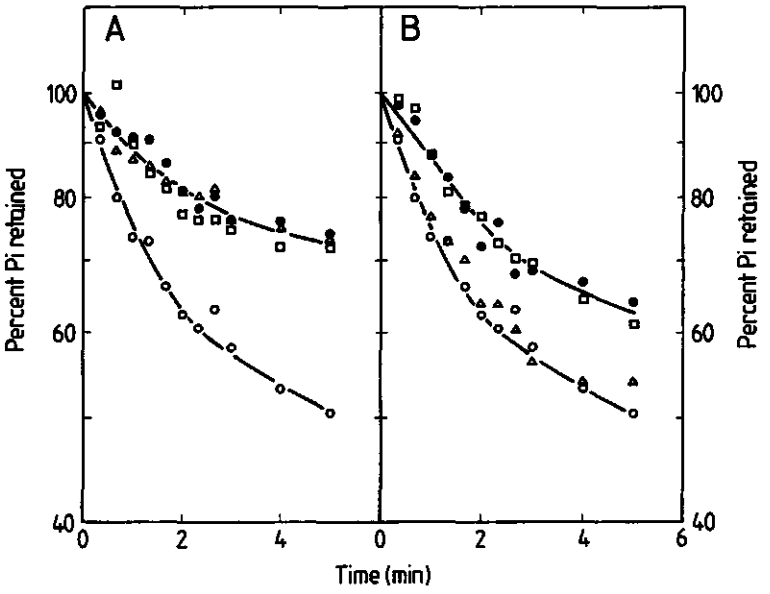

Figure 3. Efflux and exchange via Pit. Proteoliposomes in PPA buffer were loaded with 50 μM $^{32}P_i$ and 2 mM $MgSO_4$. Exit of $^{32}P_i$ was monitored following 100-fold dilution of a suspension into PPA buffer containing 20 μM CCCP and the following additions: (A) 2 mM Mg^{2+} in the absence (●) and presence of 50 μM P_i (○), 10 mM glycerol-3-P (△) or glucose-6-P (□); (B) 0.5 mM EDTA in the absence (●) and presence of 50 μM P_i (□), or 50 μM P_i in the presence of 2 mM Mn^{2+} (△) or 2 mM Mg^{2+} (○).

addition of an equimolar amount of nonlabeled magnesium or manganese phosphate to the external medium (Fig. 3). This stimulation was not observed in the presence of a 200-fold excess of glycerol-3-P, glucose-6-P (Fig. 3A) or an equimolar amount of P_i plus 0.5 mM EDTA (Fig. 3B). Besides uptake of metal phosphate, Pit obviously mediates efflux and homologous exchange of metal phosphate, the latter reaction being faster at pH 7.0, but not heterologous exchange of metal phosphate and P_i, glycerol-3-P or glucose-6-P.

Energy coupling to metal phosphate uptake

The driving force for uptake of metal phosphate was analyzed in proteoliposomes which were subjected to artificial gradients of protons and/or potassium ions. In the presence of a $\Delta\psi$ of -120 mV or a $-Z\Delta$pH of -120 mV, metal phosphate (50 μM P_i plus 10 mM Mg^{2+}) was taken up at a rate of 1.6 and 3.5 nmol/min/mg of protein, respectively. The effects of $\Delta\psi$ and ΔpH were additive. Imposition of a Δp_H+ of −240 mV resulted in metal phosphate uptake at a rate of 4.8 nmol/min/mg of protein. In the absence of a gradient, no metal phosphate uptake was observed. The metal phosphate/proton stoichiometry was determined in membrane vesicles at pH 7.8, from the steady state accumulation level of metal phosphate (50 μM P_i plus 10 mM Mg^{2+}) in the presence of 0.1 nmol nigericin/mg of protein ($\Delta p_H+ = \Delta\psi$). The $\Delta\psi$ was varied by titration with 0.01 - 0.1 nmol valinomycin/mg of protein. At thermodynamic equilibrium in the absence of a ΔpH, n equals $-Z\Delta\bar{u}_{MeHPO_4}/\Delta\psi$, in which n represents the number of protons translocated in symport with metal phosphate and $Z\Delta\bar{u}_{MeHPO_4}$ represents the transmembrane metal phosphate concentration gradient (in mV). A linear relationship was observed between $-Z\Delta\bar{u}_{MeHPO_4}$ and the $\Delta\psi$ in which n was 0.91 indicating a symport of a neutral $MeHPO_4$ complex and one proton (data not shown).

Effect of pH and Δp_H+ on metal phosphate efflux and exchange

The effect of pH on metal phosphate efflux and exchange was measured in proteoliposomes equilibrated in the presence of 150 μM P_i and 10 mM $MgSO_4$. Metal phosphate efflux and exchange occurred with pseudo-first order kinetics (Fig. 4). The metal phosphate efflux rate increased as a function of pH (half-times ($t_{½}$) of 9.4, 4.3, and 1.9 min at pH 6.3, 7.0, and 7.7, respectively) whereas the metal phosphate exchange rate was essentially pH independent ($t_{½}$ = 1.8 min). At pH values below 7.7, metal phosphate efflux was slower than metal phosphate exchange. Apparently, the release of a proton at the outer surface of the membrane is rate-limiting for metal phosphate efflux whereas in the exchange process no release of a proton is needed. In an analogous series of experiments, the effects of

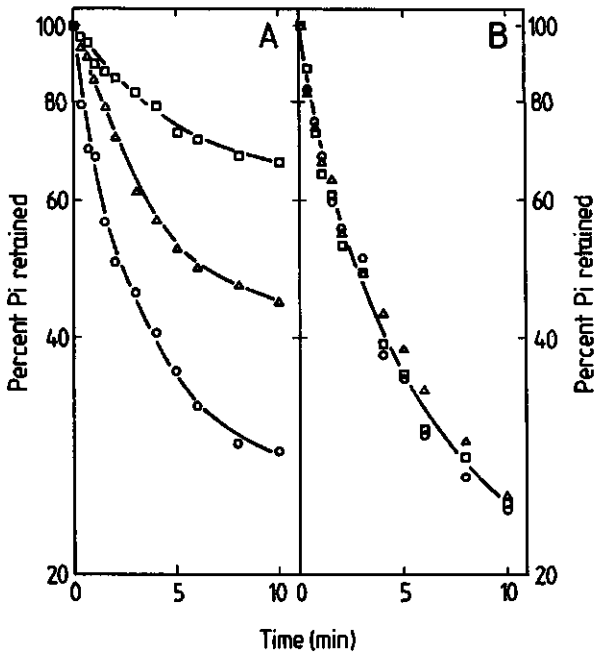

Figure 4. Effect of pH on metal phosphate efflux and exchange. Proteoliposomes were equilibrated in MPHA buffer [pH 6.3 (□), 7.0 (Δ), or 7.7 (○)] containing 150 μM $^{32}P_i$ and 10 mM Mg^{2+} and subsequently diluted 100-fold into MPHA buffer of the same pH containing 10 mM Mg^{2+} and 20 μM CCCP without (A, efflux) or with 150 μM P_i (B, exchange).

artificially imposed ion gradients on metal phosphate efflux and exchange were monitored. Imposition of a $\Delta\psi$ of -120 mV retarded the efflux rate at pH 7.0 by a factor of 3 ($t_{½}$ increased from 3.4 to 9.6 min) (Fig. 5A). Imposition of a -$Z\Delta$pH of -120 mV resulted in a 2-fold inhibition of the rate of metal phosphate release ($t_{½}$ = 6.7 min). The effects of a $\Delta\psi$ and ΔpH on metal phosphate efflux were additive. Imposition of a Δp_H+ of -240 mV retarded metal phosphate efflux more than 6-fold ($t_{½}$ = 23.2 min). The inhibition of metal phosphate efflux by a Δp_H+ is consistent with an electrogenic proton symport mechanism. Metal phosphate exchange was not affected by a ΔpH (Fig. 5B) but was retarded by the presence of a $\Delta\psi$ (Fig. 5C) ($t_{½}$ increased from 1.8 min in the absence of a $\Delta\psi$, to 3.0, 4.0, and 4.9 min in the

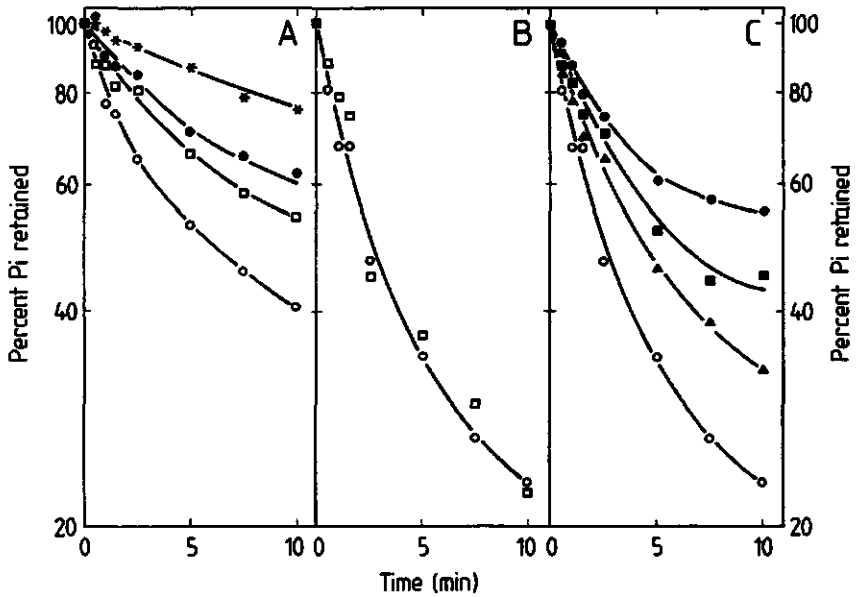

Figure 5. Effect of Δp_{H^+} on metal phosphate efflux and exchange. Proteoliposomes were equilibrated in PPA buffer containing 150 μM $^{32}P_i$ and 10 mM Mg^{2+} and diluted 100-fold into the appropriate buffers to study efflux (A) and exchange (B, C) in the presence of a $\Delta\psi$ of -40 mV (▲), -80 mV (■), -120 mV (●), a -ZΔpH of -120 mV (□), or a Δp_{H^+} of -240 mV (*), or in the absence of imposed diffusion gradients in the presence of 20 μM CCCP (○).

presence of a $\Delta\psi$ of -40, -80, and -120 mV, respectively).

Effect of pH on Δp_{H^+}-driven uptake of metal phosphate

Deprotonation of the carrier on the outside of the membrane is affected by the external pH and was found to limit the rate of metal phosphate efflux (Fig. 4). Under uptake conditions, the release of metal phosphate and proton occurs on the inside of the membrane. Internal pH effects on metal phosphate uptake may be anticipated. Δp_{H^+}-driven uptake of metal phosphate was studied as a function of the pH in membrane vesicles in which the ΔpH was dissipated by the addition of

deprotonated.

The deprotonation of the carrier protein on the outer surface of the membrane is rate-limiting for metal phosphate efflux and is affected by the external pH. In analogy with efflux, metal phosphate uptake appears to be limited by the rate of deprotonation of the carrier on the inside of the membrane. As a result, metal phosphate uptake is strongly inhibited by a low internal pH. For optimal function of Pit in cells it is therefore essential to maintain a constant alkaline pH in the cytosol. In bacteria, electrogenic uptake of K^+ accompanied by expulsion of H^+ from the cytoplasm, is an important mechanism for alkalinization of the cell's interior (3). Evidence confirming the relevance of this process for phosphate transport via Pit came from the work of Russell and Rosenberg (32, 33) who demonstrated that although potassium ions greatly stimulate Pit function in *E. coli* cells, the transport of K^+ and phosphate is linked indirectly via proton circulation.

The ordered binding model proposed for Pit shows analogies to those suggested for the LacY (14 - 16) and melibiose carriers of *E. coli* (4, 25). Unlike exchange via LacY, metal phosphate exchange is inhibited by the $\Delta\psi$ (interior negative). This inhibition may result from a decrease of the translocation rate of a positively charged ternary complex across the membrane and/or an increase in the rate of dissociation of the cosubstrates from the carrier at the inner surface of the membrane as was proposed for the melibiose carrier.

The finding of metal phosphate/proton symport in *E. coli* (this work) and *A. johnsonii* 210A (40) suggests that the transport of metal phosphates may be a general mechanism for the transport of divalent metal ions and Pi in bacteria. In *Micrococcus lysodeikticus* (8), *Acinetobacter lwoffi* (45), *Pseudomonas aeruginosa* (19) and *Bacillus cereus* (31), Pi transport in general or via a Pit-like system in particular was reported to be stimulated by Mg^{2+}. In addition, in some studies a stimulation was observed of Me^{2+} transport by Pi, *e.g.* the uptake of Mn^{2+} in *Lactobacillus plantarum* (2), and of Mg^{2+}, Ca^{2+}, Mn^{2+}, and Co^{2+} via a general divalent cation transport system in *Bacillus subtilis* (17). Interestingly, a Pit mutant of this latter organism was strongly impaired in the transport of Ca^{2+} and Co^{2+}. The mutant still elicited significant Mn^{2+} transport as a result of uptake via a second Mn^{2+}-specific high-affinity uptake system (17). In many other studies on metal

transport, the use of phosphate buffers may have masked Pi-dependent uptake of divalent cations. The mechanisms for Ca^{2+} entry in bacteria are unclear (21, 27). The transport of metal phosphate, including calcium-phosphate, via Pit provides *E. coli* with such a mechanism.

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Substrate specificity of the two phosphate transport systems of *Acinetobacter johnsonii* 210A in relation to phosphate speciation in its aquatic environment

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Summary

In natural waters and domestic waste waters in which divalent metal ions are present in excess of P_i, $H_2PO_4^-$, HPO_4^{2-} and $MeHPO_4$ prevail at pH values physiological for *Acinetobacter johnsonii* 210A (pH 5.5 - 8.0). In view of the ability of this organism to extensively accumulate P_i and divalent cations in cytoplasmic polyphosphate granules, the substrate specificity of its two P_i transport systems was studied. The constitutive, proton motive force-driven P_i carrier, previously shown to be dependent on divalent cations, plays a major role in the divalent cation and P_i flux by translocating $MeHPO_4$ rather than P_i. This notion is confirmed by the observation that divalent cations are cotransported with P_i in a 1:1 stoichiometry in proteoliposomes containing reconstituted P_i carrier protein. In contrast, the P_i repressible, periplasmic binding protein-dependent P_i transport system mediates the uptake of $H_2PO_4^-$ and HPO_4^{2-}. P_i uptake, but not $MeHPO_4$ uptake, was stimulated in cells under P_i limitation and the periplasmic P_i-binding protein has affinity for $H_2PO_4^-$ and HPO_4^{2-}, but not for $MeHPO_4$. When operating in concert, both systems enable *A. johnsonii* 210A to efficiently acquire P_i from its habitat through uptake of the predominant P_i species.

Introduction

Enhanced biological phosphorus removal from domestic waste water is based on the enrichment of activated sludge with polyphosphate-accumulating bacteria by imposition of alternating conditions of aerobiosis and anaerobiosis (11). Studies on the population structure of activated sludge have focused attention on *Acinetobacter* as being one of the important genera in this process (7, 8, 11, 22). In pure culture, *Acinetobacter johnsonii* 210A is able to accumulate P_i and Mg^{2+} or Ca^{2+} in cytoplasmic metal polyphosphate granules in the presence of excess energy and substrates (5, 31). Under anaerobic conditions, the strictly aerobic organism degrades its polyphosphate resulting in the excretion of P_i and metal ions into the medium (30). Conservation of metabolic energy during the enzymatic degradation of polyphosphate allows *A. johnsonii* 210A to use this phosphorus polymer as a source of

ATP (4, 28, 29).

P_i translocation across the cytoplasmic membrane of *A. johnsonii* 210A is mediated by two P_i transport systems. The high-affinity uptake system is a periplasmic binding protein-dependent permease which is a member of the "Traffic ATPase" family of transporters (1, 32). Its synthesis is repressed by P_i in the medium at concentrations above 10 μM (6). The constitutive, low-affinity P_i transport system is a reversible chemiosmotic carrier (32). The carrier protein has been solubilized from membrane vesicles, functionally reconstituted in proteoliposomes, and has been demonstrated to mediate P_i uptake and efflux via an electrogenic proton symport mechanism. Based on the dependence of P_i uptake on divalent cations, like Mg^{2+}, Ca^{2+}, Co^{2+} or Mn^{2+}, it was suggested that a neutral, soluble metal phosphate chelate ($MeHPO_4$) rather than P_i is the translocated solute (33).

In this study, the substrate specificity of the two P_i transport system of *A. johnsonii* 210A was examined in greater detail. The results indicate that the secondary $MeHPO_4$ transport system is a major route for the entrance and exit of divalent cations and P_i in response to aquatic environmental pertubations. The substrate specificity of the secondary $MeHPO_4$ transport system is complementary to that of the primary P_i transport system. The presence of both transport systems in *A. johnsonii* 210A allows the organism to take up the predominant P_i species from its aquatic environment.

Materials and methods

Organism and growth conditions

A. johnsonii 210A was cultured at 30 °C in a Tris-buffered medium (pH 7.0) supplemented with 20 mM sodium butyrate and 5 mM or 20 μM sodium phosphate according to the experimental needs (32).

Preparation of membrane vesicles and proteoliposomes

Membrane vesicles were prepared by osmotic lysis of high-P_i-grown cells exposed

to lithium chloride, a high concentration of lysozyme and a temperature shock (33). Solubilization of membrane vesicles with *n*-octyl-β-glucopyranoside and reconstitution of membrane proteins in proteoliposomes by detergent dilution were performed as described (33).

Transport assays

Uptake of $^{32}P_i$ (1.7 TBq/mol) or $^{45}Ca^{2+}$ (2.1 TBq/mol) in cells energized via the oxidation of butyrate was monitored as described (32) in 50 mM potassium Pipes (pH 7.0) containing 50 μg of chloramphenicol/ml. For uptake driven by an artificially imposed membrane potential ($\Delta\psi$, interior negative) or proton motive force (Δp_H+, interior negative and alkaline), proteoliposomes in 20 mM potassium Pipes (pH 7.0) containing 100 mM potassium acetate (PPA buffer) were diluted as described previously (33) into sodium Pipes-based buffers, pH 7.0. For P_i efflux, proteoliposomes in PPA buffer were loaded with ^{32}P-labeled potassium phosphate in the presence of divalent cations or EDTA by freeze-thaw-sonication (9). Loaded proteoliposomes were subsequently diluted 100-fold into PPA buffer containing a 20 μM concentration of CCCP. Transport of P_i and Ca^{2+} in cells and proteoliposomes was assayed at 30 °C by the filtration method (33). Uptake of Mg^{2+} was monitored at 30 °C by spectrofluorimetry. Proteoliposomes were loaded with 5 mM Mag-Quin-2 by freeze-thaw-sonication (9). The excitation and emission monochromator wavelengths were 335 and 490 nm with slit widths of 4 and 7.5 nm, respectively.

Periplasmic protein fractions and P_i-binding experiments

Cells were exposed to an osmotic and temperature shock procedure (in the absence of lysozyme) as described for the preparation of membrane vesicles (33). Subsequently, the cells were pelleted by centrifugation (7,000 x *g*) at 20 °C and suspended into 50 volumes of 0.1 mM $MgSO_4$ at 20 °C. After 15 min of incubation, the cells were removed by centrifugation at 4 °C. The supernatant (periplasmic protein fraction) was concentrated via ultrafiltration to about 3 mg of protein/ml

and dialyzed as described (32). For binding of P_i to the P_i-binding protein, concentrated periplasmic protein fractions were diluted 10-fold into 100 mM potassium Mes, 100 mM potassium Pipes, 100 mM potassium Hepes (pH 5.5 - 8.0) (MPH buffer) in a final volume of 0.5 ml. The MPH buffer was supplemented with EDTA or divalent cations as indicated in the legend to Fig. 5. Binding of $^{32}P_i$ (88.7 TBq/mol) was measured via a filtration method (32). The data were corrected for nonspecific binding of P_i to the nitrocellulose filters and to bovine serum albumin.

EDAX analysis of polyphosphate granules

Cells were grown at 20 °C in the medium described under "Organism and growth conditions", without Ca^{2+}, supplemented with 1 mM sodium phosphate, 1 mM potassium chloride and (i) 3 mM Mg^{2+} (high-$MgHPO_4$ medium), (ii) 0.3 mM Mg^{2+} and 3 mM Ca^{2+} (high-$CaHPO_4$ medium), or (iii) 0.3 mM Mg^{2+} and 1 mM Mn^{2+} (high-$MnHPO_4$ medium). The elemental composition of the polyphosphate granules in air-dried, unfixed, and unstained cells (5) was examined by *in situ* EDAX analysis with a Camebax MB1 scanning electron microscope and a Tracor Tn 2,000 energy dispersive X-ray spectrometer.

Calculations

The P_i species distribution in aqueous solution was calculated according to the acid dissociation constants of P_i ($\log K_a' = -12.33$, $\log K_a'' = -7.21$ and $\log K_a''' = -2.16$ (24)) and the complexation equilibrium constant of the various metal phosphate complexes (Table 1). From these constants, the concentrations of the P_i species were evaluated by a simple analytical procedure at different pH values with the assumptions that (i) $[Me^{2+}_{total}] \gg [P_{i\ total}]$ and (ii) $[Me^{2+}] \simeq [Me^{2+}_{total}]$, in which $[Me^{2+}_{total}]$ and $[P_{i\ total}]$ represent the sum of the different Me^{2+} and P_i species, respectively. For the determination of the P_i-binding constant (K_D) of the periplasmic P_i-binding protein the equilibrium expression: $K_D = ([PBP][H_2PO_4^-] + [PBP][HPO_4^{2-}])/[PBP\text{-}P_i]$, was linearized into: $1/[PBP\text{-}P_i] = (K_D/[P_{i\ total}][PBP_{total}]) + 1/[PBP_{total}]$, with the assumptions that one ligand binding site with equal affinity for

Table 1. Complexation equilibrium constants for divalent metal ions (Me^{2+}) with orthophosphate[a]

Me^{2+}	Complex	log*K*
Ca^{2+}	$CaPO_4^-$	6.46
	$CaHPO_4$	2.74
	$CaH_2PO_4^+$	1.4
Mg^{2+}	$MgPO_4^-$	4.8
	$MgHPO_4$	2.91
	$MgH_2PO_4^+$	1.2
Co^{2+}	$CoHPO_4$	3.5
Mn^{2+}	$MnHPO_4$	3.94

[a] Values are for the formation of the complex by the divalent cation and PO_4^{3-}, HPO_4^{2-} or $H_2PO_4^-$ at 25 °C and zero ionic strength. Adapted from Sillén and Martell (24), Martell and Smith (18) and Morel (21).

monobasic and dibasic P_i is present per molecule of phosphate binding protein [by analogy with the periplasmic P_i-binding protein of *Escherichia coli* which contains one P_i-binding site per molecule (17)], and that $[H_2PO_4^-] + [HPO_4^{2-}] \simeq [P_{i\ total}]$ in the pH range of 5.5 to 8.0 in the absence of divalent cations. [PBP], [PBP-P_i] and [PBP_{total}] refer to the concentration of non-liganded and liganded P_i-binding protein, and to the sum of these two species, respectively. The K_D and [PBP_{total}] were derived by plotting 1/[PBP-P_i] versus 1/[$P_{i\ total}$].

