ACETATE METABOLISM

IN

METHANOTHRIX SOEHNGENII

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Acetate metabolism

in

Methanothrix soehngenii

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
Dr. H. C. van der Plas,
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van de Landbouwuniversiteit te Wageningen.

Stellingen

1. Het meten van complexe enzymreacties in cel-vrije extracten levert meer interpretatieproblemen, dan uitkomsten op.

   Fisher, R. and Thauer, R.K. (1988) Methanogenesis from acetate in cell extracts of *Methanosarcina barkeri.* Arch. Microbiol. 151, 459-465.

2. De bevinding van Fiala en Stetter, dat *Pyrococcus furiosus* alleen H_2 en CO_2 als eindprodukten maakt, berust op onzorgvuldige analyse methoden.

   Fiala, G. and Stetter, K.O (1986) *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Arch. Microbiol. 145, 56-61.

3. Het "herontdekken" van het $g_{ave} = 1.87$ signaal in EPR spectra van het CO dehydrogenase van *Clostridium thermoaceticum* door Lindahl et. al. getuigt niet van veel bekendheid met eigen onderzoek.

   Lindahl, P.A., Münck, E. and Ragsdale, S.W. (1990) CO dehydrogenase from *Clostridium thermoaceticum.* J. Biol. Chem 265, 3873-3879.

   Ragsdale, S.W, Ljungdahl, L.G and DerVartanian, D.V. (1982) EPR evidence for nickel-substrate interaction in carbon monoxide dehydrogenase from *Clostridium thermoaceticum.* Biochem. Biophys. Res. Comm.108, 652-663.

4. De bewering van Kohler dat *Methanothrix soehngenii* "sich wenig eignet als biochemisches Untersuchungsobjekt" wordt door dit proefschrift teniet gedaan.

   Kohler, H.-P.E. (1986) Acetatkatabolismus in *Methanothrix soehngenii.* Dissertatie ETH Zürich no. 8033, p. 92.

   Dit proefschrift.

5. Resultaten, verkregen door het gebruik van P^1,P^5-Di(adenosine-5')pentafosfaat als remmer voor adenylaat kinase in gekoppelde enzym assays, dienen uiterst zorgvuldig geïnterpreteerd te worden.

   Van Groenestijn, J.W., Bentvelsen, M.M.A., Deinema, M.H. and Zehnder, A.J.B. (1989) Polyphosphate-degrading enzymes in *Acinetobacter spp.* and activated sludge. Appl.Environ. Microbiol. 55, 219-223.

6. In experimenten waarbij de hoeveelheid azijnzuur in grammen wordt afgewogen, is het gebruik van de "eenheid" mg COD (Chemical Oxygen Demand) als maat voor de hoeveelheid azijnzuur niet op zijn plaats.

    Ten Brummeler, E., Hulshoff Pol, L.W., Dolfing, J., Lettinga, G. and Zehnder, A.J.B. (1985) Methanogenesis in an UASB reactor at pH 6 on an acetate-propionate mixture. Appl. Environ. Microbiol. 49, 1472-1477.

7. Stellingen bestel je bij Overtoom.

8. Het aantal publicaties per WP-er is omgekeerd evenredig met het aantal WP-ers per vakgroep.

    Wetenschappelijk Jaarverslag 1989, Landbouw Universiteit Wageningen.

9. Bij het gebruik van de MPN methode zit men er waarschijnlijk Meestal Pijnlijk Naast.

10. Het houden van een evaluatie heeft alleen (positief) effect, wanneer zowel de gever als ontvanger op de hoogte zijn van de grondregels van evalueren.

11. De grafieken, die in de media gebruikt worden om de belangrijkste beursparameters weer te geven, dragen niet bij aan een beter begrip van deze parameters.

12. Stakingen in het openbaar vervoer treffen in het algemeen de verkeerde doelgroep.

    Stellingen behorende bij het proefschrift "Acetate metabolism in *Methanothrix soehngenii*" van M.S.M. Jetten.

    Wageningen 20 december 1991

CONTENTS

Chapter 1

General Introduction

GENERAL INTRODUCTION

Preview

In this chapter the reader will be introduced to the main aspects of the methane formation from acetate. In the first part the position of methanogenesis in the anaerobic degradation of organic matter is described. Thereafter a brief summary of the main characteristics of the methane bacteria will be given. This summary is followed by a historical overview of methanogenesis from acetate. Then an overview of the physiology and biochemistry of the acetoclastic methanogenesis is given. At the end of the introduction a short summary of the data available on the molecular biology of *Methanothrix* and an outline of this thesis are presented.

Methanogenesis

Microorganisms play an important role in the conversion of organic and inorganic matter [1]. Under anaerobic conditions organic matter is degraded by the cooperative interaction of several different physiological groups of micro-organisms [2-4]. A schematic presentation of the anaerobic degradation under methanogenic conditions is depicted in Fig. 1. The anaerobic degradation of organic matter starts with the hydrolysis of complex biopolymers into the corresponding monomers by fermentative bacteria [5,6]. These monomeric products (sugars, fatty acids and amino acids) are converted by fermentative bacteria to intermediate products like, acetate, propionate, butyrate, lactate and alcohols [7]. Most of these compounds are oxidized by obligate proton-reducing acetogenic bacteria to acetate, hydrogen and CO_2 [7].
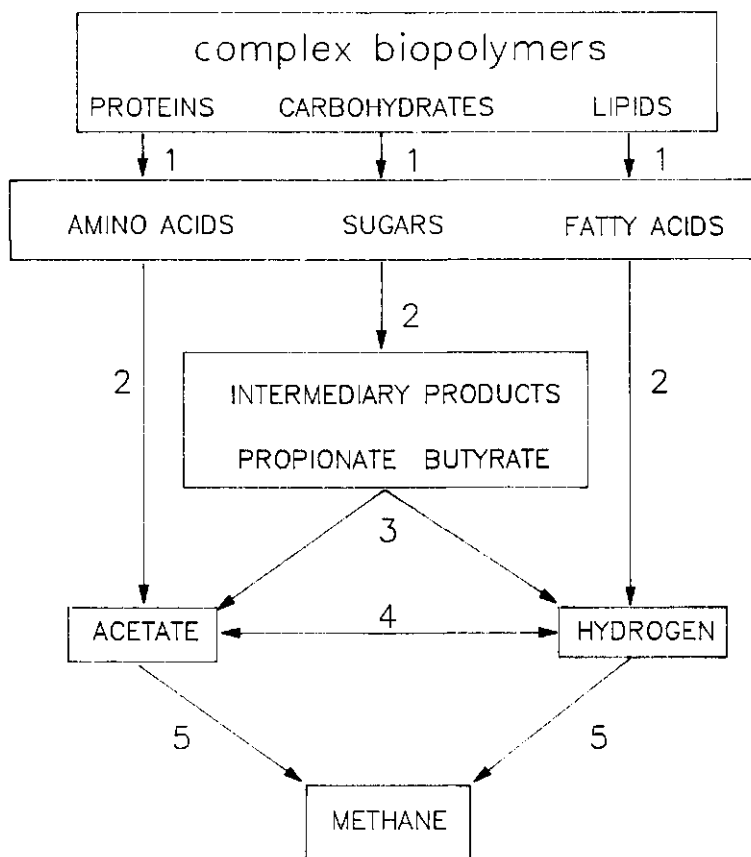
In the absence of light or electron acceptors as nitrate or sulfate terminal degradation steps are performed by the methane bacteria [8]. In this step the methane bacteria convert H_2/CO_2 and acetate to methane [1,8]. The methane bacteria play an important regulatory role in the proton and electron flow during the anaerobic degradation of organic matter [7,8]. The comsumption of hydrogen provides a low partial hydrogen pressure. This enables the obligate proton-reducing bacteria to degrade the organic acids, which are thermodynamically very unfavourable reactions [7-10]. The comsumption of acetate by the acetoclastic methanogens prevents acidification of the anaerobic ecosystem [11].