Miscellaneous

Cell protein was determined by the procedure of Lowry et al. (16). A bicinchoninic acid protein assay (25) containing 0.2% (wt/vol) SDS was used for estimation of protein concentrations in detergent extracts and proteoliposome suspensions. Bovine serum albumin was used as the standard.

Chemicals

$^{32}P_i$ (carrier-free) and $^{45}CaCl_2$ (14.8 TBq/mol) were purchased from Amersham, Buckinghamshire, UK. Cellulose nitrate filters (0.45 μm pore-size) were obtained from Schleicher und Schuell, Dassel, Germany. A Bicinchoninic Acid Protein Assay Kit and Mag-Quin-2 were supplied by Sigma and Molecular Probes, respectively. Other chemicals were reagent grade and obtained from commercial sources.

Results

Effect of pH and divalent cations on the distribution of P_i species

The distribution of the several acid and base species of P_i in solution is governed by pH. Their concentrations can be calculated using the acid dissociation constants of multistage equilibria of H_3PO_4. The predominant P_i species over the pH range 5.0 - 9.0 are $H_2PO_4^-$ and HPO_4^{2-} (Fig. 1A). However, in the presence of excess Ca^{2+} or Mg^{2+} (*e.g.* 2 mM of divalent metal ions versus 50 μM P_i) soluble metal phosphate chelates ($CaH_2PO_4^+$, $CaHPO_4$, $CaPO_4^-$ and $MgH_2PO_4^+$, $MgHPO_4$, $MgPO_4^-$) are formed of which the neutral complexes prevail at physiological pH between 5.5 and 8.0 together with $H_2PO_4^-$ and HPO_4^{2-} (Fig. 1B and 1C)[1]. At pH 7.0, about 31 and 36% of P_i is complexed into $CaHPO_4$ or $MgHPO_4$, respectively. Similar computations with Co^{2+} or Mn^{2+} at 2 mM as the divalent cation indicate that about 70 and 87% of P_i is in the $CoHPO_4$ or $MnHPO_4$ form, respectively.

Translocation of metal phosphate via the secondary phosphate transport system

Control experiments shown in Fig. 2A have verified the metal dependence of the

[1]Complexation of P_i into insoluble calcium-phosphate phases ($Ca(H_2PO4)_2$, $CaHPO_4{\cdot}2H_2O$, ß-$Ca_3(PO_4)_2$, $Ca_4H(PO_4)_3$, $Ca_5(PO_4)_3OH$, and $Ca_{10}(PO_4)_6(OH)_2$) or magnesium-phosphate phases ($MgHPO_4{\cdot}3H_2O$ and $Mg_3(PO_4)_2{\cdot}8H_2O$) was calculated to be less than 4 % of total P_i up to pH 8.0 (data not shown), using phosphate solubility equilibrium constants (21, 26, 27).

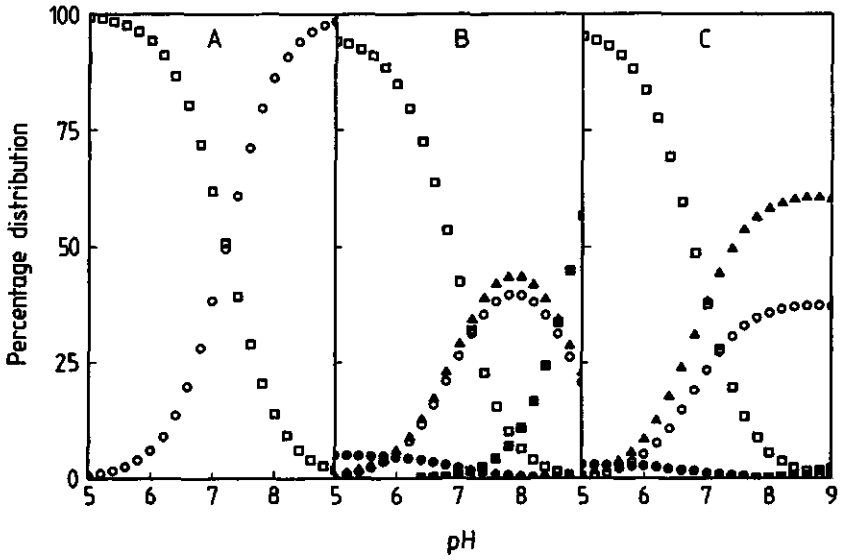

Figure 1. Distribution of phosphate species in aqueous solution as function of pH in the absence (*panel A*) and presence of Ca^{2+} (*panel B*) or Mg^{2+} (*panel C*). The total concentrations of P_i and Me^{2+} in these calculation were 50 μM and 2 mM, respectively. The lines represent the relative concentrations of the following P_i species: $H_2PO_4^-$ (□), HPO_4^{2-} (○), $MeH_2PO_4^+$ (●), $MeHPO_4$ (▲) and $MePO_4^-$ (■). The relative concentrations of H_3PO_4 and PO_4^{3-} were less than 0.14% between pH 5 and 9 (not shown).

Δp_H+-driven uptake of P_i in proteoliposomes in which P_i carrier protein of *A. johnsonii* 210A was reconstituted. In addition, P_i efflux in proteoliposomes preloaded with 50 μM P_i in which the Δp_H+ was zero (collapsed by protonophore CCCP), was enhanced by the presence of 2 mM of Mg^{2+}, Ca^{2+}, or Mn^{2+} in the internal milieu of the membranes (Fig. 2B). P_i efflux was retarded in proteoliposomes containing 0.5 mM EDTA (Fig. 2B). In view of the predominance of $MeHPO_4$ in the incubation mixture and the apparent affinity of the P_i carrier for this complex (33), the strict metal dependence of P_i uptake and efflux is in accordance with the translocation of $MeHPO_4$.

This notion prompted us to study the uptake of Mg^{2+} and Ca^{2+} in the presence and absence of P_i. Mg^{2+} transport was monitored in proteoliposomes loaded with the

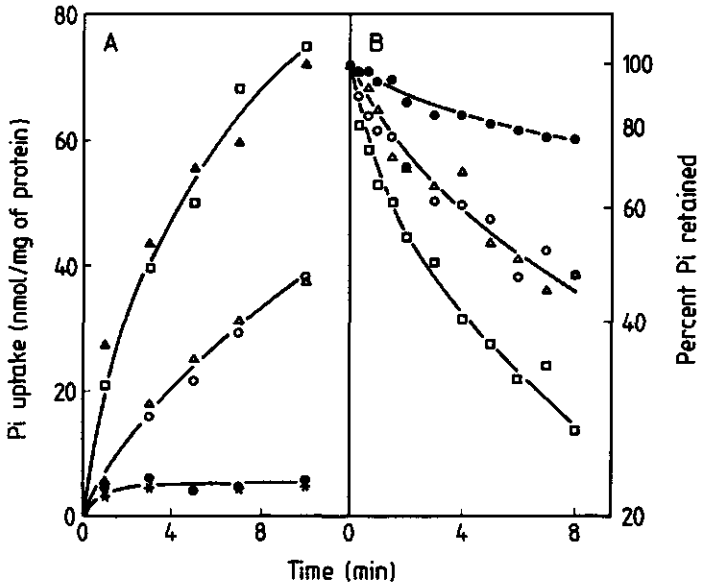

Figure 2. Effect of divalent metal ions on P_i uptake (*panel A*) and efflux (*panel B*) in proteoliposomes. Δp_H+-Driven uptake of 50 μM P_i was measured in proteoliposomes in the presence of 2 mM of Mg^{2+} (○), Ca^{2+} (△), Co^{2+} (▲), Mn^{2+} (□) or 0.5 mM EDTA (●) in the incubation mixture, or in the absence of an imposed Δp_H+ (✳). For P_i efflux, proteoliposomes in PPA buffer were loaded with 50 μM P_i and divalent cations or EDTA at concentrations as described under Δp_H+-driven uptake, and subsequently diluted 100-fold in PPA buffer without P_i, supplemented with 20 μM CCCP.

Mg^{2+} indicator Mag-Quin-2. This probe undergoes a fluorescence excitation enhancement upon binding of Mg^{2+}. The $\Delta\psi$-driven uptake ($\Delta\psi$ of -138 mV, interior negative) of Mg^{2+} at a 200 μM concentration was stimulated almost 7-fold when 5.4 mM P_i was added to the incubation mixture (data not shown). The P_i dependence of divalent cation transport was further investigated by Ca^{2+} uptake studies in proteoliposomes (Fig. 3). The rate of imposed Δp_H+-driven uptake of 100 μM $^{45}Ca^{2+}$ was low in the absence of P_i. In contrast, the accumulation of calcium in proteoliposomes was stimulated considerably when the incubation mixture was supplemented with 100 μM $^{45}Ca^{2+}$ and 100 μM $^{45}CaHPO_4$ through the additions of 5.4 mM sodium phosphate and 200 μM $^{45}Ca^{2+}$. $^{32}P_i$ uptake was measured under identical

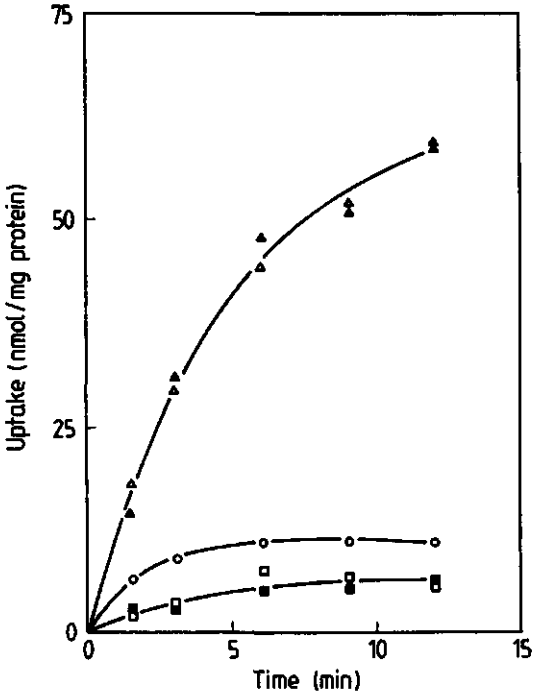

Figure 3. Cotransport of Ca^{2+} and P_i in proteoliposomes. Uptake of 100 μM $^{45}Ca^{2+}$ (○), 100 μM $^{45}Ca^{2+}$ plus 100 μM $^{45}CaHPO_4$ (△,□), or 100 μM $^{32}P_i$ plus 100 μM $CaH^{32}PO_4$ (▲,■) in the presence (○, △, ▲) and absence (□,■) of an imposed Δp_{H^+}.

conditions. The same initial rate and steady-state level of accumulation of P_i as Ca^{2+} was observed suggesting a Ca^{2+}/P_i ratio of one (Fig. 3). When 10 mM Mg^{2+} was added to the incubation mixtures, the uptake of Ca^{2+} was strongly inhibited without concomitant inhibition of P_i uptake (data not shown). These results provide evidence for the cotransport of Me^{2+} and P_i as a $MeHPO_4$ complex via the secondary P_i transport system of *A. johnsonii* 210A.

The relation between the uptake of $MeHPO_4$ and its storage in metal polyphosphate was studied in cells grown in medium containing 1 mM K^+ and about 450 μM $MgHPO_4$ (high-$MgHPO_4$ medium), 350 μM $CaHPO_4$ plus 50 μM $MgHPO_4$ (high-$CaHPO_4$ medium), or 580 μM $MnHPO_4$ plus 30 μM $MgHPO_4$ (high-

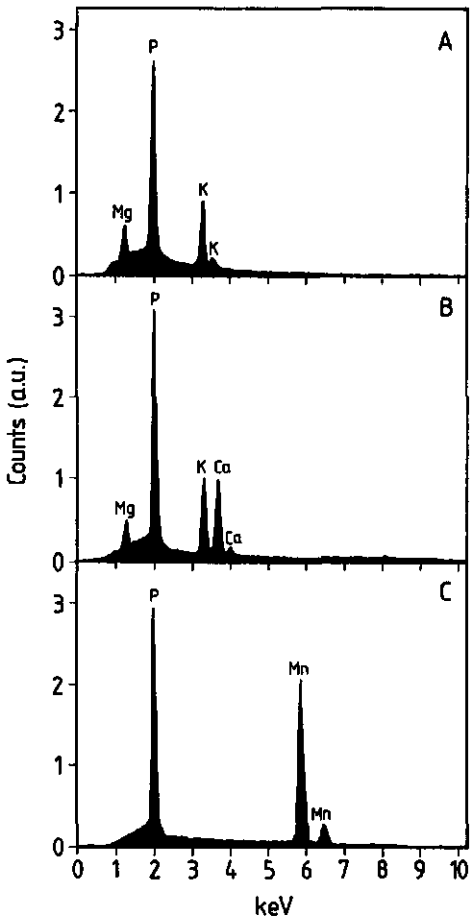

Figure 4. EDAX analysis of a large polyphosphate granule in cells of *A. johnsonii* 210A grown in high-$MgHPO_4$ (*panel A*), high-$CaHPO_4$ (*panel B*) or high-$MnHPO_4$ medium (*panel C*).

$MnHPO_4$) medium. The concentration of these complexed P_i species was considerably greater than the K_t for uptake of $MeHPO_4$ via the secondary P_i transport system (33). EDAX analysis of large granules in the three cell types revealed a strong correlation between the elemental composition of the accumulated polyphosphates and that of the transported $MeHPO_4$ species. Cells of *A. johnsonii* 210A grown in high-$MgHPO_4$ medium (Fig. 4A) contained polyphosphate granules in

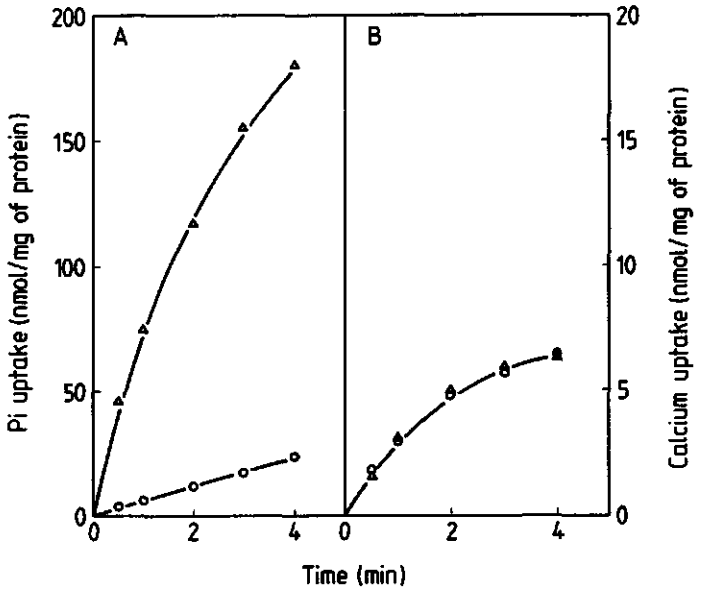

Figure 5. Uptake of P_i and $CaHPO_4$ in cells of *A. johnsonii* 210A. The uptake of 100 μM $^{32}P_i$ plus 100 μM $CaH^{32}PO_4$ (*panel A*), and of 100 μM $^{45}Ca^{2+}$ plus 100 μM $^{45}CaHPO_4$ (*panel B*) was measured in high-P_i (○) and low-P_i-grown (△) cells energized by the oxidation of 20 mM butyrate.

which Mg^{2+} and K^+ were the counterions. Ca^{2+} was found as an additional counterion in granules of cells grown in high-$CaHPO_4$ medium (Fig. 4B), whereas Mn^{2+} was strongly prevailing during growth in high-$MnHPO_4$ medium (Fig. 4C).

Translocation of mono- and dibasic P_i via the primary phosphate transport system

The uptake of 100 μM $^{32}P_i$ plus 100 μM $CaH^{32}PO_4$, and of 100 μM $^{45}Ca^{2+}$ plus 100 μM $^{45}CaHPO_4$ was measured in high- and low-P_i-grown cells (Fig. 5). $^{32}P_i$ uptake was strongly stimulated in low-P_i-grown cells. In contrast, the transport of radiolabeled calcium was identical in both cell types. These results are consistent with the translocation of $CaHPO_4$ via the constitutive secondary P_i transport system and suggest that transport of P_i, but not of $CaHPO_4$ occurs via the repressible primary P_i transport system in *A. johnsonii* 210A.

Binding of P_i to a periplasmic phosphate-binding protein is one of the initial steps in the translocation of P_i via the primary P_i transport system (12). The substrate specificity of the phosphate-binding protein of *A. johnsonii* 210A was therefore examined in periplasmic protein fractions of low-P_i-grown cells. P_i-binding studies at substrate concentrations between 0.1 and 1.5 μM yielded an apparent P_i-binding constant (K_D) of 0.59 ± 0.11 μM at pH 7.0 and a P_i-binding protein concentration of 8.4 x 10^{14} molecules/ml of concentrated periplasmic protein fraction, assuming one ligand-binding site per molecule of P_i-binding protein (see "Materials and methods"). The K_D corresponds well with the K_t value of 0.7 ± 0.2 μM previously determined for the primary P_i transport system in cells of *A. johnsonii* 210A (32). The K_D was studied as a function of pH. Although the ratio of $H_2PO_4^-$ over HPO_4^{2-} decreases about 250-fold when the pH is raised from 5.5 to 8.0, the K_D for P_i was essentially pH independent (Table 2), suggesting that the phosphate-binding protein has affinity for both monobasic and dibasic phosphate.

Its affinity for $MeHPO_4$ was investigated in P_i-binding assays (pH 7.0) containing 100 nM P_i in the absence and presence of 0.5 mM EDTA, 10 mM Mg^{2+} or 2

Table 2. Effect of pH on the $H_2PO_4^-/HPO_4^{2-}$ ratio and the K_D of the periplasmic P_i-binding protein of *A. johnsonii* 210A

pH	$H_2PO_4^-/HPO_4^{2-}$-ratio[a]	K_D (μM)[b]
5.5	51.3	0.55 ± 0.08
6.0	16.2	0.63 ± 0.11
6.5	5.1	0.48 ± 0.12
7.0	2.2	0.59 ± 0.11
7.5	0.5	0.65 ± 0.15
8.0	0.2	0.58 ± 0.10

[a] The ratio of monobasic over dibasic P_i was derived from Fig. 1A.

[b] The apparent K_D of the P_i-binding protein in periplasmic protein fractions of low-P_i-grown cells was measured at P_i concentrations between 0.1 and 1.5 μM. Values are means from two separate experiments.

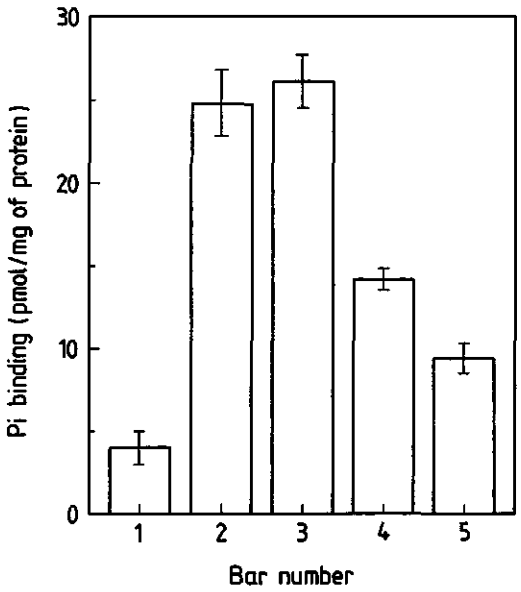

Figure 6. Effect of divalent cations on the binding of P_i to the periplasmic P_i-binding protein of *A. johnsonii* 210A. Binding of 0.1 μM P_i to concentrated periplasmic protein fractions of high-P_i-grown cells (bar 1) and low-P_i-grown cells in the absence (bar 2) and presence of 0.5 mM EDTA (bar 3), 10 mM Mg^{2+} (bar 4), or 2 mM Mn^{2+} (bar 5) was measured at pH 7.0. Each value is the mean of three separate determinations.

mM Mn^{2+}. P_i binding was not affected by EDTA, but was inhibited about 1.8 and 2.7-fold by the addition of Mg^{2+} or Mn^{2+}, respectively (Fig. 6). On the basis of equal affinity for $H_2PO_4^-$ and HPO_4^{2-} it can be calculated that at a P_i concentration of 100 nM in the absence of Me^{2+}, about 11% of the P_i-binding molecules in the incubation mixture has bound P_i. In the presence of 10 mM Mg^{2+} or 2 mM Mn^{2+} the calculated number of liganded P_i-binding molecules decreased by a factor of 2.3 and 3.6, respectively, due to $MeHPO_4$ formation with a concomitant decrease in the concentration of free P_i. These calculations agree well with the observed inhibition of P_i binding by divalent metal ions. The P_i-binding studies provide evidence for the binding of $H_2PO_4^-$ and HPO_4^{2-}, but not of $MeHPO_4$ by the phosphate-binding protein. Taken together, the results are in accordance with the translocation of both

mono- and dibasic phosphate via the primary P_i transport system of *A. johnsonii* 210A.

Discussion

The findings presented in this paper are consistent with the transport of a different set of P_i species by the two distinct P_i transport systems operating in *A. johnsonii* 210A.

Two lines of evidence provide substantial support for the translocation of a neutral $MeHPO_4$ complex via the secondary P_i transport system of *A. johnsonii* 210A. First, P_i uptake and efflux in proteoliposomes in which P_i carrier protein was reconstituted, was stimulated by various divalent cations in the following decreasing order: $Mn^{2+} > Co^{2+} \gg Ca^{2+} > Mg^{2+}$. This order is consistent with the decreasing order of the complexation equilibrium constants for $MeHPO_4$ (Table 1). Calculations using the acid dissociation constants of P_i and the complexation equilibrium constants of several metal phosphate chelates revealed the predominance of $MeHPO_4$ under the experimental conditions. During uptake, the P_i carrier exhibited a pH-independent K_t for $MeHPO_4$ in a pH range of 6.0 to 8.0 (33). The second line of evidence came from the demonstration of P_i-dependent transport of Mg^{2+} and Ca^{2+} in proteoliposomes. Excess Mg^{2+} inhibited the uptake of Ca^{2+} in the presence of P_i, but not the uptake of P_i in the presence of Ca^{2+}. Ca^{2+} and P_i were accumulated in equimolar quantities. The results presented in this and previous work (33) exclude other possible explanations for the metal dependence of P_i transport in proteoliposomes, such as binding of metal phosphate to membranes, internal precipitation of metal phosphate due to solute accumulation, or secondary effects of divalent cations on the magnitude or stability of the Δp_H+.

The binding protein-dependent, primary P_i transport system of *A. johnsonii* 210A most likely mediates the transport of HPO_4^{2-} and $H_2PO_4^-$. The uptake of $^{32}P_i$, but not of radiolabeled calcium was derepressed in cells under P_i limitation. The P_i uptake rate was about twice the calcium uptake rate in high-P_i-grown cells at substrate concentrations which saturate both P_i transport systems. These observations are consistent with the transport of P_i via the derepressible primary P_i

transport system and of $CaHPO_4$ via the constitutive secondary $MeHPO_4$ transport system, the maximal uptake rate of which is about equal to that of the primary P_i transport system in high-P_i-grown cells (32). The substrate specificity of the periplasmic P_i-binding protein of *A. johnsonii* 210A which is the initial receptor in the primary P_i transport system, was analyzed in concentrated periplasmic protein fractions. The K_D for P_i (0.59 ± 0.11 μM) closely matches the K_D (0.7 - 0.8 μM) of the periplasmic P_i-binding protein of *E. coli* (19, 20). The pH independence of the K_D and the significant inhibition of P_i binding by excess divalent cations indicate the binding of HPO_4^{2-} and $H_2PO_4^-$, but not of metal phosphate by the P_i-binding protein of *A. johnsonii* 210A. This conclusion is in agreement with X-ray diffraction studies of crystallized P_i-binding protein of *E. coli*, the structure of which reveals the atomic features responsible for P_i selectivity, either in monobasic or dibasic form (15, 17).

The primary P_i transport system of *A. johnsonii* 210A is a unidirectional uptake system for P_i (32). In contrast, the secondary $MeHPO_4$ transport system is reversible (32, 33) and provides *A. johnsonii* 210A with a major route for the entrance and exit of divalent metal ions and P_i. The activity of the latter system is closely related to the metabolism of cytoplasmic polyphosphate granules in which P_i and divalent metal ions are accumulated as was shown by EDAX analysis of these cellular inclusions. Their elemental composition reflected that of the predominant $MeHPO_4$ species in the uptake medium. These results are consistent with previous studies in *A. johnsonii* 210A (5) and other polyphosphate-accumulating microorganisms, *e.g.*, *Propionibacterium acnes* (14), *Spirillum itersonii*, *Corynebacterium diphtheriae*, *Micrococcus luteus* (34), *Micococcus lysodeikticus* (10), *Lactobacillus plantarum* (2), and *Plectonema boryanum* (3, 13), in which polyphosphate granules serve as the main sink for divalent metal ions such as Ba^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}. During polyphosphate degradation in *A. johnsonii* 210A, intracellular metal phosphate can be easily excreted via the secondary $MeHPO_4$ transport system.

Polyphosphate-accumulating *Acinetobacter* spp. can be used to efficiently remove P_i from waste water. In essence, P_i at low concentrations is removed biologically from a large volume of waste water under aerobic conditions and is concentrated through its release into a small volume of water during anaerobiosis (35).

Settled domestic waste water (pH 7.0) contains about 3 mM Ca^{2+}, 0.3 mM Mg^{2+} and 0.1 to 0.3 mM P_i (23). The presence of excess Ca^{2+} in these waters will affect P_i speciation due to extensive complexation. As a consequence, $CaHPO_4$ will prevail together with $H_2PO_4^-$ and HPO_4^{2-}. The results show that the P_i transport systems of *A. johnsonii* 210A are well adapted to the availability of $CaHPO_4$, $H_2PO_4^-$ and HPO_4^{2-} in the aquatic environment, allowing the organism to efficiently use P_i as a source of phosphorus.

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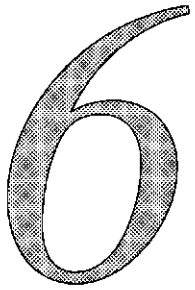
Energetics of alanine, lysine and proline transport in cytoplasmic membranes of the polyphosphate-accumulating *Acinetobacter johnsonii* strain 210A



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Summary

Amino acid transport in right-side-out membrane vesicles of *Acinetobacter johnsonii* 210A was studied. L-Alanine, L-lysine and L-proline were actively transported when a proton motive force of -76 mV was generated by the oxidation of glucose via the membrane-bound glucose dehydrogenase. Kinetic analysis of amino acid uptake at concentrations of up to 80 μM revealed the presence of a single transport system for each of these amino acids with a K_t of less than 4 μM. The mode of energy coupling to solute uptake was analyzed by imposition of artificial ion diffusion gradients. The uptake of alanine and lysine was driven by a membrane potential and a transmembrane pH gradient. In contrast, the uptake of proline was driven by a membrane potential and a transmembrane chemical gradient of sodium ions. The mechanistic stoichiometry for the solute and the coupling ion was close to unity for all three amino acids. The Na^+ dependence of the proline carrier was studied in greater detail. Membrane potential-driven uptake of proline was stimulated by Na^+ with a half-maximal Na^+ concentration of 26 μM. At Na^+ concentrations above 250 μM, proline uptake was strongly inhibited. Generation of a sodium motive force and maintenance of a low internal Na^+ concentration are most likely mediated by a sodium/proton antiporter, the presence of which was suggested by the Na^+-dependent alkalinization of the intravesicular pH in inside-out membrane vesicles. The results show that both H^+ and Na^+ can function as coupling ions in amino acid transport in *Acinetobacter* spp.

Introduction

Acinetobacter species are non-fastidious, gram-negative, strict aerobes which are ubiquitous in the environment (33). A vast number of complex catabolic pathways for the degradation of aromatic and alicyclic compounds as well as alkanes, amino acids, and related compounds in this genus are known (17). In contrast, knowledge about the transport processes which are involved in the uptake and efflux of these substances is limited. The entry of aromatic compounds into the cell is often assumed to occur by passive diffusion, but there is evidence that *Acinetobacter*

calcoaceticus possesses specific transport systems for benzoate and mandelate (8, 13, 14). The presence of a reversible L-lysine permease in *Acinetobacter johnsonii* 210A was indicated by counterflow activity of L-lysine in de-energized cells (37). In addition, L-alanine, L-glycine, L-leucine, and L-glutamate are translocated via secondary transport mechanisms in *A. calcoaceticus* (36).

A. johnsonii 210A is able to accumulate excessive amounts of phosphate as polyphosphate (35). When oxidative phosphorylation is impaired, polyphosphate is degraded and phosphate is excreted into the medium (34). Recently, membrane vesicles of *A. johnsonii* 210A were found to be an excellent model system for the study of the energetics of the secondary transport system involved in the phosphate efflux process (38). The results suggest a role for the phosphate carrier in the conservation of metabolic energy from polyphosphate degradation through the efflux of a metal phosphate complex in symport with one proton. In this way, a proton motive force can be generated in the absence of oxidative phosphorylation. This proton motive force may drive energy requiring processes, such as the uptake of amino acids.

In view of the limited insight into the bioenergetics of the transport of amino acids in *Acinetobacter* spp. and the possible energetic role of a metal phosphate efflux-induced proton motive force in the accumulation of these solutes in *A. johnsonii* 210A, we examined the presence and energetic requirements of several secondary amino acid transport systems in membrane vesicles of this organism. From the mechanism of energy coupling to the transport of amino acids in enteric and lactic acid bacteria and other microorganisms (3, 9), two different types of secondary amino acid uptake systems can be discriminated: (i) cation-amino acid symport systems, which couple amino acid translocation to the proton or sodium motive force and (ii) antiporters which couple uptake of an amino acid to the efflux of a product. Here, it is demonstrated that the alanine and lysine carriers of *A. johnsonii* 210A both translocate their solute in symport with one H^+. In contrast, the proline permease is absolutely dependent upon Na^+ at micromolar concentrations and catalyzes Na^+-proline symport. The low internal sodium concentration necessary for optimal proline uptake is achieved by the activity of an Na^+/H^+ antiporter.

Materials and methods

Cell growth and preparation of membrane vesicles

A. johnsonii 210A was grown at 30 °C with vigorous aeration in a Tris-buffered minimal salts medium (pH 7.0) supplemented with 20 mM sodium butyrate and 5 mM sodium phosphate (37). Right-side-out membrane vesicles were prepared by osmotic lysis of cells exposed to LiCl, a high concentration of lysozyme, and a temperature shock (38). Membrane vesicles were suspended in 50 mM potassium Pipes (pH 7.0) supplemented with 10 mM $MgSO_4$ at a final concentration of about 15 mg of protein per ml, rapidly frozen, and stored under nitrogen. For the preparation of inside-out membrane vesicles, the method of Ambudkar et al. (1) was modified. Cells were harvested in the mid exponential phase by centrifugation (8,000 x *g*, 10 min) and washed twice in lysis buffer [10 mM potassium Pipes, 10 mM potassium Hepes, 10% (vol/vol) glycerol, 0.5 mM dithiothreitol (pH 8.0)]. The cells were suspended at 5 volumes per g of wet cells in lysis buffer to which 10 μg of deoxyribonuclease I and 10 μg of ribonuclease per ml were added and lysed by one passage through a French pressure cell at 8,000 lb/in^2. After 10 min of incubation at 20 °C, unbroken cells and cell debris were removed from the lysate by centrifugation (8,000 x *g*, 10 min). Inside-out membrane vesicles were pelleted by centrifugation (100,000 x *g*, 2 h), suspended in lysis buffer at a final concentration of about 8 mg of protein per ml, rapidly frozen and stored under nitrogen. All centrifugations were performed at 4 °C.

Solute transport

Solute uptake in right-side-out membrane vesicles driven by a proton motive force (Δp_H+, interior negative and alkaline) generated by glucose oxidation via the membrane-bound glucose dehydrogenase was measured essentially as described previously (38). Membrane vesicles were washed and suspended in 50 mM potassium Pipes (pH 7.0) or 20 mM potassium Mes, 20 mM potassium Pipes, 20 mM potassium Hepes (pH 5.0 to 8.0) supplemented with 10 mM $MgSO_4$. To start

uptake, ^{14}C-labeled proline, alanine, or lysine was added to the membrane vesicle suspensions at a concentration of 1.95, 3.23, or 1.62 μM, respectively, unless indicated otherwise. Transport was terminated by dilution of 100 μl of reaction mixture with 2 ml of ice-cold 0.1 M KCl and filtration on a 0.45 μm cellulose nitrate filter. Filters were washed once with 2 ml of ice-cold 0.1 M KCl. The radioactivity retained on the filters was measured by liquid scintillation spectrometry.

For uptake driven by an artificially imposed membrane potential ($\Delta\psi$, interior negative), transmembrane pH gradient (ΔpH, interior alkaline), transmembrane chemical gradient of sodium ions (ΔpNa, interior low), or combinations thereof, right-side-out membrane vesicles were equilibrated overnight at 4 °C in 20 mM potassium Mes, 20 mM potassium Pipes (pH 6.5 or 7.0) supplemented with 100 mM potassium acetate and 10 mM $MgSO_4$, concentrated to about 17 mg of protein per ml, and diluted as described previously (38) and in the text (see below). Potassium- and sodium-free dilution buffers were titrated with choline hydroxide. All dilution media were supplemented with 10 mM $MgSO_4$ and radiolabeled substrates at the concentrations described above. Subsequent steps were as described for Δp_H+-driven uptake. A specific internal volume of 3 μl per mg of membrane protein was used for calculation of the intravesicular concentration of the solute (18, 30).

Determination of Δp_H+

The $\Delta\psi$ (interior negative) in right-side-out membrane vesicles energized by glucose oxidation was determined as described before (38) from the distribution of the lipophilic cation TPP^+, with a TPP^+-selective electrode (29). The $\Delta\psi$ was calculated from the steady-state level of TPP^+ accumulation and corrected for concentration-dependent, nonspecific binding of TPP^+ to the membranes (20). The ΔpH (interior alkaline) in right-side-out membrane vesicles was calculated from the increase in $\Delta\psi$ after addition of nigericin, assuming a complete interconversion of ΔpH into $\Delta\psi$ (11).

Determination of internal pH

The internal pH in inside-out membrane vesicles was estimated from the fluorescence of the pH indicator BCECF trapped in the intravesicular space (23). The membrane vesicles were loaded directly with BCECF by lysis of cells as described above under "Cell growth and preparation of membrane vesicles" in lysis buffer supplemented with 40 μM BCECF. BCECF-loaded membrane vesicles were washed in a 1,000-fold volume of 10 mM potassium Pipes, 10 mM potassium Hepes (pH 8.0) supplemented with 10 mM $MgSO_4$ and suspended in this buffer to about 0.2 mg of protein per ml. A Δp_H+ (interior positive and acid) was generated in the membrane vesicles at 25 °C by the addition of 3 mM potassium ATP (pH 8.0). Changes in the intravesicular pH were monitored by continuous recording of BCECF fluorescence, with excitation and emission wavelengths of 502 and 526 nm, respectively, and slit widths of 5 nm. The fluorescence signal was averaged over time intervals of 0.3 s and calibrated in the pH range from 6 to 9 by measuring the fluorescence as a function of pH in the presence of 1 nmol of valinomycin and nigericin per mg of protein (internal pH equals external pH).

Other methods

Protein content was determined by the method of Lowry et al. (21) with bovine serum albumin as the standard.

Chemicals

Cellulose nitrate filters were supplied by Schleicher und Schuell, Dassel, Germany. L-[U-^{14}C]alanine (6.9 TBq/mol), L-[U-^{14}C]lysine monohydrochloride (11.8 TBq/mol), and L-[U-^{14}C]proline (9.5 TBq/mol) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, UK. BCECF was obtained from Sigma Chemicals Co., St. Louis, MO, USA. Other chemicals were reagent grade and obtained from commercial sources.

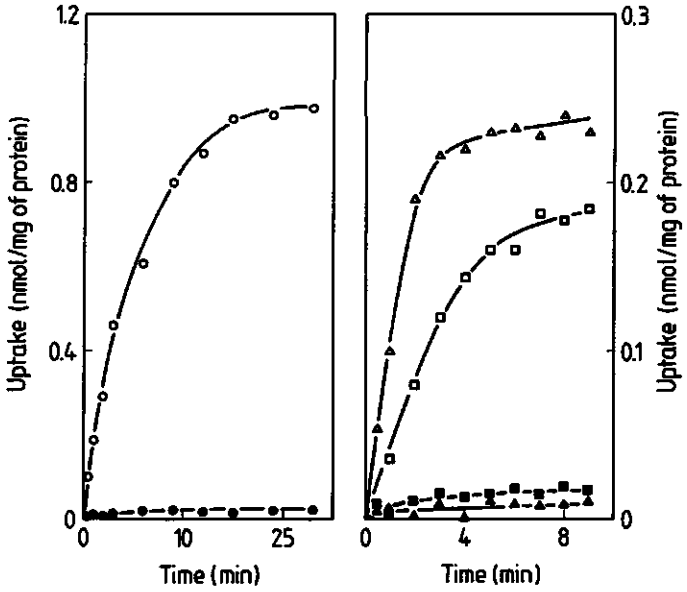

Figure 1. Transport of lysine, alanine and proline in right-side-out membrane vesicles. Uptake of lysine (○,●), alanine (□,■), and proline (△,▲) was measured in the presence (open symbols) and absence (solid symbols) of glucose plus PQQ.

Results

Kinetics and specificity of amino acid uptake

L-Alanine, L-lysine, and L-proline were accumulated about 20-, 200- and 40-fold, respectively, in right-side-out membrane vesicles of *A. johnsonii* 210A in 50 mM potassium Pipes (pH 7.0) supplemented with 10 mM $MgSO_4$, in which a Δp_H+ of -76 mV was generated by glucose oxidation via the membrane-bound glucose dehydrogenase (Fig. 1).

The dependence of the transport activity on the amino acid concentration, as evaluated from the initial rates of linear uptake over the first 30 s at substrate concentrations of between 0.3 and 80 μM, followed monophasic Michaelis-Menten kinetics. The uptake of these amino acids showed apparent K_t values of less than 4

Table 1. Kinetic parameters of alanine, lysine, and proline uptake in right-side-out membrane vesicles[a]

amino acid	K_t (μM)	V_{max} (nmol of amino acid/min/mg of protein)
L-alanine	3.0 ± 0.9	0.09 ± 0.02
L-lysine	1.3 ± 0.2	0.32 ± 0.07
L-proline	2.5 ± 0.8	0.21 ± 0.06

[a] Kinetic data were determined by linear regression analysis of Lineweaver-Burk plots. Values are means from three separate experiments.

μM and maximal velocities of 0.1 to 0.3 nmol/min/mg of protein (Table 1). The Δp_H+-driven accumulation of lysine was not affected when a 100-fold excess of unlabeled L-ornithine or L-arginine was added to the incubation mixture at the start of the uptake experiment or after a steady state level of lysine accumulation had been reached (data not shown).

Energetics of amino acid transport

The Δp_H+-driven uptake of alanine, lysine, and proline was affected differently by the external pH. Alanine and lysine transport showed an optimum activity at medium pHs of between 6 and 7, whereas the proline uptake rate increased with increasing pH up to an optimum of above pH 8.0 (Fig. 2A). Comparison of the amino acid uptake rate and the Δp_H+ in the course of the pH dependence (Fig. 2B) showed a clear correlation between these parameters for alanine and lysine transport but suggested the influence of other parameters on proline uptake.

The mode of energy coupling to the transport of alanine, proline, and lysine was studied in greater detail in right-side-out membrane vesicles in which artificial ion diffusion gradients were generated. A $\Delta\psi$ (interior negative) of -120 mV was created by diluting potassium-loaded membrane vesicles 100-fold into a potassium-free buffer in the presence of the potassium ionophore valinomycin. Dilution of potassium-loaded membrane vesicles into a sodium buffer resulted in the generation

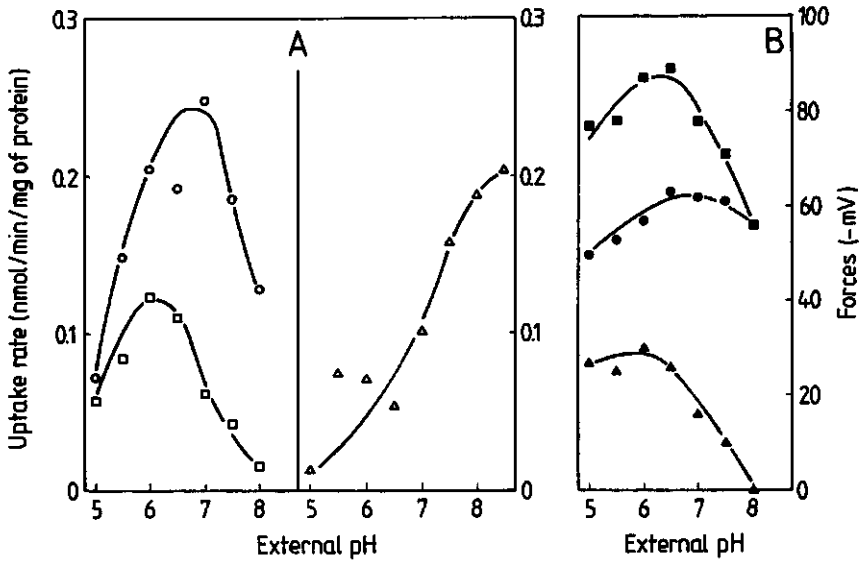

Figure 2. Effect of pH on the rate of Δp_H+-driven uptake of amino acids (A) and the magnitude and composition of the Δp_H+ (B) in right-side-out membrane vesicles. Initial velocities of uptake of lysine (○), alanine (□), and proline (△) were determined over the first 30 s. The $\Delta\psi$ (●), -$Z\Delta$pH (▲), and Δp_H+ (= $\Delta\psi$ - $Z\Delta$pH) (■) were determined in parallel assays under the same conditions used to measure amino acid uptake.

of an inwardly directed -$Z\Delta$pNa of -120 mV. A -$Z\Delta$pH (interior alkaline) of -120 mV was imposed by diluting acetate-loaded membrane vesicles 100-fold into a solution containing Mes. Alanine and lysine were accumulated in response to an artificially imposed ΔpH or $\Delta\psi$ (Fig. 3A and B). When a combination of both gradients was applied, the highest alanine and lysine uptake was measured. $\Delta\psi$- and ΔpH-driven uptake of these amino acids was additive. In contrast, an imposed ΔpNa could not drive the uptake of alanine and lysine, and the Δp_H+-driven uptake was not affected by the simultaneous imposition of a ΔpNa. Proline accumulated in response to an artificial $\Delta\psi$ (Fig. 3C). Uptake was stimulated significantly when a ΔpNa was applied. The highest uptake of proline was measured after imposition of a sodium motive force (Δp_{Na}+). A ΔpH imposed alone or on top of a $\Delta\psi$ or Δp_{Na}+ did not affect the uptake of proline. The results point to the translocation of alanine,

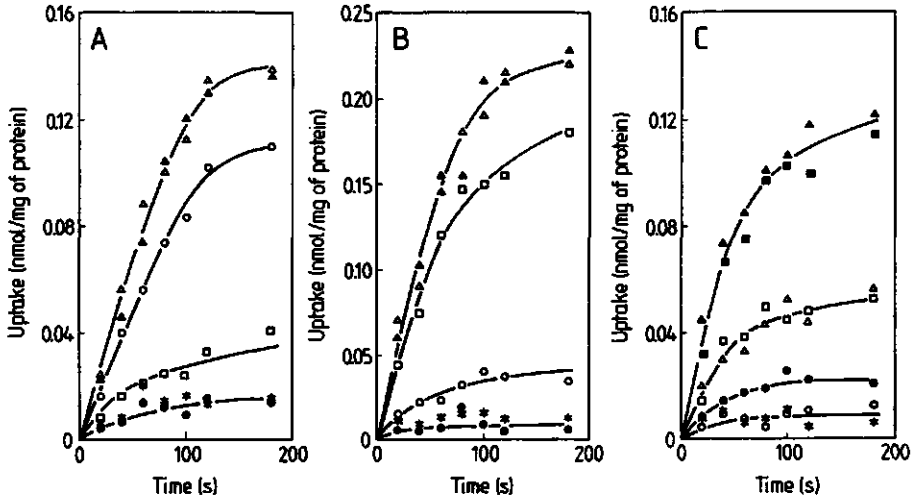

Figure 3. Effect of artificially imposed ion diffusion gradients on amino acid transport in right-side-out membrane vesicles. The uptake of alanine (A) and lysine (B) at pH 6.5 and of proline (C) at pH 7.0 was measured in the absence (✳) and presence of a $\Delta\psi$ (interior negative) (□), ΔpH (interior alkaline) (○), ΔpNa (interior low) (●), Δp_{H^+} (△), Δp_{Na^+} (■), or Δp_{H^+} and ΔpNa (▲).

lysine, and proline via electrogenic processes with H^+ as the symported ion for alanine and lysine, and Na^+ as symported ion for proline.