Acetate is quantitatively the most important intermediate in the anaerobic degradation of organic matter [11,12]. It is the most abundant precursor of methane formation. Seventy precent of the methane in anaerobic digestors is derived from acetate [13]. In sediments, flooded soils and rice-fields also 60-80 % of the methane is formed from acetate [3,14-17]. Further the conversion rate of acetate by methanogenic bacteria is proposed to be the rate limiting step in the degradation of soluble organic matter under anaerobic conditions [18]. The study of acetoclastic methanogens, therefore, is of relevance to our understanding of anaerobic processes and their optimal application in treatment of waste water from various sources.

Methanogenic bacteria

Methanogenic bacteria have been isolated from nearly every habitat in which anaerobic degradation of organic material takes place, such as waste water digestors, fresh water and marine sediments, and intestinal tracts

3

complex biopolymers

PROTEINS   CARBOHYDRATES   LIPIDS

↓ 1   ↓ 1   ↓ 1

AMINO ACIDS   SUGARS   FATTY ACIDS

2

INTERMEDIARY PRODUCTS

PROPIONATE   BUTYRATE

2   2

3

ACETATE ←———— 4 ————→ HYDROGEN

5   5

METHANE

Fig. 1 : Anaerobic degradation of soluble organic matter under methanogenic conditions

(1): Hydrolytic degradation of biopolymers
(2): Fermentation of monomers
(3): Oxidation by obligate proton reducing bacteria
(4): Acetogenic hydrogen conversion
(5): Methanogenesis

of man and animals [20-24].

axonomically, methane bacteria comprise of a rather diverse group. Balch et al. proposed classification into three orders and six families on the basis of morphological differences : Methanobacteriales (Methanobacteriaceae and Methanothermoceae), Methanococcales (Methanococceae) and Methanomicrobiales (Methanomicrobiaceae, Methanosarcinaceae and Methanoplanaceae) [25]. Although, taxonomically rather diverse, the methane bacteria were reorganized in a coherent group on the basis of their physiological similarities [26,27].

Methane bacteria produce methane from a limited number of compounds. The classical one- and two-carbon compounds are listed in Table I. Nearly all methanogenic bacteria use H_2/CO_2, whereas many species are also able to utilize formate [28]. Until now only two genera of methane bacteria are described which can use acetate as carbon and energy source : *Methanosarcina* and *Methanothrix* [28]. Very recently it was shown that some methane bacteria are also able to use primary and secondary alcohols [29,30].

Methanogenic bacteria show clear differences by comparison with other procaryotes [25]. The cell wall consists of proteins, glycoproteins or contains pseudomurein instead of murein [31,32]. The membranes do not contain glycerol-esters of fatty acids, but are composed of the glycerolethers of squalens or other isoprenoid hydrocarbons [33,34]. Methane bacteria use a number of unique coenzymes in their metabolism [35,36]. Further differences were observed in the nucleotide patterns of the 16S rRNA, the base composition of the tRNA and the length of the genome [37-39]. On the basis of all these differences Woese et al. classified the methane bacteria to the domain of the archae [40]. In the next section an overview is presented of the history of methane formation from acetate.