Na^+ dependency of the proline transport system

The effect of Na^+ on the transport of lysine, alanine, and proline was investigated. Because of the low levels of Na^+ contamination in the inorganic salts used for the preparation of buffers and leeching of Na^+ from glassware, the "sodium-free" buffers used in the experiments described above contained up to 150 μM Na^+. Na^+ contamination in buffers could be reduced to concentrations of less than 10 μM by the use of polypropylene containers and tubes for buffer preparation and uptake measurements, respectively, and by titration of buffers with choline hydroxide instead of potassium hydroxide. Right-side-out membrane vesicles were equilibrated in 50 mM potassium Pipes (pH 7.0) containing 10 mM $MgSO_4$ and Na^+ at

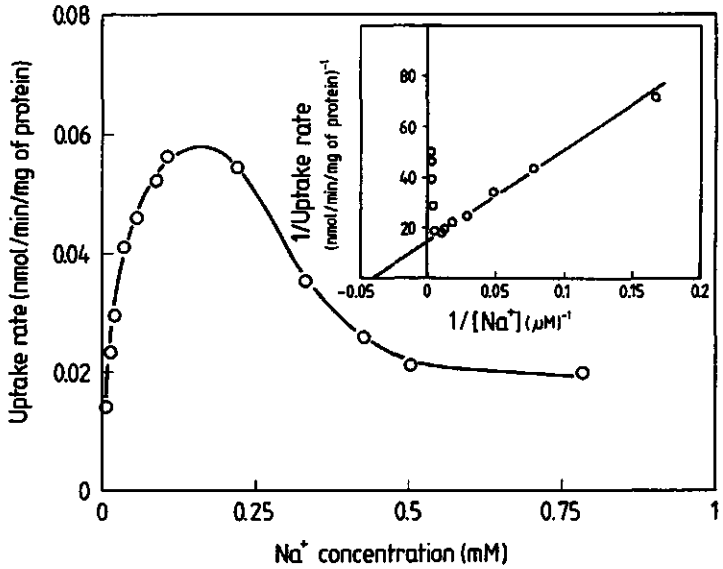

Figure 4. Effect of Na^+ concentration on the $\Delta\psi$-driven uptake of proline in right-side-out membrane vesicles. The initial transport rates were determined over the first 20 s of uptake. The external Na^+ concentration was measured by atomic absorption spectrometry. The data were corrected for the Na^+ concentration. (Inset) Double reciprocal plot of the data.

concentrations ranging from about 40 μM (no Na^+ added) to 750 μM. A $\Delta\psi$ of -162 mV was imposed by a 500-fold dilution of the membrane vesicles in 50 mM choline Pipes (pH 7.0) containing 10 mM $MgSO_4$ and Na^+ at concentrations ranging from about 6 μM (no Na^+ added) to 750 μM. During $\Delta\psi$-driven uptake of the amino acids, the internal sodium concentration was about equal to the external one at Na^+ concentrations of more than 40 μM. All actual Na^+ concentrations in the dilution buffer were measured by atomic absorption spectrometry. Imposed $\Delta\psi$-driven uptake of proline was stimulated by considerably low concentrations of Na^+ (Fig. 4). A double-reciprocal plot of Na^+ concentration versus the initial velocity of amino acid transport over the first 20 s of uptake revealed a half-maximal Na^+ concentration of about 26 μM (Fig. 4, inset). At Na^+ concentrations higher than about 250 μM, inhibition of uptake was observed. In contrast, imposed $\Delta\psi$-driven

uptake of alanine and lysine was not affected by Na^+ (not shown).

Mechanistic stoichiometry of energy coupling

The cation/amino acid stoichiometry was determined from the steady state accumulation levels of alanine, proline, and lysine in right-side-out membrane vesicles in 50 mM potassium Pipes (pH 7.0) containing 10 mM $MgSO_4$, in which a $\Delta\psi$ of -82 mV was generated by glucose oxidation in the presence of 0.1 nmol each of monensin and nigericin per mg of protein. Steady state levels of amino acid accumulation were reached in 25 min for lysine and in 10 min for alanine and proline (Fig. 1). In the absence of a ΔpH or ΔpNa, solute/cation symport occurs according to the equation $-Z\Delta p_{solute} = (n + m)\ \Delta\psi$, in which $-Z\Delta p_{solute}$ represents the transmembrane concentration gradient of the solute (in millivolts) and n is the number of cations translocated in symport with one molecule of solute with charge m. Calculation of n yielded a value of 0.92 for alanine, 0.94 for lysine, and 1.12 for proline. Together, these results strongly suggest that alanine and lysine are transported in symport with one proton, whereas proline is transported in symport with one sodium ion.

Na^+/H^+ exchange activity

In membrane vesicles of *A. johnsonii* 210A, glucose oxidation via glucose dehydrogenase is functionally linked to redox reaction-coupled primary H^+ translocation by the respiratory chain and results primarily in the generation of a Δp_H+ (37). In order to evaluate the role of an Na^+/H^+ antiporter in the conversion of the Δp_H+ into the $\Delta p_{Na}+$, evidence for the presence of such an antiporter was sought in inside-out membrane vesicles loaded with the fluorescent pH probe BCECF. After the addition of 3 mM potassium ATP to inside-out membrane vesicles, the fluorescence was quenched, reflecting the formation of a ΔpH (interior acid) through proton pumping via the H^+-ATPase (Fig. 5). The fluorescence intensity recovered rapidly after the dissipation of the Δp_H+ by the addition of valinomycin plus nigericin (each at 1 nmol/mg of protein). The fluorescence signal in membrane vesicles was not affected

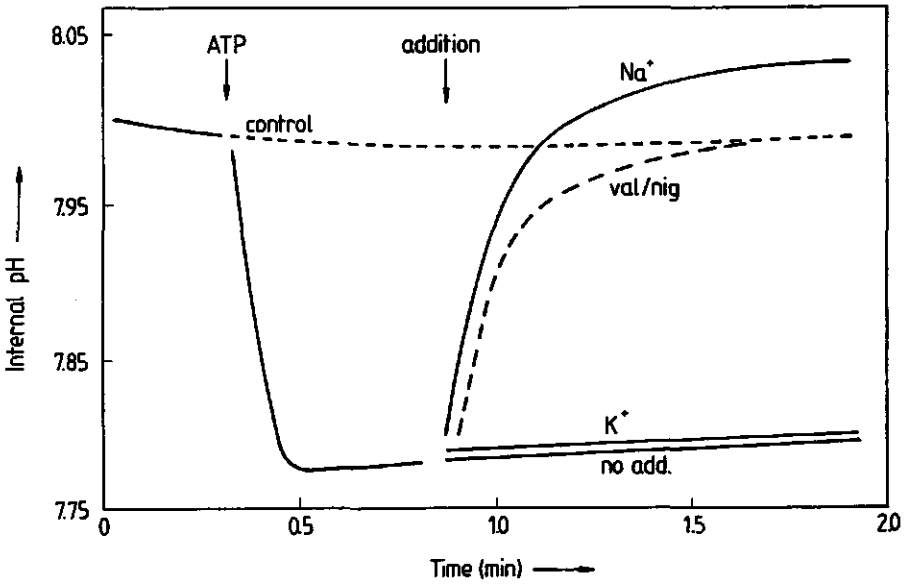

Figure 5. Na^+-dependent alkalinization of the internal pH in inside-out membrane vesicles. BCECF fluorescence quenching was initiated by the addition of 3 mM potassium ATP at the time indicated by the first arrow. A control measurement was performed in which ATP was replaced by an equal volume of buffer (dashed line labeled control). At the time indicated by the second arrow, 5 mM KCl, 1 nmol each of valinomycin and nigericin per mg of protein, or 5 mM NaCl was added to the membrane vesicles, or no addition was made (no add.).

by the addition of 5 mM K^+ to the external milieu. However, a rapid alkalinization of the intravesicular pH was observed after the addition of 5 mM Na^+. This exchange of H^+ for Na^+ indicated the presence of a sodium/proton antiporter in cytoplasmic membranes of *A. johnsonii* 210A.

Discussion

In this study, the transport of alanine, lysine, and proline was analyzed in right-side-out membrane vesicles of *A. johnsonii* 210A. Kinetic analysis of the uptake of these amino acids at substrate concentrations in the range from 0.3 to 80 μM gave

evidence for the presence of single, high-affinity carriers for each of these solutes.

Lysine and alanine were actively accumulated in right-side-out membrane vesicles in which a Δp_H+ was generated by glucose oxidation. Uptake was also observed in response to an artificially imposed Δp_H+. An imposed membrane potential and ΔpH, but not a ΔpNa, could act separately as a driving force for the uptake of these solutes. In contrast to alanine, which is more or less neutral at physiological pHs for *A. johnsonii* (pH 5 to 8), lysine is positively charged because of the high pK_a of its ϵ-amino group (pK_a = 10.5). Consequently, the membrane potential is expected to be a main driving force for lysine accumulation. Comparison of (i) the rates of Δp_H+-driven uptake of alanine and lysine in membrane vesicles at a Δp_H+ of different magnitudes and compositions in the course of the pH dependence and (ii) the uptake of these solutes in response to an artificially imposed membrane potential or ΔpH confirm that lysine transport is biased towards the $\Delta\psi$.

Analysis of the steady-state accumulation levels of $\Delta\psi$-driven uptake of alanine and lysine, and the magnitude of $\Delta\psi$ suggest the transport of these amino acids with an H^+/solute stoichiometry of one. The lack of inhibition of Δp_H+-driven lysine uptake in membrane vesicles by excess arginine and ornithine in competition experiments and the lack of heterologous lysine:arginine and lysine:ornithine exchange demonstrate (i) the high specificity of the lysine carrier of *A. johnsonii* 210A for lysine and (ii) the absence of a lysine/ornithine/arginine antiporter (LAO system), as has been observed in *Escherichia coli* (28), *Lactococcus lactis* (12, 27), and *Pseudomonas aeruginosa* (39). In addition, osmotic shock-insensitive lysine uptake in cells of *A. johnsonii* 210A (37) indicates that a binding protein-dependent system is not involved in the uptake of lysine. Together, the results point to the presence of one lysine-specific permease in *A. johnsonii* 210A grown in synthetic minimal medium, which mediates the translocation of lysine in symport with one proton. This system is possibly related to the Δp_H+-driven lysine carrier of *L. lactis* (12) and to the *lysP* gene-encoded lysine-specific permease of *E. coli* (28, 31).

Although rapid Δp_H+-driven uptake of proline was observed in right-side-out membrane vesicles energized by glucose oxidation, a detailed analysis of the energetics of transport through imposition of artificial diffusion gradients suggested the involvement of the $\Delta\psi$ and ΔpNa but not of the ΔpH as a driving force for uptake.

Determination of the proline/Na^+ stoichiometry indicates the translocation of proline together with one Na^+. The absolute dependence of proline transport on Na^+ could be demonstrated only when special precautions were taken to reduce the sodium contamination in the uptake medium to 6 μM. Under these conditions, the rate of proline uptake was very low and stimulated by an increase in the (external) sodium concentration, with a half-maximal Na^+ concentration of 26 μM. Without these precautions, the amount of contaminating Na^+ in the uptake medium greatly exceeded the low apparent K_t value of the proline carrier for sodium ions, resulting in the observation of active uptake of proline in the absence of added sodium ions. On the other hand, Na^+ concentrations above 250 μM ($[Na^+]_{inside} \simeq [Na^+]_{outside}$) were inhibitory for proline transport. By analogy with internal catalytic pH effects on proton symport systems (10, 19, 38), this observation may reflect the inhibition by high internal sodium ion concentrations of Na^+ dissociation from the proline carrier protein at the inner surface of the membrane. Strict regulation of the internal Na^+ concentration during the uptake of proline via a sodium symport mechanism will be essential for retaining optimal proline transport via this carrier.

The Na^+-dependent alkalinization of the intravesicular pH of inside-out membrane vesicles of *A. johnsonii* 210A at alkaline pH suggests the presence of a sodium/proton antiporter which may have functions similar to those of the *nhaA* gene-encoded system of *E. coli*: (i) regulation of internal pH (4, 25), (ii) energy buffering (5), (iii) generation of a Δp_{Na}+ (7), and (iv) maintenance of a low internal sodium concentration (24). The activity of NhaA was found to increase at least 100-fold when the pH was increased from 6.6 to 8.5 (26). A similar pH regulation of the NhaA-like system of *A. johnsonii* 210A could explain the increase in the rate of Δp_{Na}+-driven uptake of proline with increasing pH in right-side-out membrane vesicles energized by glucose oxidation.

The proline permease of *A. johnsonii* 210A resembles the major proline permease (PPI) of *E. coli* (16, 32) and *Salmonella typhimurium* (6). *E. coli* and *S. typhimurium* contain a second secondary proline transport system, designated PPII. This system has a low affinity for proline and glycine-betaine (K_t for proline 130 μM) (2), is activated by a hyperosmotic shift (22), and is involved in osmoregulation (15). Since the experimental conditions used in this study hardly allow the

detection of a PPII-like system in membrane vesicles of *A. johnsonii* 210A, the presence of this system remains to be established.

The results presented in this article demonstrate the presence of high-affinity secondary transport systems for L-alanine, L-lysine, and L-proline in *A. johnsonii* 210A. The alanine and lysine carriers translocate their solute in symport with a proton, whereas the proline permease mediates Na^+/proline symport. Although the role of amino acid transport systems in amino acid-auxotrophic organisms, such as lactic acid bacteria, is obviously to supply amino acids for growth, *Acinetobacter* species are able to fulfill the amino acid demand of the cell by de novo synthesis. Unlike lactic acid or enteric bacteria, acinetobacters are found in environments that are normally subject to considerable variation in composition, temperature, and oxygen supply (33). High-affinity uptake systems, *e.g.*, for amino acids, will enable *Acinetobacter* spp. to scavenge the environment for traces of metabolizable substrates and to recapture endogenous compounds leaking out of the cell. Retention of metabolites will become particularly important when oxidative phosphorylation is impaired in these aerobic organisms. In the polyphosphate-accumulating *A. johnsonii* strain 210A, generation of a Δp_H+ via efflux of a neutral metal phosphate ($MeHPO_4$) complex in symport with a proton may contribute to the survival of the cell under such conditions.

Acknowledgements

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7

Generation of a proton motive force by the excretion of metal phosphate in the polyphosphate-accumulating *Acinetobacter johnsonii* strain 210A

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Summary

The strictly aerobic, polyphosphate-accumulating *A. johnsonii* strain 210A degrades its polyphosphate when oxidative phosphorylation is impaired. The endproducts of this degradation, divalent metal ions and phosphate, are excreted as a neutral metal phosphate ($MeHPO_4$) chelate via the electrogenic $MeHPO_4/H^+$ symport system of the organism. The coupled excretion of $MeHPO_4$ and H^+ in *A. johnsonii* 210A can generate a proton motive force. In membrane vesicles and deenergized cells, a membrane potential of about -70 mV and transmembrane pH gradient of about -8 mV were formed in response to an imposed outwardly directed $MeHPO_4$ concentration gradient of 120 mV (initial value). The $MeHPO_4$ efflux-induced proton motive force could drive energy requiring processes, such as the accumulation of L-proline and L-lysine, and the synthesis of ATP via the membrane-bound F_0F_1 H^+-ATPase. *In vivo* ^{31}P-NMR studies of polyphosphate degradation in anaerobic cell suspensions revealed the presence of a considerable outwardly directed phosphate gradient across the cytoplasmic membrane, corresponding to a $MgHPO_4$ concentration gradient of at least 100 mV. This $MgHPO_4$ concentration gradient was maintained for several hours. Thus, energy recycling by $MeHPO_4/H^+$ efflux will contribute significantly to the overall production of metabolic energy from the degradation of polyphosphate in *A. johnsonii* 210A.

Introduction

Activated sludge in wastewater treatment plants is enriched with polyphosphate-accumulating bacteria, *e.g.* from the strictly aerobic genus *Acinetobacter*, when alternating aerobic and anaerobic conditions are applied (12, 14). The polyphosphate metabolism of one of these strains, *Acinetobacter johnsonii* 210A, has been studied in detail (6, 9, 10, 44, 48, 52). In the presence of excess energy and substrates, *A. johnsonii* 210A accumulates large amounts of P_i and metal ions as metal polyphosphate in granules in the cytosol. Under these conditions, the organism is able to take up the predominant P_i species from its aquatic environment by the concerted operation of two P_i transport systems (49). An ATP-driven, periplasmic binding

protein-dependent system catalyzes the unidirectional uptake of $H_2PO_4^-$ and HPO_4^{2-} (K_t for P_i of 0.7 μM) (49, 52). The synthesis of this system is repressed by P_i at medium concentrations above 10 μM (9, 49). In addition, the organism possesses a constitutive secondary transport system mediating the uptake of a neutral $MeHPO_4$ chelate via an electrogenic proton symport mechanism (K_t for $MeHPO_4$ of 7.7 μM) (49, 50, 52). This chelate is formed by complexation of HPO_4^{2-} and divalent cations like Mg^{2+}, Ca^{2+}, Co^{2+} or Mn^{2+} (52).

Two enzymes are involved in the degradation of polyphosphate in *A. johnsonii* 210A: (i) polyphosphatase, and (ii) polyphosphate:AMP phosphotransferase (44). Polyphosphatase catalyzes the hydrolysis of polyphosphate to P_i (8, 44). The activity of this enzyme enables the organism to use metal polyphosphate as a source of P_i and divalent cations when the environmental concentrations of these nutrients are limiting (45, 47). Polyphosphate:AMP phosphotransferase catalyzes the phosphorylation of AMP to ADP with polyphosphate as phosphoryl donor (7, 44). The subsequent conversion by adenylate kinase of two molecules of ADP into one molecule of AMP and ATP, enables *A. johnsonii* to regenerate AMP for the phosphotransferase reaction and to use its polyphosphate as a source of ATP when oxidative phosphorylation is impaired, *e.g.* under anaerobic conditions (43, 46). During the degradation of metal polyphosphate in *A. johnsonii* 210A, P_i and metal divalent ions are excreted into the environment via the secondary $MeHPO_4$ transport system (47, 49, 52).

Besides the direct synthesis of ATP via the polyphosphate:AMP phosphotransferase/adenylate kinase pathway, *A. johnsonii* 210A may conserve metabolic energy from polyphosphate degradation by the reversed process of $MeHPO_4$ uptake via the secondary $MeHPO_4$ transport system (27, 49, 50). During $MeHPO_4$ uptake the energy of the electrochemical proton gradient is converted into the energy of a chemical $MeHPO_4$ gradient, whereas during $MeHPO_4$ efflux the energy of a chemical $MeHPO_4$ gradient may be converted back into the energy of an electrochemical proton gradient (50). In this paper, experimental support is given for this energy recycling mechanism. Energy transduction to electrogenic $MeHPO_4/H^+$ efflux was studied in deenergized cells and membrane vesicles of *A. johnsonii* 210A. In addition, *in vivo* ^{31}P-NMR was used to examine polyphosphate degradation and

$MeHPO_4$ efflux in cells under physiological conditions.

Materials and methods

Cells growth and preparation of membrane vesicles

A. johnsonii 210A was grown at 30 °C in a Tris-buffered medium (pH 7.0) supplemented with 20 mM sodium butyrate and 5 mM or 20 μM sodium phosphate for cultivation of high-P_i and low-P_i-grown cells, respectively (49). Cells were harvested in midexponential phase by centrifugation (7,000 x *g*, 10 min). Membrane vesicles were prepared by osmotic lysis of high-P_i grown cells exposed to lithium chloride, a high concentration of lysozyme and a temperature shock (50).

Polyphosphate degradation in cells

High P_i-grown cells containing polyphosphate granules and low P_i-grown control cells in which polyphosphate granules were absent, were washed twice in ice-cold 50 mM Tris-HCl (pH 7.8). To permeabilize the outer membrane, cells were given an EDTA treatment as described previously (49). Subsequently, cells were washed in ice-cold 50 mM Tris-HCl (pH 7.8) and resuspended in this buffer to about 5.5 mg of protein per ml. The cell suspensions were transferred to Hungate tubes, flushed for 10 min with oxygen-free N_2, and incubated anaerobically at 30 °C.

$MeHPO_4$ efflux from preloaded cells and membrane vesicles

High-P_i grown cells were deenergized by incubation for 12 h at 30 °C in 20 mM potassium Pipes (pH 7.0) supplemented with 10 mM $MgSO_4$, 2.5 mM α-dinitrophenol and 50 μg chloramphenicol per ml (49). Depletion of endogenous energy reserves was followed in time by measuring the endogenous respiration rate of the cells. Cells were treated with EDTA (49), washed extensively and resuspended to a concentration of 0.5 mg protein per ml in 20 mM potassium Pipes (pH 7.5) containing 10 mM $MgSO_4$ and 50 μg chloramphenicol per ml (Buffer A). Cells were

equilibrated overnight at 4 °C in Buffer A supplemented with 5 mM potassium phosphate (pH 7.5) [about 3 mM $MgHPO_4$ (52)]. The cell suspensions were concentrated to about 14 mg protein per ml. An outwardly directed $MeHPO_4$ concentration gradient of 120 mV was imposed by diluting preloaded cells 100-fold into 20 mM potassium Pipes (pH 7.5) containing 50 μg chloramphenicol per ml (Buffer B). Deenergized cells equilibrated in Buffer A without added P_i served as a control. Membrane vesicles in 10 mM potassium Pipes (pH 7.5) supplemented with 10 mM $MgSO_4$ (Buffer C) were equilibrated in the presence and absence of 5 mM potassium phosphate (pH 7.5), and diluted in 10 mM potassium Pipes (pH 7.5) (Buffer D) as described for deenergized cells.

Determination of membrane potential ($\Delta\psi$)

The $\Delta\psi$ (interior negative) in cells was determined from the distribution of the lipophilic tetraphenylphosphonium ion (TPP^+), using a TPP^+-selective electrode (36). For the determination of the $\Delta\psi$ in cells during polyphosphate degradation, 800 μl of 50 mM Tris-HCl (pH 7.8) supplemented with 5 mM potassium cyanide and 5 μM TPP^+, was added to the TPP^+ electrode vessel. The vessel was sealed with a rubber septum. The buffer inside the vessel was flushed for 10 min with oxygen-free N_2. Anaerobic cell samples of 200 μl were injected into the vessel after which TPP^+ accumulation was monitored. The induction of a $\Delta\psi$ by $MeHPO_4$ efflux in deenergized cells was monitored with a TPP^+-electrode by diluting $MeHPO_4$-loaded cells 100-fold in Buffer B supplemented with 4 μM TPP^+. The ability of deenergized cell to generate a proton motive force (Δp_H+) by the oxidation of glucose under aerobic conditions was checked by measuring the $\Delta\psi$ (interior negative) as described previously (49). For calculations, an internal cell volume of 3 μl per mg of protein was used (4, 18, 41). Measurements were corrected for concentration-dependent, nonspecific probe binding according to the model of Lolkema et al. (21). For qualitative measurements of a $MeHPO_4$ efflux-induced $\Delta\psi$ in membrane vesicles, $MeHPO_4$-loaded membrane vesicles were diluted 100-fold in Buffer D supplemented with 9 μM of the membrane potential indicator 3,3'-dipropylthiocarbocyanine iodide [$DiSC_3(5)$]. The $\Delta\psi$-dependent fluorescence quenching

was measured at excitation and emission wavelengths of 637 nm and 667 nm, respectively, with slit withs of 10 nm. The fluorescence signal was averaged over time intervals of 0.3 s and calibrated in a $\Delta\psi$ range of -44 mV to -90 mV by measuring the fluorescence as a function of an artificially imposed potassium diffusion potential (50).

Determination of internal pH

The internal pH in cells and membrane vesicles was estimated from the fluorescence of the pH indicator BCECF entrapped in the intracellular or intravesicular space. $MeHPO_4$-loaded and control cells were loaded with BCECF by an acid shock treatment as described by Molenaar et al. (28), washed five times with 1 ml Buffer A with or without 5 mM potassium phosphate, respectively, and resuspended in these buffers to about 5 mg protein per ml. The cells containing BCECF (as judged from the yellow colour of cell pellets) after treatment with the smallest amount of HCl, were used for further experiments. Membrane vesicles were loaded with BCECF and $MeHPO_4$ by freeze-thaw-sonication (13). An outwardly directed $MeHPO_4$ gradient was imposed in $MeHPO_4$ /BCECF-loaded cells and membrane vesicles as described under "$MeHPO_4$ efflux from preloaded cells and membrane vesicles". Changes in the internal pH during $MeHPO_4$ efflux were monitored by continuous recording of BCECF fluorescence as described (51).

Amino acid transport assays

The uptake of 1.62 μM L-lysine and 1.95 μM L-proline in membrane vesicles was measured via the filtration method (17) as described (52).

^{31}P-NMR spectroscopy of intact cells

Low-P_i-grown cells were washed and suspended to about 7 mg protein per ml in 150 mM potassium phosphate (pH 7.0) supplemented with 5 mM $MgSO_4$. To allow the synthesis of polyphosphate, cells were kept under continuous aeration with

oxygen at 21 °C. After 60 min of incubation, cells were washed twice in 50 mM Tris-Cl (pH 7.0) and resuspended to about 15 mg protein per ml in 50 mM Tris-Cl (pH 7.8) supplemented with 10 mM potassium chloride and 2 mM $MgSO_4$. Subsequently, the cell suspension was kept in a 10 mm NMR tube and gassed with argon using an air-lift system. ^{31}P-NMR experiments were recorded using a 10 mm broadband probehead in a Bruker AMX500 spectrometer operating at 202.45 MHz for phosphorus. Spectra were acquired at 25 °C, without proton decoupling, using a 45° flip angle and 5.8 s repetition delay in 16 K data points. Phosphorus resonances were referenced with respect to external 85% H_3PO_4.

Miscellaneous

P_i and total phosphorus, degraded to P_i by preliminary persulfate digestion (1), were determined colorimetrically by the ascorbic acid method (1). The presence of polyphosphate granules in cells was evaluated by light-microscopy after staining according to Neisser (15). For the determination of intracellular ATP concentrations, cells were extracted with perchloric acid by the method of Otto et al. (29). ATP in neutralized extracts was determined with a firefly bioluminescence assay (LUMIT). The respiration rate of cells was measured with a Clark-type oxygen electrode. Protein was determined by the method of Lowry et al. (22) using bovine serum albumin as the standard.

Chemicals

$DiSC_3(5)$ and BCECF were obtained from Sigma Chemicals Co., St. Louis, MO, USA. The firefly bioluminescence NRB/LUMIT-PM kit was purchased from Lumac, Omnilabo, Breda, The Netherlands. L-[U-^{14}C]lysine (6.9 TBq/mol) and L-[U-^{14}C]proline (9.5 TBq/mol) were supplied by the Radiochemical Centre, Amersham, Buckinghamshire, UK. Other chemicals were reagent grade and obtained from commercial sources.

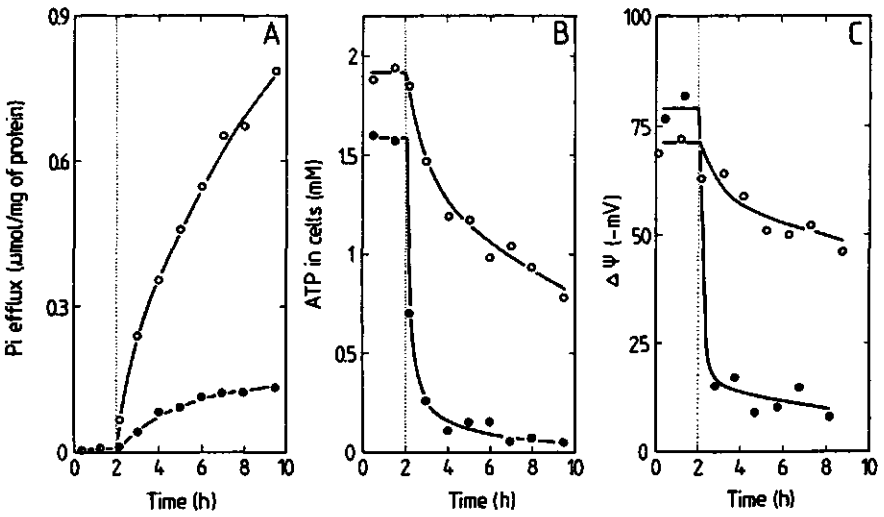

Figure 1. Conservation of metabolic energy from polyphosphate degradation in *A. johnsonii* 210A. The P_i efflux rate (*panel A*), internal ATP concentration (*panel B*), and $\Delta\psi$ (*panel C*) were monitored in cells with (○) and without (●) polyphosphate during an aerobic period of 2 h, followed by an anaerobic period of 8 h.

Results

Conservation of metabolic energy from polyphosphate degradation

The experiments shown in Fig. 1 demonstrate the ability of *A. johnsonii* 210A to conserve metabolic energy from the degradation of polyphosphate. In this study, high-P_i and low-P_i-grown cells were used. High-P_i-grown cells showed a relatively high phosphorus accumulation level of 4.1 µmol P per mg of protein due to the presence of one or two large metal polyphosphate granules in the cytoplasm. In contrast, low-P_i-grown control cells were devoid of these granules and contained only 0.8 µmol phosphorus per mg of protein. During aerobic incubation for 2 h in the absence of an exogenous carbon and energy source, high-P_i-grown cells hardly excreted $MeHPO_4$. Their cellular ATP level and $\Delta\psi$ were similar to those observed in low-P_i-grown cells. The aerobic incubation period was followed by an anaerobic one. Under the latter condition, high-P_i-grown cells rapidly degraded polyphosphate,

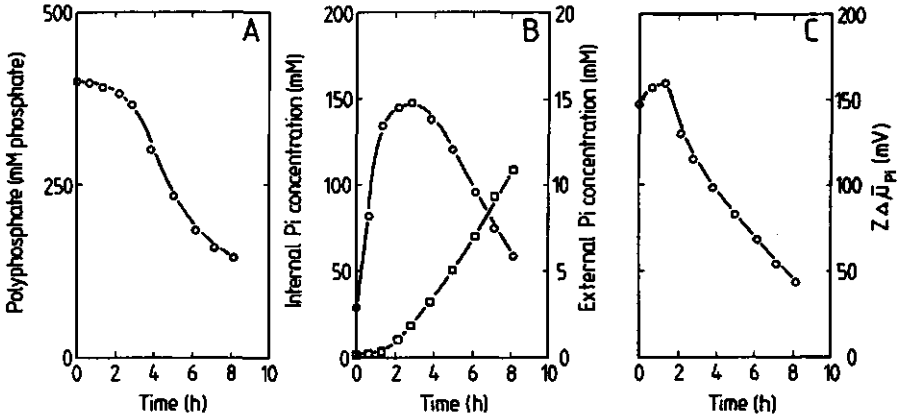

Figure 2. *In vivo* ^{31}P-NMR analysis of the degradation of polyphosphate in an anaerobic cell suspension of *A. johnsonii* 210A. The concentrations of polyphosphate (*panel A*), and internal (○) and external (□) P_i (*panel B*) were determined from the fractional integrated intensities in each resonance of interest and total phosphorus content. The outwardly directed P_i concentration gradient ($Z\Delta\bar{u}_{P_i}$) (*panel C*) was calculated from the data presented in panel B.

resulting in the excretion of $MeHPO_4$ at an initial rate of about 3 nmol per min per mg of protein (Fig. 1A). High-P_i-grown cells were able to maintain a significant intracellular ATP concentration and $\Delta\psi$ for at least eight hours under these conditions, whereas in low-P_i-grown cells the levels of these parameters strongly decreased within one hour (Fig. 1B and 1C).

P_i gradient during degradation of polyphosphate

Polyphosphate degradation was studied in *A. johnsonii* 210A using *in vivo* ^{31}P-NMR (Fig. 2). During the first 8 h of anaerobiosis, about 65% of soluble polyphosphates was degraded (Fig. 2A). Strikingly, intracellular P_i accumulated up to 150 mM in the course of polyphosphate degradation (Fig. 2B). Since the external P_i concentration remained below 11 mM, an outwardly directed P_i gradient ($Z\Delta\bar{u}_{P_i}$) of 100 to 160 mV was maintained for 5 h (Fig. 2C). In view of (i) the important role of polyphosphate in the production of metabolic energy during anaerobiosis, (ii) the large

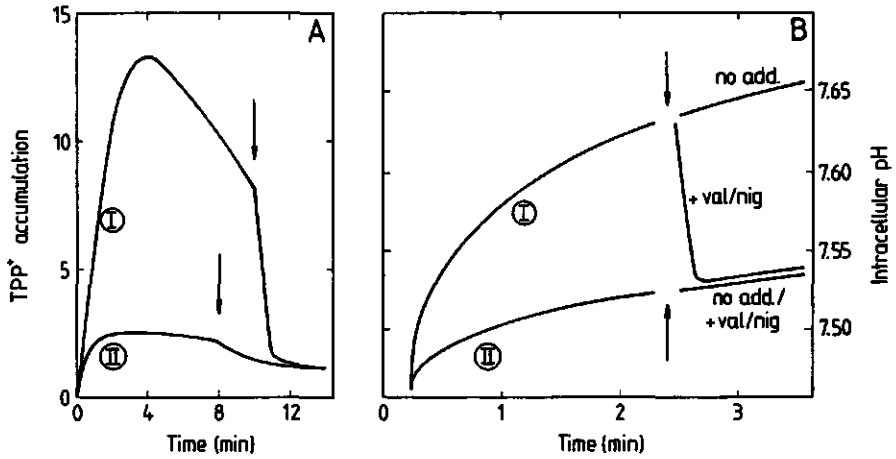

Figure 3. Effect of $MeHPO_4/H^+$ efflux on the accumulation of TPP^+ (*panel A*) and the intracellular pH (*panel B*) in deenergized cells of *A. johnsonii* 210A. Measurements were performed in $MeHPO_4$-loaded cells in the presence of an outwardly directed $MeHPO_4$ gradient (initial value of 120 mV) (*Trace I*), and in unloaded cells (*Trace II*). At the arrow, 2 nmol each of valinomycin and nigericin per mg of cell protein was added.

outwardly directed P_i gradient which is maintained during polyphosphate degradation, and (iii) the presence of a phosphate carrier which mediates the translocation of $MeHPO_4$ via an electrogenic H^+ symport mechanism, it was of interest to study energy transduction coupled to $MeHPO_4$ efflux in *A. johnsonii* 210A.

$MeHPO_4$ efflux in deenergized cells

In order to study the recycling of metabolic energy by $MeHPO_4/H^+$ efflux in the absence of polyphosphate metabolism, cells of *A. johnsonii* 210A were depleted of polyphosphate and other endogenous energy reserves by aerobic incubation in the presence of the uncoupler α-dinitrophenol. As was shown previously (49), the cells retain the secondary $MeHPO_4$ transport system in an active form during the deenergization procedure, and remain readily energizable after removal of dinitrophenol.

In deenergized cells, $MeHPO_4/H^+$ efflux was coupled to the generation of a

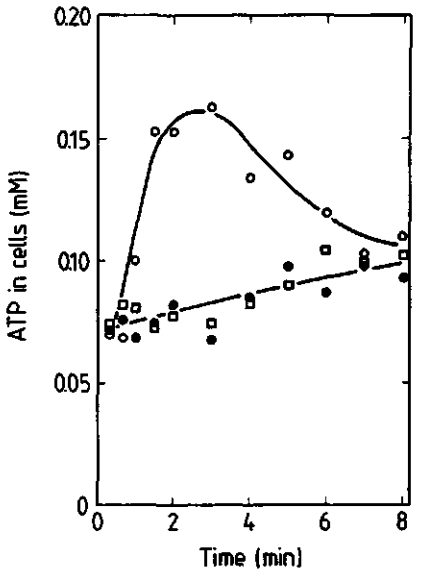

Figure 4. Effect of a $MeHPO_4$ efflux-induced Δp_H+ on the synthesis of ATP in deenergized cells of *A. johnsonii* 210A. ATP concentrations were determined in $MeHPO_4$-loaded (□) and unloaded cells (●) in the absence of a $MeHPO_4$ concentration gradient, and in $MeHPO_4$-loaded cells in which an outwardly directed $MeHPO_4$ gradient (initial value of 120 mV) was imposed artificially (○).

Δp_H+. Thus, the lipophilic cation TPP^+ was accumulated 16-fold when an outwardly directed $MeHPO_4$ gradient (initial value of -120 mV) was imposed artificially by dilution of deenergized, $MeHPO_4$-loaded cells into $MeHPO_4$-free buffer (Fig. 3A). The maximum TPP^+ accumulation level suggested the generation of a $\Delta\psi$ of about -73 mV. No significant accumulation of TPP^+ was observed in unloaded cells or in loaded cells in which the Δp_H+ was dissipated by valinomycin plus nigericin (each 2 nmol per mg of protein) (Fig. 3A). To monitor the changes in the intracellular pH by $MeHPO_4/H^+$ efflux, deenergized cells were loaded with the fluorescent pH indicator BCECF. A rapid alkalinization of the internal milieu was observed when an outwardly directed $MeHPO_4$ gradient (initial value of 120 mV) was imposed (Fig. 3B). Addition of valinomycin plus nigericin (each 2 nmol per mg of protein) resulted in the decrease of the internal pH to baseline levels observed in unloaded

cells. With a constant external pH during $MeHPO_4$ efflux, the degree of alkalinization of the cytoplasmic pH equalled a transmembrane pH gradient ($-Z\Delta pH$) of about -8 mV under these conditions.

The Δp_H+ induced by $MeHPO_4/H^+$ efflux in deenergized cells could drive the synthesis of ATP from endogenous ADP and P_i via the membrane-bound H^+-ATPase (Fig. 4). ATP levels remained very low in control cells in which a $MeHPO_4$ gradient was absent, *e.g.* through dilution of $MeHPO_4$-loaded cells into buffers containing $MeHPO_4$ at a concentration equimolar to the internal one, or of unloaded cells into $MeHPO_4$-free buffer. A significant synthesis of ATP was observed when $MeHPO_4$-loaded cells were diluted 100-fold into $MeHPO_4$-free buffer. This synthesis is transient due to the rapid decrease of the $MeHPO_4$ gradient.

$MeHPO_4$ efflux in membrane vesicles

In membrane vesicles, the mechanism of energy coupling to secondary transport of solutes can be studied in the absence of their metabolism by cytoplasmic enzymes. Membrane vesicles therefore offer an excellent model system to study energy transduction coupled to $MeHPO_4/H^+$ efflux in *A. johnsonii* 210A. The generation of a $MeHPO_4$ efflux-induced $\Delta\psi$ was monitored in membrane vesicles using the fluorescent $\Delta\psi$-indicator $DiSC_3(5)$ (Fig. 5A). Upon imposition of an outwardly directed $MeHPO_4$ gradient (initial value of 120 mV) in membrane vesicles, a rapid fluorescence quenching of $DiSC_3(5)$ was observed corresponding to a $\Delta\psi$ of about -63 mV (Fig. 5A). The quenched fluorescence signal was elevated to the baseline level observed in unloaded membrane vesicles after dissipation of the Δp_H+ by valinomycin plus nigericin (each 1 nmol per mg of protein). The formation of a ΔpH by $MeHPO_4/H^+$ efflux was demonstrated by continuous recording of the fluorescence intensity of BCECF trapped within the membrane vesicles (Fig. 5B). Imposition of an outwardly directed $MeHPO_4$ gradient (initial value of 120 mV) resulted in an alkalinization of the intravesicular pH and the generation of a $-Z\Delta pH$ of -8 mV. A decrease of the BCECF fluorescence intensity down to the baseline level of unloaded membrane vesicles was observed upon addition of valinomycin plus nigericin (each 1 nmol per mg of protein). These results clearly demonstrate

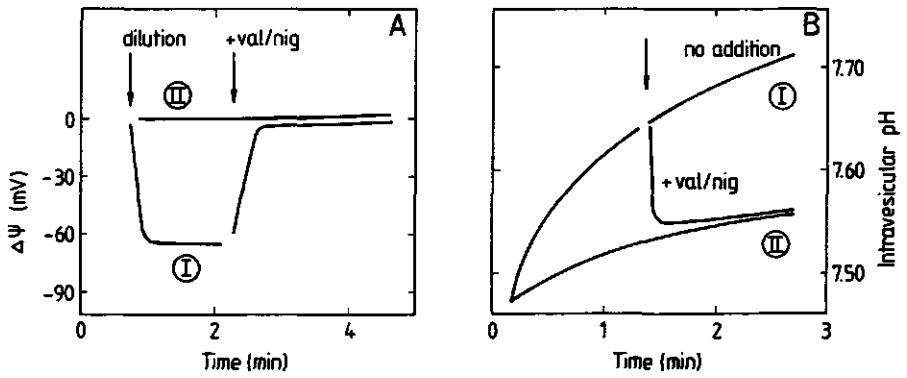

Figure 5. Generation of a $\Delta\psi$ (*panel A*) and ΔpH (*panel B*) by $MeHPO_4/H^+$ efflux in membrane vesicles of *A. johnsonii* 210A. Measurements were performed in $MeHPO_4$-loaded membrane vesicles in the presence of an outwardly directed $MeHPO_4$ gradient (initial value of 120 mV) (*Trace I*), and in unloaded membrane vesicles (*Trace II*). At the arrow, 1 nmol each of valinomycin and nigericin per mg of protein was added to $MeHPO_4$-loaded membrane vesicles.

the generation of both components of the Δp_H+ by the electrogenic excretion of $MeHPO_4$ and H^+.

Membrane vesicles of this organism contain several cation-amino acid transport systems which couple amino acid translocation to the Δp_H+ (51). Significant levels of L-lysine and L-proline accumulation were observed in $MeHPO_4$-loaded membrane vesicles which were diluted 100-fold in $MeHPO_4$-free buffer (Fig. 6). Accumulation of the amino acids was not observed (i) upon dissipation of the $MeHPO_4$ efflux-induced Δp_H+ by valinomycin plus nigericin (each 1 nmol per mg of protein), and (ii) in the absence of an outwardly directed $MeHPO_4$ gradient, *e.g.*, when $MeHPO_4$-loaded vesicles were diluted in buffers containing $MeHPO_4$ at a concentration equimolar to the internal one, or when unloaded membrane vesicles were diluted in $MeHPO_4$-free buffer. These experiments show that a $MeHPO_4$ efflux-induced Δp_H+ can drive the uptake and accumulation of solutes in *A. johnsonii* 210A.

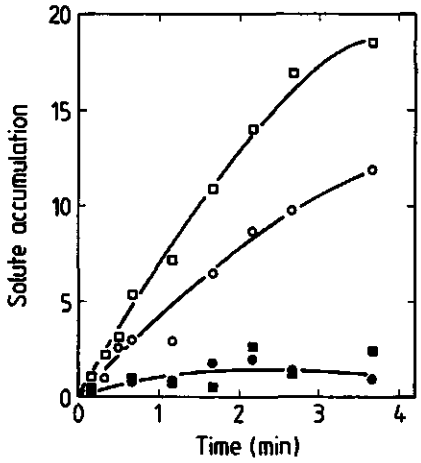

Figure 6. $MeHPO_4$ efflux-induced accumulation of amino acids in membrane vesicles of *A. johnsonii* 210A. Accumulation of L-proline (○,●) and L-lysine (□,■) was monitored in $MeHPO_4$-loaded membrane vesicles in the presence of an outwardly directed $MeHPO_4$ gradient (initial value of 120 mV) (open symbols), and in unloaded membrane vesicles (closed symbols). The accumulation of amino acids in other control experiments (see text) was comparable to that in unloaded membrane vesicles.