Historic overview of the methane formation from acetate

After the discovery of methane (aria infiammabile) by A. Volta in 1776, it took another century before the microbial origin of the methane formation from decomposing plant material was demonstrated by Béchamp (1868) and Popoff (1873) [41-43]. The methane production from acetate was firstly described by Hoppe-Seyler in 1876 [44]. Even before the classical thermodynamical concepts were totally formulated, he noticed the difficulties of the energy conservation in acetoclastic methanogenesis. Extensive description of acetoclastic methanogenic bacteria occurred in the first decade of this century by Söhngen (1906) [45]. He described two different organisms from enrichment cultures on acetate; a gram-negative sarcina and a non-motile rod shaped bacterium which often formed long filaments [46]. His cultures were lost after the end of his work. Barker conducted in 1936 further studies and got again enrichments of the bacteria described by Söhngen [47]. He named them *Methanosarcina methanica* and *Methanobacterium soehngenii* [48]. Barker was not able to obtain these bacteria in pure culture. It was Schnellen in 1947, who isolated the first methanogen in pure culture : *Methanosarcina barkeri* [49]. However, after some taxonomic studies the sarcina was lost. New isolates of *Methanosarcina* were obtained and described from 1974 on [50,51].

The non-motile rod "*Methanobacterium soehngenii*" was described in various enrichment cultures on butyrate, benzoate and lignin [10,52-54]. But it lasted untill 1980 before the bacterium was obtained in pure culture by Zehnder and coworkers [55]. They renamed the bacterium *Methanothrix soehngenii*, with as type strain, strain Opfikon [56,57]. Thereafter several *Methanothrix* strains have described including the

Table I : Substrates used by methanogenic bacteria

			$\Delta G'_o$ (kJ/mol CH_4)
$4 H_2 + CO_2$	---------->	$CH_4 + 2H_2O$	-130
$4 HCOOH$	---------->	$CH_4 + 3 CO_2 + 2H_2O$	-120
$4 CO + 2H_2O$	---------->	$CH_4 + 3 CO_2$	-186
CH_3COOH	---------->	$CH_4 + CO_2$	- 32
$CH_3OH + H_2$	---------->	$CH_4 + H_2O$	-113
$4 CH_3OH$	---------->	$3 CH_4 + CO_2 + 2H_2O$	-103
$4 CH_3NH_2 + 2H_2O$	---------->	$3 CH_4 + CO_2 + 4 NH_3$	- 74

$\Delta G'_o$ = Gibbs free energy at pH 7, 25 °C, 1 atm and 1 M concentration.

Table II : Comparison of the physiological parameters of *Methanosarcina spp.* and *Methanothrix spp.*

Organism		*Methanosarcina*	*Methanothrix*
Morphology		Sarcina-type	Rod-shaped
		Clumps	Long filaments
Physiology		Generalist	Specialist
Substrates		Hydrogen, Methanol Methylamines, Acetate	Acetate
μ_{max}	$(days)^{-1}$	0.3	0.1
doubling time	(days)	0.5-2	1-12
Yield	(g dw/mol Ac)	2.1	1.4
K_s	(mM)	3.0	0.5
Threshold	(mM)	0.2 - 1.2	0.007 - 0.07

mesophilic *Methanothrix soehngenii* strain FE, and *Methanothrix concilii* GP-6 and the thermophilic strains *Methanothrix* CALS-1 and *Methanothrix thermoacetophila* [58-61]. Although immunological studies with antisera showed only a weak relationship between the different *Methanothrix* species, Touzel unified the strains Opfikon, FE and GP-6 into one species, *Methanothrix soehngenii*, on the basis DNA-DNA hybridization studies [58,62]. However, recently it has been proposed that these three *Methanothrix* species should be included in the genus *Methanosaeta* [63]. Since the discussion to maintain the name of *Methanothrix* is still in progress, throughout this thesis the name *Methanothrix soehngenii* will be used to avoid any confusion.

Physiology and biochemistry of acetoclastic methanogenesis

Only two genera of methane bacteria are known which degrade acetate; *Methanosarcina* and *Methanothrix*. The acetoclastic metabolism of *Methanosarcina* recieved considerable attention in the past decade, but hardly any atttention was paid to the enzymology and biochemistry of *Methanothrix* [64]. In next part of the introduction the relevant knowledge about the acetoclastic methanogenesis is summarized.