Discussion

A. johnsonii 210A is a strictly aerobic, non-fermentative bacterium. When oxidative phosphorylation is impaired, it degrades the metal polyphosphate which was accumulated under aerobic conditions. The results of this investigation demonstrate that during this degradation, the organism is able to maintain its Δp_H+ and intracellular ATP at levels comparable to those observed under aerobic conditions. Two mechanisms for the conservation of metabolic energy from polyphosphate degradation have been suggested in *A. johnsonii* 210A: (i) the direct synthesis of ATP from polyphosphate via the polyphosphate:AMP phosphotransferase/adenylate kinase pathway (43, 46), and (ii) the generation of a Δp_H+ by the excretion of $MeHPO_4$, a major endproduct of metal polyphosphate degradation, together with H^+ (49, 50).

Recently, the energetics and mechanism of the secondary $MeHPO_4$ transport system of *A. johnsonii* 210A were examined in detail. The $MeHPO_4$ carrier cataly-

zes the coupled movement of a neutral $MeHPO_4$ chelate and a proton via an electrogenic symport mechanism (50, 52). Thus, the driving force for $MeHPO_4$ translocation via this transport system is the sum of forces supplied by the Δp_H+ (= $\Delta\psi$ - $Z\Delta$pH) and the $MeHPO_4$ concentration gradient ($Z\Delta$p$MeHPO_4$): Δp_H+ + $Z\Delta$p$MeHPO_4$. A steady state is reached when Δp_H+ = -$Z\Delta$p$MeHPO_4$. During $MeHPO_4$ uptake, the Δp_H+ will exceed the -$Z\Delta$p$MeHPO_4$, whereas $MeHPO_4$ efflux occurs when the -$Z\Delta$p$MeHPO_4$ exceeds the Δp_H+. In previous work, the generation of a Δp_H+ by electrogenic $MeHPO_4/H^+$ efflux was indirectly indicated by (i) the stimulation of $MeHPO_4$ efflux from cells by the uncoupler α-dinitrophenol and by *N,N'*-dicyclohexylcarbodiimide, an inhibitor of the membrane-bound H^+-ATPase (43), (ii) the enhancement by protonophore CCCP of $MeHPO_4$ efflux from proteoliposomes containing reconstituted $MeHPO_4$ carrier protein (50), (iii) the retardation of $MeHPO_4$ efflux from proteoliposomes by an artificially imposed ΔpH and/or $\Delta\psi$ (50).

The results presented in this study further corroborate the generation of a Δp_H+ by $MeHPO_4/H^+$ efflux. Thus, the generation of a $\Delta\psi$ by the efflux of $MeHPO_4$ efflux was demonstrated directly by the fluorescence quenching of the $\Delta\psi$-probe $DiSC_3(5)$ in membrane vesicles, and by the accumulation of TPP^+ in deenergized cells under these conditions. In both systems, imposition of an outwardly directed $MeHPO_4$ gradient of 120 mV (initial value) resulted in the generation of a $\Delta\psi$ of about -60 to -70 mV. The generation of a transmembrane pH gradient by $MeHPO_4/H^+$ efflux was shown by the fluorescence enhancement of the pH-probe BCECF which was entrapped in the lumen of the membrane vesicles and deenergized cells. With the external pH remaining fairly constant during $MeHPO_4$ efflux, a -$Z\Delta$pH of about -8 mV was built up upon imposition of a $MeHPO_4$ gradient of 120 mV (initial value).

The $MeHPO_4$ efflux-induced Δp_H+ could be coupled to different metabolic energy requiring processes as summarized in Fig. 7. In membrane vesicles of *A. johnsonii* 210A, $MeHPO_4/H^+$ efflux could drive the accumulation of L-proline and L-lysine. Recent transport studies in membrane vesicles of this organism revealed the presence of single, high-affinity lysine and proline carriers which mediate the electrogenic symport of lysine and a proton, and of proline and a sodium ion,

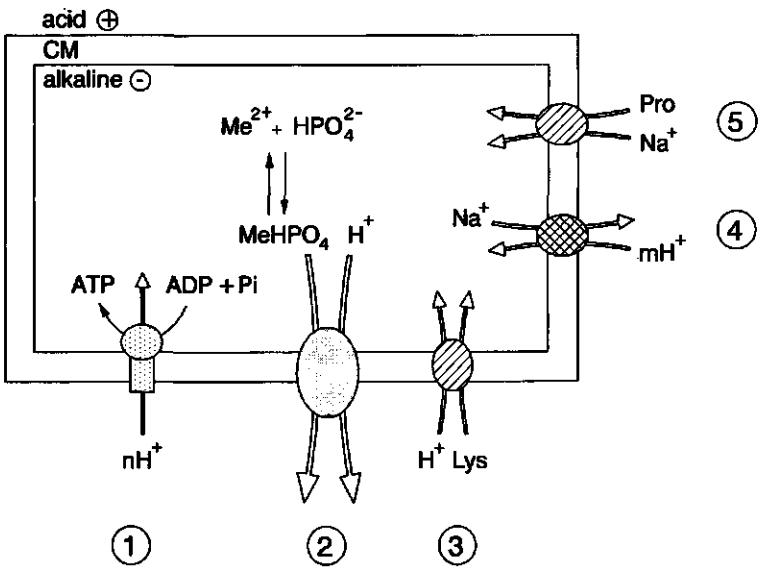

Figure 7. Schematic presentation of the coupling of $MeHPO_4/H^+$ efflux to different energy requiring processes in *A. johnsonii* 210A. Cytoplasmic membrane, L-lysine, L-proline, protons and sodium ions are indicated by *CM*, *LYS*, *PRO*, H^+ and Na^+, respectively. 1, H^+-ATPase; 2, secondary $MeHPO_4/H^+$ carrier; 3, lysine/proton symporter; 4, sodium/proton antiporter; 5, proline/sodium symporter.

respectively (51). The Na^+ contamination in the uptake buffers (up to 150 μM) greatly exceeds the low K_t of the proline carrier for Na^+ (K_t = 26 μM Na^+). Na^+ is therefore present in sufficient amounts to allow Na^+/proline symport (51). In addition, *A. johnsonii* 210A possesses a Na^+/H^+ antiporter which converts the Δp_H+ into a $\Delta p_{Na}+$ (51). In view of the driving forces for lysine and proline uptake, $2\Delta\psi$ - $Z\Delta$pH and $\Delta\psi$ - $Z\Delta$pNa, respectively, the almost 2-fold higher accumulation level of lysine compared to that of proline points to an effective generation of a $\Delta\psi$ by $MeHPO_4/H^+$ efflux under the experimental conditions. This conclusion is consistent with the direct measurements of the composition and magnitude of the $MeHPO_4$ efflux-induced Δp_H+ in membrane vesicles and deenergized cells. Besides solute accumulation, the $MeHPO_4$ efflux-induced Δp_H+ could drive the synthesis of ATP via the membrane-bound H^+-ATPase in deenergized cells of *A. johnsonii* 210A. The

results from these *in vitro* studies demonstrate the potential of $MeHPO_4/H^+$ efflux as an energy recycling mechanism in *A. johnsonii* 210A (Fig. 7).

^{31}P-NMR was used for *in vivo* studies of P_i gradients formed by the degradation of polyphosphate in anaerobic cell suspensions of *A. johnsonii* 210A. The concentration variations in internal P_i were much greater in magnitude and range than those in the external medium. The cells were able to maintain an outwardly directed P_i gradient of 100 to 160 mV for 5 h. Due to the high internal concentrations of Mg^{2+} (up to 40 mM (6)) and P_i (up to 150 mM) during the degradation of magnesium polyphosphate, the intracellular $MgHPO_4$ concentration will have reached saturating levels of about 20 to 30 mM (52). Thus, a substantial outwardly directed $MgHPO_4$ concentration gradient (of at least 100 mV) was present during the first 3 hours of polyphosphate degradation, allowing $MeHPO_4/H^+$ efflux to be an effective energy conserving mechanism.

Under conditions of polyphosphate synthesis, the $MeHPO_4$ carrier and the ATP and binding protein-dependent P_i uptake system of *A. johnsonii* 210A enable the organism to efficiently acquire P_i from its habitat through uptake of the predominant P_i species (49, 52). However, the latter transport system has to be inactivated during the degradation of polyphosphate to prevent waste of ATP by re-accumulation of P_i which was previously released as $MeHPO_4$ via the secondary $MeHPO_4$ transporter. Inactivation of the primary P_i uptake system may be exerted through *trans*-inhibition by the high internal P_i concentration which is established during the degradation of polyphosphate. *Trans*-inhibition has been described for the major potassium transport system of *Enterococcus faecalis* (3), the potassium transport systems TrkA and Kup of *Escherichia coli* (34), the P_i uptake system of *Lactococcus lactis* (32), and the Ugp and phosphate specific transport (Pst) systems (11, 25) of *E. coli*. In *E. coli*, a second mechanism of control of the P_i flux through the Pst system involves the regulation of protein synthesis by the Phosphate (Pho) regulon (37). This regulon probably uses a transmembrane signal transduction mechanism in which the complex between external P_i with its binding protein is sensed and further transduced by the two-component regulatory system *PhoR/PhoB* (33). The primary P_i uptake system of *A. johnsonii* 210A strongly resembles the Pst system of *E. coli*, and there is evidence for the existence of a Pho regulon in *A. johnsonii*

210A (49, 52). *Trans*-inhibition of the primary P_i uptake system in *A. johnsonii* 210A by internal P_i may thus complement the repression of protein synthesis by external P_i.

In their "energy recycling model", Michels et al. (27) proposed that excretion of metabolic endproducts via an ion symport system may lead to the generation of an electrochemical ion gradient across the cytoplasmic membrane, thus providing metabolic energy to the cell. In recent years evidence has been presented for H^+-linked carrier-mediated excretion of lactate in *L. lactis* (30, 31), *E. coli* (42) and *E. faecalis* (38, 39), and Na^+-linked excretion of succinate in *Selenomonas ruminantium* (26). Carrier-mediated excretion of acetate has been suggested in *Syntrophomonas wolfei* (24), *Desulfovibrio desulfuricans* (24), several other sulfate reducers (16, 35), *Acetobacter woodii* (5), and some methanogens (40). The results presented in this paper show that metabolic energy can be conserved as well by the excretion of inorganic endproducts. A Δp_H+ is generated by the electrogenic excretion of $MeHPO_4$ and H^+ via the secondary $MeHPO_4$ transport system of *A. johnsonii* 210A. This carrier may be one example of a new class of bacterial porters whose operation involves the translocation of $MeHPO_4$ rather than P_i. In addition, recent studies on the phosphate inorganic transport (Pit) system in *E. coli* have identified this permease as an electrogenic $MeHPO_4/H^+$ symporter (53). The Pit system in *Bacillus subtilis* (19) and a transport system for divalent cations in the manganese-polyphosphate-accumulating *Lactobacillus plantarum* (2) may translocate $MeHPO_4$ as well. Energy recycling by $MeHPO_4/H^+$ efflux may therefore be a more general energy conserving mechanism in polyphosphate-accumulating microorganisms. Recycling of metabolic energy by the excretion of inorganic endproducts may also have interesting implications for the efflux of NH_4^+ by *Ureoplasma* species (23, 41), and the excretion of sulfate by *Thiobacillus* species and other sulfur oxidizing bacteria (20).

In conclusion, *A. johnsonii* 210A is able to use metal polyphosphate as a source of metabolic energy during anaerobiosis by (i) the direct synthesis of ATP via the polyphosphate:AMP phosphotransferase/adenylate kinase pathway and (ii) the generation of a Δp_H+ via the coupled excretion of $MeHPO_4$ and H^+. Polyphosphatase may enhance the latter energy recycling mechanism by providing the

$MeHPO_4$ efflux process with a continuous supply of P_i and divalent metal ions. As a consequence of energy recycling by $MeHPO_4$ excretion, less ATP has to be hydrolysed via the H^+-ATPase to generate a Δp_H+ when oxidative phosphorylation is impaired. Conservation of metabolic energy from metal polyphosphate degradation may enable *A. johnsonii* 210A to survive alternating aerobic/anaerobic conditions as encountered in certain natural habitats and wastewater treatment plants.

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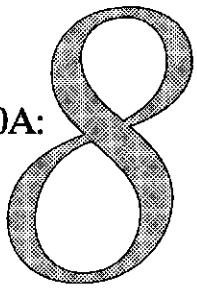
Phosphate transport in *Acinetobacter johnsonii* 210A: mechanisms, perspectives and implications
General dicussion



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1. Phosphate inorganic transport (Pit) system

The mechanism and energetics of the proton motive force-dependent phosphate inorganic transport (Pit) systems in *Acinetobacter johnsonii* 210A and *Escherichia coli* were investigated (Chapters 2 to 5). In *E. coli*, Pit is the major secondary uptake system for P_i, but its transport mechanism has been studied less extensively than that of the P_i-linked antiporters for glycerol-3-P (GlpT) and glucose-6-P (UhpT) (for review, see 41) or the binding protein-dependent phosphate specific transport system (Pst) (for review, see 64). The transport of the phosphate anion via these four systems has generally been interpreted in terms of the translocation of mono- and/or dibasic phosphate. Thus, the periplasmic binding protein which is the initial receptor in the Pst system shows affinity for $H_2PO_4^-$ and HPO_4^{2-} (36, 39). GlpT and UhpT belong to a family of P_i-linked antiporters which mediate electroneutral homologous exchange of $H_2PO_4^-$ or organic phosphate anions, or heterologous exchange of both substrates (41). In addition, it is generally asssumed that HPO_4^{2-} is the phosphate ion species which is transported via Pit (58, 60). However, the work described in this thesis suggests that the Pit system in *E. coli* and *A. johnsonii* may represent a new class of bacterial porters whose operation involves the transport of a neutral, soluble $MeHPO_4$ complex rather than P_i (Chapters 3 to 5). This surprising aspect of phosphate transport by Pit is not immediately evident. Instead it has been successfully masked since divalent metal ions and P_i are usually simultaneously present under the experimental conditions. Cotransport across biomembranes of divalent metal ions and P_i via metal phosphate chelates may thus be a more common phenomenon in prokaryotic and eukaryotic cells.

1.1 Substrate specificity

Since the idea of $MeHPO_4/H^+$ symport arose during studies of P_i transport by the metal polyphosphate-accumulating *A. johnsonii* strain 210A, it is useful to summarize relevant information from that work. Transport studies in whole cells gave kinetic evidence for the presence of two phosphate transport systems which showed a strong analogy to the Pst and Pit system of *E. coli* (Chapter 2). Pit in *A. johnsonii*

210A was found to be a constitutive phosphate/arsenate transport system with an apparent K_t for P_i of about 9 μM and for arsenate of about 11 μM at pH 7.0. The system is strongly inhibited by the uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Chapters 2 and 3).

A. johnsonii Pit has been characterized in membrane vesicles and proteoliposomes in which the carrier protein was reconstituted (Chapter 3). During proton motive force-driven uptake, the apparent K_t for P_i increased from about 5 μM at pH 8.0 to 24 μM at pH 6.0. Although this result would be consistent with a specificity for HPO_4^{2-}, further studies pointed to the translocation of a neutral $MeHPO_4$ complex. Thus, P_i uptake via *A. johnsonii* Pit was strictly dependent on the presence of divalent cations, like Mg^{2+}, Ca^{2+}, Mn^{2+} or Co^{2+}. Similar observations were reported for a citrate permease in *Bacillus subtilis* mediating the transport of a Me^{2+}-citrate complex (9, 73). Calculation of the concentrations of several P_i species under the experimental conditions indicated that at pH 7.0, 31% (Ca^{2+}) to 87% (Mn^{2+}) of P_i was present as a neutral $MeHPO_4$ complex (Chapters 3 to 5). The stimulation of P_i uptake by the divalent metal ions correlated well with the concentrations of $MeHPO_4$ in the incubation mixtures. Moreover, a re-evaluation of the kinetic data for *A. johnsonii* Pit revealed a pH-independent apparent K_t for $MeHPO_4$ of 7.9 μM. Finally, control measurements excluded other possible explanations for the metal-dependence of P_i transport via *A. johnsonii* Pit, such as binding of $MeHPO_4$ to membranes, internal precipitation of $MeHPO_4$ due to solute accumulation, or secondary effects of divalent cations on the magnitude or stability of the artificially imposed proton motive force in proteoliposomes (Chapter 5).

Additional experiments have been carried out to elucidate the substrate specificity and transport mechanism of the Pit system in *A. johnsonii* 210A (Chapters 3 and 5). *A. johnsonii* Pit appears to operate in a similar way as that of *E. coli* Pit (Chapter 4). Proton motive force-driven P_i transport in proteoliposomes in which Pit carrier protein from *E. coli* was reconstituted, was found to be strictly divalent cation-dependent. The apparent K_t for $MeHPO_4$ of 8.8 μM, is very similar to the one obtained for Pit in *A. johnsonii* 210A. Specificity for $MeHPO_4$ was tested further by studying the proton motive force-driven transport of Mg^{2+} and Ca^{2+} in proteoliposomes in the presence and absence of P_i. Besides a divalent cation-

dependent uptake of P_i, such studies demonstrated (i) P_i-dependent uptake of Mg^{2+} and Ca^{2+}, (ii) inhibition by Mg^{2+} of Ca^{2+} uptake in the presence of P_i, but not of P_i uptake in the presence of Ca^{2+}, and (iii) equimolar transport of Ca^{2+} and P_i. Verification that $MeHPO_4$ but not P_i, was the authentic substrate of *E. coli* Pit came from transport experiments performed in the absence of a proton motive force. Measurements of solute transport via exchange and efflux reactions allowed an easy experimental control over *cis* and *trans* compartments. Under these conditions, Pit of *E. coli* could mediate efflux and homologous exchange of $MeHPO_4$, but not heterologous exchange of $MeHPO_4$ and the substrates for the GlpT and UhpT system: P_i, glycerol-3-P or glucose-6-P.

Presently, the evidence is most simply interpreted by the translocation of $MeHPO_4$, but not of P_i, via the Pit systems of both *E. coli* and *A. johnsonii* 210A. The previously observed Mg^{2+}-dependence of Pit function in *E. coli* cells (44, 56) and its membrane vesicles (34) most likely reflected the translocation of $MgHPO_4$ via this system. Earlier conclusions with respect to HPO_4^{2-} being the physiological substrate of Pit of *E. coli* Pit (58, 60) can be related to the almost identical pH dependency of $MeHPO_4$ and HPO_4^{2-} in aqueous solutions.

1.2 Transport mechanism

Artificial imposition of ion diffusion gradients in proteoliposomes containing Pit proteins confirmed previous work in membrane vesicles of *E. coli* (34). Both a membrane potential and a pH gradient can drive $MeHPO_4$ transport through this system. A detailed analysis of the steady-state accumulation level of membrane potential-driven uptake of $MeHPO_4$ indicated the translocation of a (neutral) $MeHPO_4$ complex in symport with one proton (Chapters 3 and 4).

The mechanism of $MeHPO_4/H^+$ symport via Pit has been deduced from the pH and proton motive force-dependency of $MeHPO_4$ uptake, efflux and exchange (Chapter 3, 4). In the present discussion, these facts will not be recited, but readily incorporated into two models. In the cellular model, the overall transport reactions are conveniently summarized (Fig. 1). $MeHPO_4$ uptake occurs in symport with a proton (Fig. 1A). During $MeHPO_4$ efflux the transport reaction is reversed, resulting

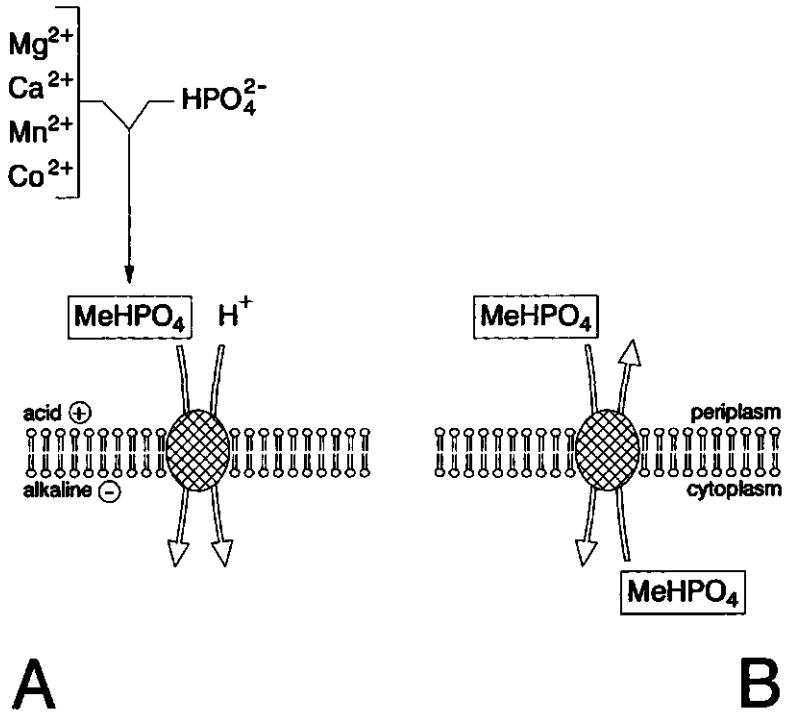

Figure 1. Cellular model showing overall transport reactions via Pit. (A) Proton motive force-driven uptake of $MeHPO_4$. (B) Homologous $MeHPO_4$ exchange.

in the generation of a proton motive force. In homologous exchange of $MeHPO_4$ no net translocation of protons takes place (Fig. 1B). In the kinetic model (Fig. 9, Chapter 3), the vectorial translocation of $MeHPO_4$ across the cytoplasmic membrane can be considered as a cyclic process in which binding and dissociation of $MeHPO_4$ and proton on the outer and inner surface of the membrane occur via an ordered mechanism. Thus, the process of efflux involves protonation of the carrier protein on the inner surface of the membrane followed by binding of $MeHPO_4$. The loaded carrier protein reorients its binding sites to the outer surface of the membrane after which $MeHPO_4$ is released first from the carrier, followed by the loss of the catalytic proton. A conformational change of the empty carrier restores the initial

orientation of the binding sites. The carrier protein remains protonated during homologous $MeHPO_4$ exchange.

Since the dissociation of the catalytic proton from the Pit protein appears to be rate-limiting for $MeHPO_4$ transport, changes in the internal and external pH lead to major alterations in catalytic activity (Chapter 2, 3). During $MeHPO_4$ efflux, the carrier protein is deprotonated on the outer surface of the membrane. Inhibition of this deprotonation step by low external pH, retards the $MeHPO_4$ efflux rate. For $MeHPO_4$ uptake, a similar catalytic internal pH effect was found which controls the rate of deprotonation of the carrier on the inner surface of the membrane (apparent pK of 7.2, Chapter 4).

Pit catalyzes completely reversible transport reactions under deenergized conditions. However, unlike the exchange reactions mediated via other proton symporters (22, 30) or the P_i-linked antiporters UhpT and GlpT (41), the homologous exchange of $MeHPO_4$ via Pit is inhibited by the membrane potential. This difference in behaviour may play an important role in the intriguing paradox that the Pit system of *E. coli*, being a secondary "phosphate" transporter, mediates an apparent unidirectional uptake of phosphate in cells under physiological conditions (24, 58, 74).

1.3 Implications

The finding of $MeHPO_4/H^+$ symport in *A. johnsonii* 210A and *E. coli* suggests that this reaction may be a general mechanism for the transport of divalent metal ions and P_i (or arsenate) in bacteria. P_i transport is stimulated by Mg^{2+} in *Micrococcus lysodeikticus* (25), *Acinetobacter lwoffi* (76), *Pseudomonas aeruginosa* (37) and *Bacillus cereus* (61). There is evidence for the presence of a Pit-like P_i permease in all these organisms (58). Furthermore, in some studies a stimulation was observed by P_i of Me^{2+} transport, *e.g.* the uptake of Mn^{2+} in the manganese polyphosphate-accumulating *Lactobacillus plantarum* (6), and of Mg^{2+}, Ca^{2+}, Mn^{2+}, and Co^{2+} via a general divalent cation transport system in *Bacillus subtilis* (31). Interestingly, a Pit mutant of this latter organism was strongly impaired in the transport of Ca^{2+} and Co^{2+}. The mutant still elicited significant Mn^{2+} transport as a result of uptake via a

second Mn^{2+}-specific high-affinity uptake system (31).

Research on prokaryotic calcium transport systems lags behind that in eukaryotes. Although information is available on bacterial Ca^{2+} efflux systems, the mechanisms for Ca^{2+} entry are unclear (40). The transport of $MeHPO_4$, including the calcium phosphate complex, via Pit provides *E. coli* with such a mechanism. Pit may be functionally linked to the Ca^{2+}/nH^+ antiporter of this organism. Thus, a chemiosmotic circuit for divalent cations can be envisaged in which Pit mediates the entrance of P_i and divalent cations, whereas the antiporter catalyzes the proton motive force-driven extrusion of Ca^{2+} and other divalent cations like Mn^{2+}, Sr^{2+}, or Ba^{2+} (17) in order to maintain low concentrations of these ions in the cytosol. The relationship between Pit and a presumed phosphate-dependent calcium/proton antiporter (4) in *E. coli* remains to be established.

Transport of metal phosphates may also be encountered in eukaryotic cells. In many biological systems, P_i transport is linked to the cellular metabolism and transport of divalent cations. Divalent cations stimulate the uptake of P_i across the plasma and vacuolar membranes in *Saccharomyces cerevisiae* and other lower eukaryotes (33, 35, 48). Furthermore, Pi is known to have a large influence on Ca^{2+} transport by mitochondria isolated from a range of tissues and species (16, 19, 38, 45, 55, 75). The influx of Mg^{2+} in rat hepatocytes has recently been suggested to occur by sodium motive force-driven Mg^{2+}/P_i cotransport (27). Clearly, the weight of the current evidence necessitates the continued consideration of the impact of divalent metal ions on P_i transport processes and *vice versa*. In addition, it provides an adequate base to encourage further experimentation on the topic.

2. Phosphate specific transport (Pst) system

Besides the Pit system, *A. johnsonii* 210A possesses a P_i permease showing analogy to the phosphate specific transport (Pst) system of *E. coli*. Like its *E. coli* counterpart, *A. johnsonii* Pst is a P_i-repressible, shock-sensitive transporter which acts in conjunction with a periplasmic P_i-binding protein (Chapter 2, 5). The system has a relatively high affinity for the solute. The apparent P_i-binding constant (K_D) of the binding protein of 0.6 μM P_i corresponds well to the apparent K_t of 0.7 μM P_i for

P_i transport via the Pst system. These values are in close agreement with those previously determined for the Pst of *E. coli* (42-44, 59, 74).

2.1 Substrate specificity

In this thesis, evidence has been presented for the translocation of $H_2PO_4^-$ and HPO_4^{2-}, but not of $MeHPO_4$ via the Pst system of *A. johnsonii* 210A. Firstly, the uptake of P_i, but not of $MeHPO_4$ was derepressed in cells under P_i limitation. Secondly, the relative P_i and Me^{2+} uptake rates (2:1) in high-P_i-grown cells at solute concentrations that saturate Pit and Pst, were consistent with the translocation of P_i via Pst and of $MeHPO_4$ via Pit, the maximal uptake rate of which equals that of Pst under these conditions (Chapter 2). Thirdly, the specificity of Pst for P_i was further corroborated by studies on the substrate specificity of the P_i-binding protein, whose activity represents an initial step in solute translocation via Pst. Thus, the K_D of the P_i-binding protein was essentially pH independent in the pH range 5.5 to 8.0, a range in which the ratio of $H_2PO_4^-$ over HPO_4^{2-} varied about 250-fold. Moreover, a decrease in the concentration of free P_i by the formation of a soluble $MeHPO_4$ complex reduced P_i binding. The reduction level could be predicted by calculations assuming the presence of one binding site on the P_i-binding protein with equal affinity for $H_2PO_4^-$ and HPO_4^{2-}. The substrate specificity of the P_i-binding protein of *A. johnsonii* 210A is in agreement with X-ray crystallographic studies of the P_i-binding protein of *E. coli*. These studies predict the binding of mono- and divalent P_i by the binding protein (39).

2.2 Energy coupling

An *in vivo* study of energy coupling to the Pst system of *A. johnsonii* 210A has indicated that ATP or some form of phosphate bond energy derived thereof, is responsible for driving the permease (Chapter 2). This conclusion is consistent with (i) the presence of a conserved ATP-binding motif in the *pstB* gene encoding a putative hydrophilic, membrane-associated ATP-binding subunit of the Pst complex in *E. coli* (20, 29, 67), and (ii) recent *in vitro* studies on the similarly organized his-

tidine and maltose permeases in *E. coli* which provide direct evidence for the role of ATP hydrolysis in the energization of solute uptake via periplasmic binding protein-dependent transport systems (5, 21, 28, 52).

2.3 Regulation of transport

Several mechanisms are involved in the regulation of the flux of P_i through Pst. As was outlined in Chapter 1, the synthesis of Pst protein in *E. coli* is regulated by external P_i via a control of gene expression by the Pho regulon. Thus, the synthesis of Pst complex and other proteins involved in the assimilation of P_i is induced in cells under P_i limitation to ensure a sufficient supply of P_i. The 10-fold increase in maximal uptake rate of *A. johnsonii* Pst under these conditions, together with the induction of periplasmic alkaline phosphatase activity (14, Chapter 2 and 5) and the synthesis of an outer membrane protein (35.5 kDa)[1] which may be functionally related to the PhoE pore protein of *E. coli* (51), point to the existence of a Pho regulon in *A. johnsonii* 210A.

A second mechanism of control of the P_i flux through Pst involves the regulation of the activity of Pst transport systems via *trans*-inhibition by internal P_i. *Trans*-inhibition is the equivalent of feedback inhibition of enzyme activity by endproducts in many biosynthetic pathways. ATP-driven permeases have the ability to accumulate solutes in the cell against large concentrations gradients (up to 10^5) which exceed the thermodynamic limits of secondary transporters set by the proton motive force (assuming a H^+/solute stoichiometry of one). The presence of thousands of polyelectrolytes and small metabolites in the cytoplasm poses serious problems of solubility, and the maintenance of low metabolite concentrations may be a compelling reason for citing control at solute accumulation via primary uptake systems. Thus, *trans*-inhibition functions as a "safety-brake" that prevents solute accumulation to unacceptably high levels (54). Already in 1971, Medveczky and Rosenberg (44) observed the negative effect of an increasing intracellular P_i pool in *E. coli* cells on the rate of P_i transport via the Pst system. Since its discovery, this

[1]Van Veen HW, unpublished observation.

type of control of transport activity has been largely neglected in studies on P_i regulation of Pst of *E. coli* due to a strong emphasis on the molecular mechanisms of the Pho regulon. Recent work of Poolman et al. (54) with *Lactococcus lactis* subsp. *lactis* ML3 and of Brzoska et al. (18) with *E. coli* confirmed the relevance of *trans*-inhibition in the regulation of phosphate bond energy-driven P_i permeases.

Besides regulation of Pst by internal P_i, also regulation occurs by the intracellular H^+ concentration (60, 62, 63). This property is shared with the primary P_i permease of *L. lactis* subsp. *lactis* ML3 (54) and many other phosphate bond energy-driven transport systems (3, 15, 53). Thus, the activities of these systems decrease with decreasing internal pH. In contrast to the *catalytic* pH effects observed on Pit activity, internal pH effects on Pst transport are likely to be *allosteric*, being brought about by putative regulatory domains that interact with protons from the cytosol (53). In view of the regulation of Pst and Pit by the internal pH, it is essential to maintain a constant alkaline pH in the cytoplasm for optimal function of both systems. K^+ transport plays an important role in pH homeostasis in bacteria: inward electrogenic movement of K^+ is used to decrease the membrane potential during redox reaction-coupled primary H^+ expulsion by the respiratory chain (2, 7). These ion translocation processes lead to an increased transmembrane pH gradient and, consequently to a higher internal pH (2, 7, 15). Evidence confirming the relevance of these processes for phosphate transport via Pst and Pit came from the work of Russell and Rosenberg (62, 63) who demonstrated that potassium ions greatly stimulate Pst and Pit function in *E. coli* cells, but that the transport of K^+ and phosphate are linked indirectly via proton circulation. Similar underlying transport processes may explain the observed stimulation by K^+ of P_i uptake in cells of *A. johnsonii* 210A (70) in which both Pit and Pst are functionally present.

3. Pit, Pst and the metabolism of polyphosphate

3.1 Polyphosphate synthesis

Under aerobic conditions, *A. johnsonii* 210A and other *Acinetobacter* spp. accumulate excess P_i and divalent metal ions as a metal polyphosphate chelate in

cytoplasmic granules. This property of *Acinetobacter* can be used to efficiently remove P_i from waste water (Chapter 1). Calculations on the P_i speciation in settled domestic waste water, in which calcium ions are present in excess of P_i, indicate the predominance of $CaHPO_4$, HPO_4^{2-} and $H_2PO_4^-$ (Chapter 5). In view of the substrate specificity of Pit and Pst, the concerted operation of these systems allows *A. johnsonii* 210A to take up the prevailing P_i species from such habitats. The P_i regulation of Pst enables the organism to cope with constant fluctuations in environmental P_i, thus ensuring a supply of P_i for the synthesis of polyphosphate and other cell constituents. Pst is a unidirectional P_i uptake system whereas Pit is a reversible $MeHPO_4$ transporter. The latter system provides the cell with a major route for the simultaneous entrance or exit of P_i and divalent cations. As a consequence, $MeHPO_4$ translocated via Pit strongly affects the elemental composition of metal polyphosphate granules (Chapter 5). Besides for *Acinetobacter*, this observation may be relevant for other polyphosphate-accumulating microorganisms among which *Propionibacterium acnes* (32), *Spirillum itersonii*, *Corynebacterium diphtheriae*, *icrococcus luteus* (72), *M. lysodeikticus* (26), *L. plantarum* (6), and *Plectonema boryanum* (8), in which polyphosphate granules serve as the main sink for divalent metal ions such as Ba^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}. The cotransport of P_i and heavy metal ions via Pit, and their subsequent incorporation in metal polyphosphate granules may be used as a biological mechanism for the concentration of heavy metal ions and the removal of these compounds from polluted waters. The important role of K^+ and divalent cations in (i) the maintenance of optimal Pit and Pst function, and (ii) the metabolism of polyphosphates (10-13, 71) may provide a rationale for their requirement in the enhanced biological phosphorus removal process (57).

3.2 Anaerobiosis and polyphosphate degradation

Under aerobic conditions, energy conservation in *A. johnsonii* 210A and other strictly aerobic bacteria is based on (i) primary H^+ translocation by the respiratory chain for the generation of a proton motive force and the maintenance of an alkaline cytoplasmic pH, and (ii) proton motive force-coupled synthesis of ATP via

the membrane-bound F_0F_1 H^+-ATPase. In view of the central role of the proton motive force in energy transducing processes, such as the preservation of solute gradients across the cytoplasmic membrane, this energy intermediate is essential for the vitality of the cell. In the absence of oxygen, aerobic bacteria may use the H^+-ATPase in the hydrolytic direction as an alternative H^+ extrusion pump to generate a proton motive force. However, the lack of effective mechanism(s) for the synthesis of ATP under these conditions seriously limits the ability of most strictly aerobic bacteria to survive anaerobic periods.

An adaptation of microorganisms to short interruptions in the supply of metabolic energy is the regulation of solute transport by the internal pH. During impaired electron transfer in aerobic bacteria, the internal pH will decrease. The inhibition of Pit, Pst and other solute transport systems under these conditions will reduce the consumption of metabolic energy (proton and sodium motive force, and ATP), thus preserving it for maintenance purposes (1, 53).

In addition, *A. johnsonii* 210A is well equipped to survive prolonged periods of anaerobiosis (up to 5 h) due to its ability to use polyphosphate as source of metabolic energy (Chapter 7). Two mechanisms are involved in this process: (i) the direct sythesis of ATP via the polyphosphate:AMP phosphotransferase/adenylate kinase pathway (69, 70), and (ii) the generation of a proton motive force via the coupled excretion of $MeHPO_4$ and H^+ via Pit (Chapter 7). The $MeHPO_4$ efflux-induced proton motive force could be coupled to different metabolic energy requiring processes (Fig. 7, Chapter 7) such as the synthesis of ATP, the accumulation of L-lysine via an electrogenic H^+/lysine symport system and the accumulation of L-proline via an electrogenic Na^+/proline symport system (Chapter 6). Evidence was obtained for the presence of a sodium/proton antiporter in *A. johnsonii* 210A which will participate in sodium-coupled solute accumulation through conversion of the proton motive force into a sodium motive force (Chapter 6). *In vivo* studies of polyphosphate degradation in *A. johnsonii* 210A by ^{31}P-NMR suggested the presence of a substantial, outwardly directed $MgHPO_4$ gradient which allows $MeHPO_4/H^+$ efflux to be an effective metabolic energy conserving mechanism. Polyphosphatase may be involved in this energy recycling mechanism by providing Pit with a continuous supply of $MeHPO_4$. Re-accumulation of P_i via

Pst with a concomitant hydrolysis of ATP may be prevented by *trans*-inhibition of this permease by the high internal P_i concentration (up to 150 mM) resulting from the degradation of polyphosphate.

Fermentative bacteria continuously excrete relatively large quantities of lactic acid and other organic acids into the environment. In their "energy recycling model", Michels and co-workers (47) proposed that electrogenic efflux of these organic endproducts in symport with protons could significantly contribute to the generation of a proton motive force. Evidence has been obtained for carrier-mediated H^+-linked excretion of lactate in lactic (49, 50, 65, 66) and enteric bacteria (68), and Na^+-linked excretion of succinate in *Selenomonas ruminantium* (46). Other organic acids, like acetate, may be excreted via carrier-mediated processes as well (23). The observations in *A. johnsonii* 210A extend the "energy recycling model" to the excretion of inorganic endproducts (Chapter 7). In view of the evidence for cotransport of P_i and divalent cations in many other biological systems, energy recycling by the excretion of $MeHPO_4$ may be a general mechanism for the conservation of metabolic energy in polyphosphate-accumulating microorganisms. In addition, the energy recycling model may have implications for the energy metabolism of microorganisms excreting ammonia, sulfate or other inorganic endproducts.

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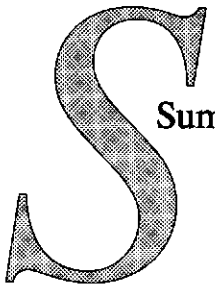


The biological removal of phosphorus from waste water is an attractive method to control eutrophication of surface waters. The process is currently perceived to depend on the provision of alternate stages in which the activated sludge is subjected to anaerobic and aerobic conditions. A characteristic feature of such plant is that P_i, after being released from biomass in the anaerobic stage, is reincorporated into biomass during aeration, together with part or all of the influent P_i. Analysis of the population structure of activated sludge has focussed attention on the strictly aerobic, gram-negative genus *Acinetobacter* as being one of the important genera in enhanced biological phosphorus removal. However, due to the lack of insight into the relevant physiological processes in these microorganisms our understanding of the mechanisms of enhanced biological phosphorus removal is only superficial.

The project of this thesis was initiated to study the mechanisms and regulation of P_i uptake and efflux in the polyphosphate-accumulating *Acinetobacter johnsonii* 210A. The nature of polyphosphates and the enzymology of their metabolism have been a subject of previous studies with *A. johnsonii* 210A and other *Acinetobacter* spp. Chapter 1 presents a review of these investigations and those concerning the molecular mechanisms of P_i transport in prokaryotes. The results described in this thesis show that *A. johnsonii* 210A is well adapted to the environmental conditions encountered in activated sludge plants through (i) the efficient acquisition of the predominant P_i species from its habitat, and (ii) the ability to survive prolonged periods of anaerobiosis, by using polyphosphate as a source of metabolic energy

when oxidative phosphorylation is impaired.

P_i is taken up in *A. johnsonii* 210A against a concentration gradient by energy-dependent, carrier-mediated processes (Chapter 2). Kinetic analysis of P_i uptake in cells grown under P_i limitation, revealed the presence of two P_i transport systems with an apparent K_t for P_i of 0.7 and 9 μM. The high-affinity permease could be classified as an ATP- and periplasmic binding protein-dependent P_i uptake system. Induction of this system under P_i limitation, and the ability to maintain a low internal P_i by the synthesis of polyphosphate enable the organism to reduce the P_i concentration in the environment to micromolar levels. The low-affinity system is a constitutive secondary P_i transport system involved in P_i uptake and efflux.

P_i transport via the secondary transport system was studied in membrane vesicles and proteoliposomes in which the carrier protein was successfully reconstituted (Chapter 3). These model systems allow detailed studies on the mechanism of P_i transport without the interference of polyphosphate metabolism or other cellular processes. P_i uptake is strongly dependent on the presence of divalent metal ions, such as Mg^{2+}, Ca^{2+}, Mn^{2+}, or Co^{2+}. These cations form a $MeHPO_4$ complex with up to 87% of the P_i present in the incubation mixtures, suggesting that divalent cations and P_i are cotransported via a $MeHPO_4$ complex. $MeHPO_4$ uptake is driven by the proton motive force with an mechanistic $MeHPO_4/H^+$ stoichiometry of one. The pH dependence of various modes of facilitated diffusion processes, such as efflux, exchange, and counterflow catalyzed by the $MeHPO_4$ carrier suggests that H^+ and $MeHPO_4$ binding and release to and from the carrier protein occur via an ordered mechanism.

In view of the similarities between P_i transport in cells of *A. johnsonii* 210A and *Escherichia coli*, a more extensively studied organism (Chapter 2), the mechanism and energetics of the phosphate inorganic transport (Pit) system of *E. coli* were investigated (Chapter 4). P_i and metal transport studies in proteoliposomes containing reconstituted Pit protein identified Pit as a $MeHPO_4/H^+$ symport system. The effects of pH and the proton motive force on the different modes of $MeHPO_4$ transport are consistent with the ordered binding model proposed for the $MeHPO_4$ transporter in *A. johnsonii* 210A.

Chapter 5 describes the substrate specificity of the two P_i transport systems in

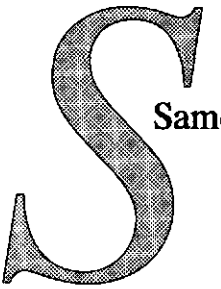
A. johnsonii 210A in relation to P_i speciation in the aquatic environment. In natural waters and domestic waste water in which divalent metal ions are present in excess of P_i, the species $H_2PO_4^-$, HPO_4^{2-} and $MeHPO_4$ prevail at physiological pH values for *Acinetobacter* (pH 5.5 to 8.0). The transport of $MeHPO_4$ by the secondary P_i transport system is demonstrated in proteoliposomes by the (i) divalent cation-dependent uptake and efflux of P_i, (ii) P_i-dependent uptake of Ca^{2+} and Mg^{2+}, (iii) equimolar transport of P_i and Ca^{2+}, and (iv) inhibition by Mg^{2+} of Ca^{2+} uptake in the presence of P_i, but not of P_i uptake in the presence of Ca^{2+}. The transport of $MeHPO_4$ is closely related to the metabolism of cytoplasmic polyphosphate granules in which P_i and divalent cations are accumulated. $H_2PO_4^-$ and HPO_4^{2-} are translocated by the primary P_i uptake system. P_i uptake, but not $MeHPO_4$ uptake, was stimulated in cells under P_i limitation. The periplasmic P_i-binding protein showed affinity for $H_2PO_4^-$ and HPO_4^{2-}, but not for $MeHPO_4$.

Chapter 6 demonstrates the presence of high-affinity secondary transport systems for L-lysine, L-alanine and L-proline in *A. johnsonii* 210A. The lysine and alanine carriers translocate their solute in symport with one proton. In contrast, the proline carrier is strictly dependent on the presence of Na^+ ions and mediates Na^+/proline symport. The low internal Na^+ concentration, necessary for optimal proline uptake, is achieved by a sodium/proton antiporter. High-affinity systems will enable the organism to scavenge the environment for traces of metabolizable substrates and to recapture endogenous compounds leaking out of the cell.