Physiology

In Table II an overview is presented of the physiological properties of both *Methanosarcina* and *Methanothrix*. Although not strictly a physiological property the table starts with the comparison of the morphological differences between the two genera [31,32,54-62,65,66]. The two acetoclastic methane bacteria have developped different strategies for growth on acetate. *Methanosarcina* is metabolically versatile [64]. It appears to be

a generalist, capable of growing on several different substrates including, H_2/CO_2, methanol, methylamines and acetate [13,67]. The bacterium grows on acetate as sole energy source with a doubling time of about one day and a growth yield of 2 g dry weight / mol acetate. Its affinity for acetate is rather low (K_s = 3-5 mM) [13,67]. *Methanothrix* species differ from *Methanosarcina* species in that they use only acetate as energy source. The growth rate is low (doubling time 2-12 days) and the growth yield is low (1.4 g dry weight / mol acetate) [54,55]. However, its affinity for acetate is rather high (K_s = 0.5 mM) [55].

The minimum threshold concentration of acetate utilization of *Methanosarcina* and *Methanothrix* was investigated by several research goups [68,69]. It was shown that *Methanothrix* reached lowest acetate concentrations (<10 μM), where *Methanosarcina* did not consume acetate under concentrations of 0.2-1.2 mM. The kinetic parameters (μ_{max} , K_s and threshold) of *Methanothrix* and *Methanosarcina* are depicted in Fig. 2. From this figure it can be seen that *Methanothrix* is favoured in ecosystems with acetate concentrations between 0 and 1 mM [4,68,69].

Biochemistry

Although *Methanothrix* and *Methanosarcina* produce methane from acetate their metabolism shows some differences [64]. The general scheme of the acetoclastic pathway is depicted in Fig. 3. The conversion of acetate starts with the activation of acetate to acetyl-CoA [64,70,71]. Since both organisms have different kinetic parameters for growth on acetate, it is not surprising that they have developped different mechanisms for the activation of acetate [64]. In *Methanothrix* acetate is activated by the enzyme acetyl-CoA synthetase [70]. The enzyme activates acetate to acetyl-CoA, with
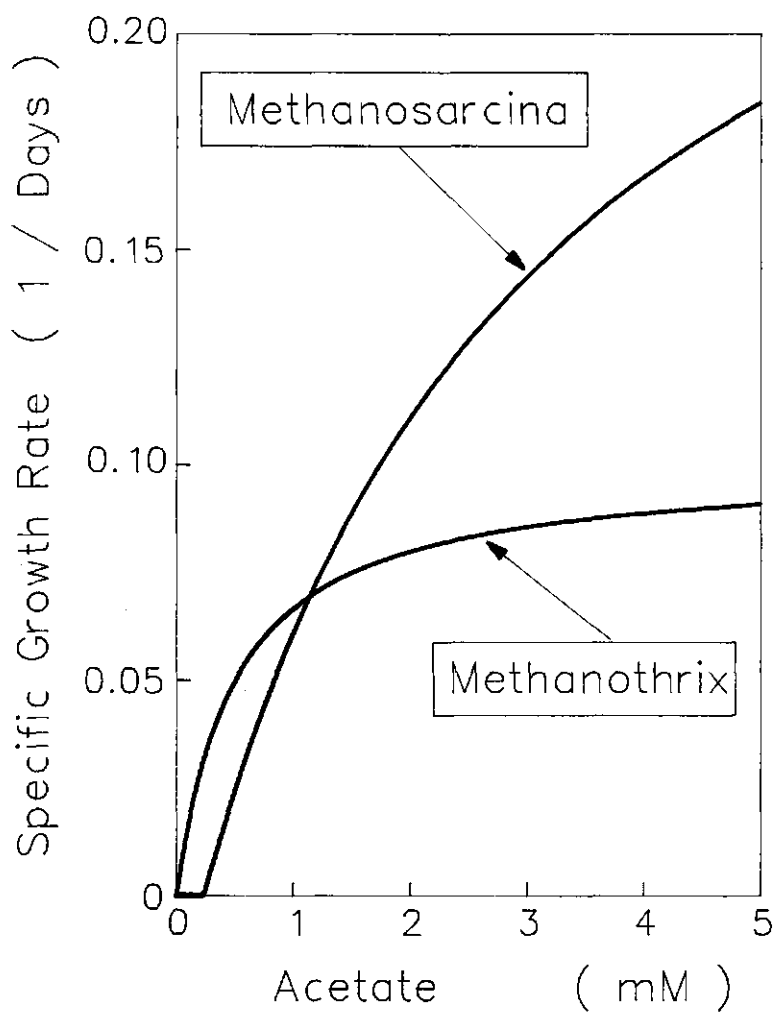
7