Retention of metabolites will become particularly important for survival when oxidative phosphorylation is impaired in *A. johnsonii* 210A. In Chapter 7, evidence is presented for the ability of the organism (i) to use polyphosphate as a source of metabolic energy during anaerobiosis, (ii) to maintain a considerable, outwardly directed $MeHPO_4$ gradient across the cytoplasmic membrane during the degradation of polyphosphate, and (iii) to generate a proton motive force by the excretion of $MeHPO_4$ and H^+ via the $MeHPO_4$ carrier. This $MeHPO_4$ efflux-induced proton motive force can drive energy-requiring processes such as the accumulation of lysine and proline, and the synthesis of ATP. Conservation of metabolic energy from polyphosphate degradation may enable *A. johnsonii* 210A to survive alternating aerobic/anaerobic conditions as encountered in natural habitats and

wastewater treatment plants.

The significance of the here described findings for the cotransport of P_i and divalent metal ions across biomembranes and the recycling of metabolic energy in microorganisms by the excretion of inorganic endproducts is discussed in Chapter 8.



Samenvatting

Waar gaat dit proefschrift over?

Een proefschrift bevat vaak een groot aantal pagina's die gewoon onleesbaar zijn voor een buitenstaander. Zelfs met het grootste enthousiasme lukt het je dan nauwelijks om een ander te vertellen waar de promovendus eigelijk mee bezig is geweest. In dit geval zou dat kunnen uitmonden in: "iets met fosfaattransport of zo...". Daarom nu een samenvatting in normaal Nederlands.

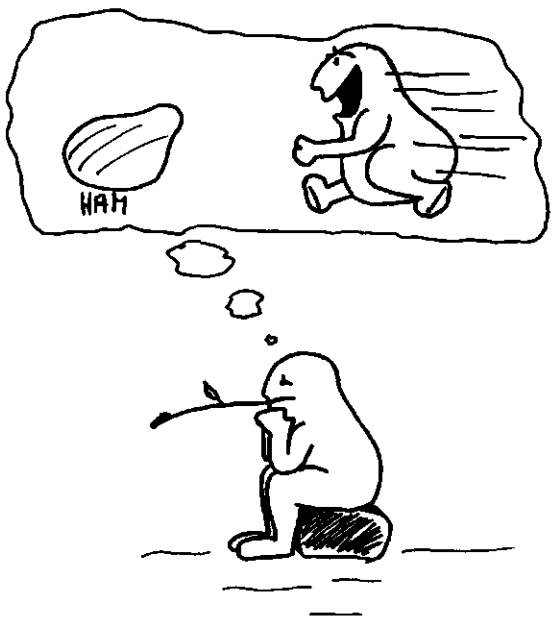
Fosfaat en het milieu

Iedereen heeft ze gezien, die wasmiddelenreclames op de TV. De vrolijke huisman slaakt de kreet: "fosfaat-vrij, dus beter voor het milieu...". Zijn stralende glimlach overtuigt ons direct van het feit dat een goed milieu goed is voor onszelf. Tegelijkertijd vergeten we vaak de vraag te stellen wat fosfaat nu eigenlijk met het milieu te maken heeft. Om die vraag te kunnen beantwoorden moeten we eerst iets meer weten over de tussenliggende schakel: micro-organismen.

Micro-organismen

Micro-organismen zijn hele kleine wezentjes die je vaak met het blote oog niet kunt zien. Ze zitten overal, op en in onszelf, in de lucht, in huis, op straat, in sloten en

rivieren. Alhoewel micro-organismen vaak een negatieve bijklank hebben en menig persoon onpasselijk zal worden bij het idee zelf volgeplakt te zitten met deze beestjes, zijn zij essentieel voor het leven op aarde. Wij danken ons bestaan aan hen. Soms zijn ze onze vijand, bijvoorbeeld bij infectieziekten, maar meestal zijn ze een goede buur. Net als wij, hebben micro-organismen een grote hobby: eten. Voor een gezond lichaam zijn verschillende bouwstenen nodig, zoals koolstof, stikstof, zwavel en fosfor. Terwijl de mens het voordeel heeft naar de supermarkt te kunnen gaan om te kopen naar behoefte, is het voor een micro-organisme maar afwachten wat er aan zijn neus voorbij komt. Het bezit nauwelijks het vermogen zijn omgeving aan te passen aan zijn wensen. Zo kan het voorkomen dat niet alle bouwstenen in voldoende mate aanwezig zijn voor groei. Op zo'n moment zit een micro-organisme op een houtje te bijten en na te denken over de hamvraag hoe te overleven (Figuur 1).



Figuur 1. In de afwezigheid van voedsel zit het micro-organisme op een houtje te bijten en na te denken over de hamvraag hoe te overleven.

Eutrofiëring

Fosfor is een van de voedingsstoffen die van nature weinig aanwezig is in meren, sloten en rivieren. Wanneer fosfaat wordt toegevoegd door lozing van verontreinigd rioolwater zijn het in eerste instantie vooral algen die heel snel gaan groeien. Met een duur woord heet dit proces eutrofiëring. Door de algengroei wordt het water heel troebel, je kunt de bodem niet meer zien. Door afsterving van algen en waterplanten wordt het water zuurstofarm. Vissen verdwijnen en het water gaat stinken door rottingsprocessen waarin andere micro-organismen een rol spelen. Gezien het bovenstaande is het dus belangrijk om het rioolwater zo fosfaatarm mogelijk te maken voordat het weggegooid wordt in de natuur.

Biologische fosfaatverwijdering

Het gebruik van fosfaatvrije wasmiddelen draagt bij aan de vermindering van de hoeveelheid fosfaat in ons afvalwater. De normen die de overheid stelt zijn echter streng. We moeten daarom het fosfaatgehalte in ons afvalwater nog verder verlagen. Sommige micro-organismen kunnen ons daarbij helpen. Zo zijn er bijvoorbeeld bacteriën gevonden, behorende tot de soort *Acinetobacter*, die veel meer fosfaat naar binnen werken dan nodig is voor de groei. Dit extra fosfaat wordt in deze gulzigaards geaccumuleerd in de vorm van lange fosfaat ketens: het polyfosfaat. Dit polyfosfaat wordt samen met metaal-ionen opgeslagen in polyfosfaatkorrels die ronddrijven in de cel. Na een goede maaltijd in fosfaatbevattend afvalwater zitten de beestjes tot de nok toe vol met deze polyfosfaatkorrels, terwijl het fosfaatgehalte in hun omgeving sterk is verlaagd. De volle bacteriën kunnen nu worden verzameld zodat fosfaatarm afvalwater overblijft. Deze zuiveringsmethode waarin bacteriën nuttig werk voor ons verrichten, wordt biologische fosfaatverwijdering genoemd.

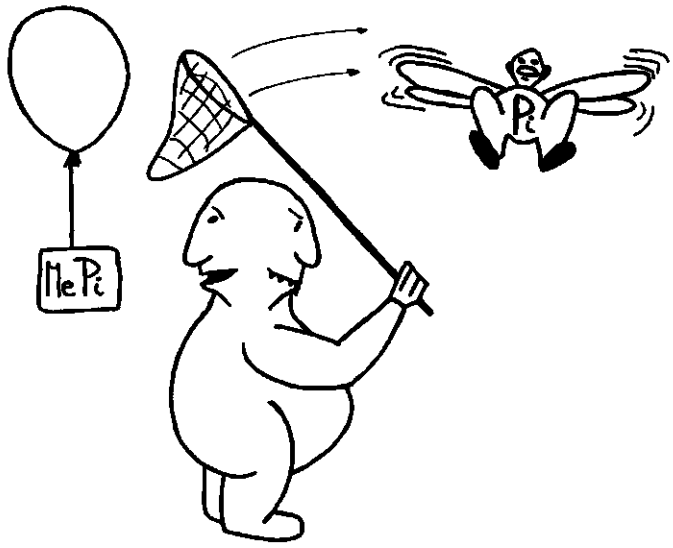
Polyfosfaat-accumulerende acinetobacters

De arbeid die acinetobacters verrichten kost energie. Net als wij, haalt de acinetobacter adem omdat zuurstof in zijn lichaam een belangrijke rol speelt bij het vrijmaken van

bruikbare energie uit voedsel. Onderzoekers hebben ontdekt dat er iets raars gebeurt als je polyfosfaat-bevattende acinetobacters in een omgeving brengt zonder zuurstof: zij breken polyfosfaat af tot fosfaat, en scheiden fosfaat uit in hun omgeving. Het werd zelfs nog eigenaardiger, toen men ontdekte dat zo'n zuurstofloze periode heel gunstig is voor het proces van biologische fosfaatverwijdering. Polyfosfaat-accumulerende acinetobacters worden talrijker in een waterzuiveringsinstallatie waarin een zuurstofrijke periode wordt afgewisseld met een zuurstofarme periode. Maar, hoe overleeft de bacterie zonder zuurstof? Dit proefschrift gaat over de vraag wat er gebeurt tijdens de afbraak van polyfosfaat in acinetobacters. Kan de bacterie polyfosfaat als bron van energie gebruiken? Hoe scheidt de bacterie fosfaat uit? Maar ook, hoe komt de bacterie aan het fosfaat dat nodig is voor de opbouw van polyfosfaat?

De "mond" van acinetobacters

Een bacterie heeft niet één mond, maar wel duizenden tegelijkertijd die over het hele lichaam zijn verspreid. Het is dan ook heel moeilijk voor een bacterie om zijn mond dicht te houden, vooral omdat zijn mondjes met verschillende taken bezig zijn. Terwijl de eerste mond zich bijvoorbeeld verslikt in de opname van boterzuur molekulen, een tweede een aminozuur molekuul het lekkerst vindt, is een derde mond weer gespecialiseerd in de opname van een andere voedingstof, zoals bijvoorbeeld fosfaat. Heeft onze polyfosfaat-accumulerende modelbacterie *Acinetobacter johnsonii* 210A een mond voor fosfaat? Ja, en niet één maar twee verschillende soorten (Figuur 2) ! De eerste soort (de "metaal-fosfaatmondjes") is met een vast aantal aanwezig. De soort krijgt het fosfaat relatief moeilijk te pakken, ook omdat feitelijk niet fosfaat zélf maar een metaal-fosfaat complex herkend wordt. Bij een grote hoeveelheid fosfaat en metaal ionen in het milieu is dat geen probleem. Als de fosfaatconcentratie echter erg laag wordt (minder dan 0.1 milligram per liter), komt er nauwelijks nog metaal-fosfaat via deze mondjes naar binnen. Daar heeft het beestje iets op gevonden. Bij lage fosfaatconcentraties vergroot de acinetobacter het aantal exemplaren van een tweede soort mond (de "fosfaatmondjes-met-arm") die met een arm, vrij fosfaat uit het milieu wegvangt en naar zich toebrengt. Dat gaat veel gemakkelijker maar het kost ook meer energie en daar wil de bacterie spaarzaam mee omgaan.



Figuur 2. Fosfaatopname in *Acinetobacter johnsonii* 210A. De bacterie bezit twee verschillende soorten monden voor fosfaat. Alhoewel er van elke soort nu maar één is getekend, komen de mondjes in werkelijkheid met duizenden tegelijkertijd voor over het hele lichaam. Het "metaal-fosfaatmondje" (links) kan metaal-fosfaat complexen maar moeilijk te pakken krijgen. Het "fosfaatmondje-met-arm" (rechts) vist met een soort arm, fosfaat weg uit het milieu. De bacterie moet zich inspannen om metaal-fosfaat en fosfaat te pakken te krijgen, en vast te leggen in polyfosfaat. De energie die daarvoor nodig is, haalt de bacterie uit voedsel en zuurstof.

Fosfaatopname kost energie

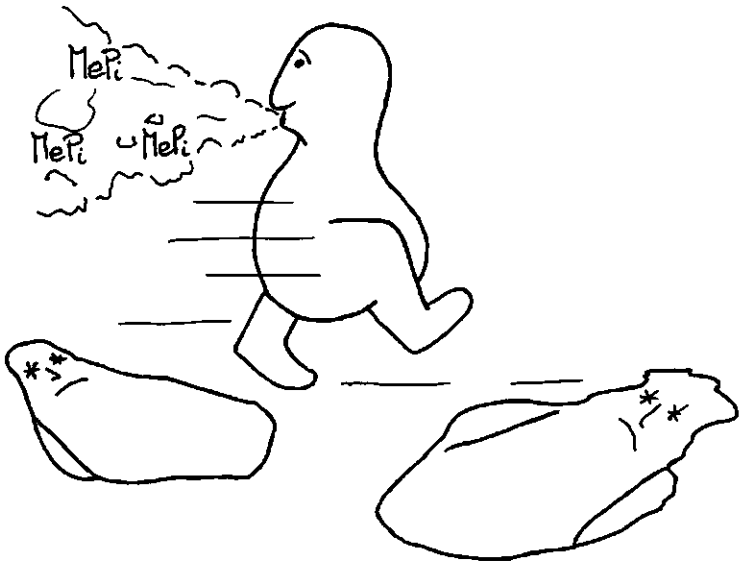
Een acinetobacter is een heel eenvoudig organisme. Het bestaat maar uit een enkele cel. De samenstelling van de vloeistof in zo'n cel is heel anders dan die van het omringende milieu. Zo is de fosfaatconcentratie in de cel vaak veel hoger en constanter dan die erbuiten. Het organisme moet energie investeren om dit verschil in stand te houden. Bij de opname van metaal-fosfaat complexen via de "metaal-fosfaatmondjes" wordt deze energie geleverd via een protonenpomp. Deze pomp zit net als de mondjes van de bacterie in een dun vliesje om de cel, dat het celmembraan wordt genoemd. De protonenpomp is continu bezig om protonen (H^+) vanuit de binnenzijde van de cel naar

buiten te pompen ten koste van energie die verkregen wordt uit voedsel en zuurstof. Hierdoor wordt het aantal protonen aan de buitenzijde van de cel veel groter dan aan de binnenzijde. Die protonen willen graag weer naar binnen, maar het celmembraan verhindert de doorgang. Protonen kunnen echter wel worden opgenomen via de "metaal-fosfaatmondjes", mits een metaal-fosfaat complex meegevoerd wordt naar binnen. Zo'n complex voelt zich dan als op een topdag in de V&D. Als je eenmaal in de stroom winkelende mensen bent terecht gekomen kun je alleen nog maar meelopen en is er geen omkeren meer aan. Zo drijft de protonenstroom de opname van metaal-fosfaat. De "fosfaatmondjes-met-arm" werken volgens een andere methode. Hier staat een portier bij de binnenkant van de deur, in de vorm van een energierijke verbinding die ATP wordt genoemd. Splitsing van ATP rukt de fosfaatklant dan naar binnen en zorgt ervoor dat de gast niet meer langs deze weg terug kan. Over een hartelijk welkom gesproken....

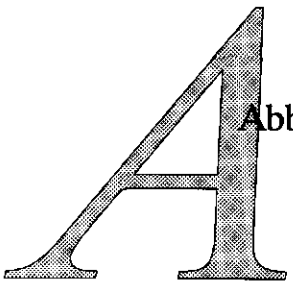
Fosfaatafgifte levert energie.

We weten nu al het een en ander over acinetobacters. Ze kunnen polyfosfaat maken dat samen met metaal ionen wordt opgeslagen in korrels in de cel. Acinetobacters bezitten mondjes voor de opname van metaal-fosfaat complexen en vrij fosfaat. We kunnen zeggen dat de vorming van polyfosfaat niet goedkoop is voor de bacterie. Niet alleen de opname van (metaal-)fosfaat vraagt nogal wat energie. Ook voor de vorming van polyfosfaat uit fosfaat zal de cel diep in zijn energie-beurs moeten tasten. Tenslotte hebben we kunnen lezen dat acinetobacters bruikbare energie uit voedsel kunnen vrijmaken wanneer zuurstof aanwezig is. Wat gebeurt er met de bacterie bij afwezigheid van zuurstof? Gaat de acinetobacter dan direct dood? Nee, want onder deze omstandigheden wordt het polyfosfaat als energieleverancier gebruikt. Het polyfosfaat wordt gesplitst, waarbij metaal-fosfaat via de "metaal-fosfaatmondjes" uitgescheiden wordt in het milieu. Deze mondjes werken nu dus in de omgekeerde richting. De energie die nodig was voor de opname van metaal-fosfaat wordt daardoor deels weer teruggewonnen. Ook de energie die direct vrijkomt tijdens splitsing van polyfosfaatketens kan mogelijk door de bacterie worden benut. Door het gebruik van polyfosfaat als energiereserve behouden polyfosfaat-accumulerende acinetobacters hun

levensvatbaarheid onder omstandigheden waar andere bacteriën al snel het loodje leggen (Figuur 3). De wereld van de micro-organismen is hard en onverbiddelijk, maar wél bijzonder interessant.



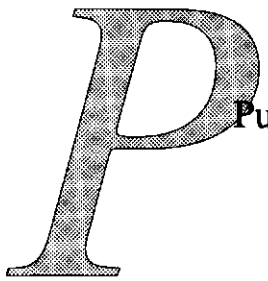
Figuur 3. Polyfosfaat-afbraak en fosfaat-afgifte in *A. johnsonii* 210A. Het "metaal-fosfaatmondje" kan metaal-fosfaat complexen naar binnen werken, maar ook naar buiten. Tijdens de afbraak van polyfosfaat spuugt het metaal-fosfaat complexen uit. Het "fosfaatmondje-met-arm" zorgt alleen voor de opname van fosfaat. Deze mond is stil tijdens de afbraak van polyfosfaat. Polyfosfaat-afbraak en fosfaat-afgifte leveren de bacterie energie. Hierdoor kan *A. johnsonii* 210A een periode zonder zuurstof overleven, terwijl andere bacteriën dan al snel het loodje leggen.



Abbreviations

$\Delta\psi$	: transmembrane electrical potential difference
$Z\Delta pH$	: transmembrane proton gradient (in millivolt)
Δp_H+	: proton motive force ($\Delta p_H+ = \Delta\psi$ - $Z\Delta pH$)
$Z\Delta pNa$	: transmembrane chemical gradient of sodium ions (in millivolt)
$\Delta p_{Na}+$	: sodium motive force ($\Delta p_{Na}+ = \Delta\psi$ - $Z\Delta pNa$)
ADP	: adenosine 5'-diphosphate
AMP	: adenosine 5'-monophosphate
ATP	: adenosine 5'-triphosphate
BCECF	: 2',7'-bis-(2-carboxyethyl)-5[and -6]-carboxyfluorescein
CCCP	: carbonyl cyanide 3-chlorophenylhydrazone
DCCD	: *N,N'*-dicyclohexylcarbodiimide
$DiSC_3(5)$	: 3,3'-dipropylthiadicarbocyanine iodide
DNP	: α-dinitrophenol
DTT	: dithiothreitol
EDAX	: energy dispersive X-ray micro-analysis
EDTA	: ethylenediaminetetraacetate
Hepes	: 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid
$MeHPO_4$	: metal phosphate complex
Mes	: 2-(*N*-morpholino)ethanesulfonic acid
P_i	: inorganic phosphate
Pipes	: piperazine-*N,N'*-bis(2-ethanesulfonic acid)

PolyP	: polyphosphate
PQQ	: 4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]-quinoline-2,7,9-tricarboxylic acid
SDS	: sodium dodecyl sulphate
TCA	: trichloroacetic acid
TPP^+	: tetraphenylphosphonium ion
Tris	: 2-amino-2-(hydroxymethyl)-1,3-propandiol



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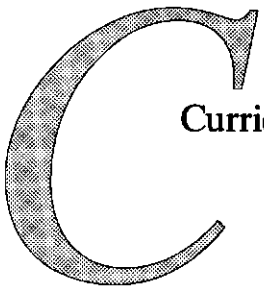

Curriculum vitae

In de vroege ochtend van 1 augustus 1963 werd Hendrik Willem van Veen geboren te Kerensheide, gemeente Beek, Limburg. Na een verhuizing in 1969 naar Beek werd hij zozeer aangetrokken door het wel en wee van zijn nieuwe microgroentetuin (1 m^2) dat de gedachte opkwam om "later" naar de toenmalige Landbouw Hogeschool in Wageningen te gaan. Na het ontvangen van het einddiploma Atheneum-B aan de Scholengemeenschap St. Michiel te Geleen, werd in 1981 begonnen met de studie Moleculaire Wetenschappen (biologische oriëntatie) in Wageningen. In mei 1985 werd het kandidaatsexamen met lof afgelegd. Het daarna volgende doctoraalprogramma omvatte 3 hoofdvakken, Biochemie (De Kok/Veeger), Moleculaire Genetica (Kusters/Harmsen/Visser) en Microbiologie (Kortstee/Zehnder). Deze studiefase werd afgesloten met een stage bij E.I. DuPont de Nemours and Co., Inc., Wilmington, Delaware, USA (Weimer/Marrs) waar Riks enthousiasme voor microbiële fysiologie verder kon worden uitgebouwd. Het ingenieursdiploma werd in maart 1988 met lof behaald. Een maand later begon een nieuw leven in inspirerend groen bij de krijgsmacht. Tijdens deze periode werd de auteur volop de gelegenheid geboden zich verder te verdiepen in de moleculaire microbiologie. Van juni 1989 tot december 1993 werkte Rik als onderzoeker in opleiding bij de vakgroep Algemene Microbiologie van de Landbouwuniversiteit Wageningen. Het onderzoek dat hij in deze periode heeft verricht aan fosfaattransport in *Acinetobacter johnsonii* werd afgerond met de vervaardiging van het proefschrift dat nu voor u ligt. Sinds januari 1994 is Rik als post-doc werkzaam bij de vakgroep

Microbiologie van de Rijksuniversiteit Groningen, waar hij onderzoek verricht aan multidrugresistentie in melkzuurbacteriën.

Hend**rik** Willem van Veen was born on August 1 1963 in Beek in the province of Limburg (Netherlands). He attended St. Michiel high school (Atheneum-B) in Geleen (1975-1981). In 1981 he began his microbiological studies ("Molecular Sciences") at the Wageningen Agricultural University where he received his bachelors degree with honours in May 1985. Rik continued at the same university with majors in biochemistry, molecular genetics and microbiology. A practical period was carried out at E.I. DuPont de Nemours and Co., Inc., Wilmington, Delaware, USA. In March 1988, he received his masters degree with honours. After having served in the army, he started his PhD study in June 1989 at the Department of Microbiology of the WAU. This study on phosphate transport in *Acinetobacter johnsonii* was finished in december 1993 with this thesis. Rik will continue to work in the field of bioenergetics and solute transport. Since January 1994, he has a post-doc position at the Department of Microbiology, University of Groningen, where he is studying bacterial multidrug resistance.