Fig. 2 : Relation between specific growth rate (μ) and acetate
concentration for *Methanosarcina spp.* and *Methanothrix spp.*.

concomitant hydrolysis of one ATP to AMP and PP$_i$. *Methanosarcina* uses acetate kinase and phosphotransacetylase to activate acetate [71]. Via acetyl-phosphate, acetyl-CoA is formed at the expense of one ATP, which is hydrolyzed to ADP and P$_i$. Both enzyme systems have been purified from the respective organisms [72-74]. The properties of the Acetyl-CoA synthetase are described in chapter 3 and are compared with those of the acetate kinase and phosphotransacetylase of *Methanosarcina*. The kinetic properties of both enzyme systems support again the hypothesis that *Methanosarcina* predominates in systems with high acetate concentrations, whereas *Methanothrix* is dominant in environments with low acetate concentrations [4,68,69,75].

In the acetate activation to acetyl-CoA by *Methanothrix* one ATP is cleaved to AMP and PP$_i$. The AMP is converted to ADP at the expense of one other ATP by the enzyme adenylate kinase [74]. The PP$_i$ formed is hydrolyzed to two P$_i$ by the action of an inorganic pyrophosphatase. A sum of these reactions leads to the suggestion that for acetate activation in *Methanothrix* two ATP are needed [64]. The possible result of this high energy input to activate its substrate, may be the relatively low growth yield and growth rate of *Methanothrix* compared to *Methanosarcina*, who only invests one ATP in acetate activation. Since it is believed that methane formation from acetate can only yield one ATP, it is difficult to envisage how *Methanothrix* is able to grow at all [64]. One possible site of energy conservation may be coupled to the hydrolysis of pyrophosphate. To investigate the possibility that the energy of the PP$_i$ bound could be used to drive endergonic reactions, the pyrophosphatase was isolated from *Methanothrix*. The properties and purification of the pyrophosphatase from *Methanothrix* are described in chapter 4.

In chapter 5 the interconversion of adenine nucleotides by *Methanothrix* during acetate consumption is described and compared to results obtained from experiments with other (methane) bacteria.

After acetate activation, the formed acetyl-CoA is supposed to be cleaved into C$_1$-units, one at the oxidation level of methanol (methyl-group), the other at the oxidation level of CO (carbonyl-group) [64]. The methyl-group is reduced to methane, the electrons for the reduction are derived from the oxidation of CO-moiety [76-79]. The carbon-carbon cleavage and the oxidation of the carbonyl-group are probably catalyzed by the same enzyme: CO dehydrogenase (CDH) [80,81]. CDH is present in both *Methanosarcina* and *Methanothrix* and constitutes up to 5 % of the soluble cell protein of these bacteria [82-86]. The enzymes have similar subunit composition $(\alpha\beta)_2$ and molecular masses (200 kDa) [82-86]. The enzyme contains Ni and Fe/S clusters [82-86].

The CO:methylviologen oxidoreductase activity of *Methanothrix* was found to be insensitive to oxygen, whereas other CDH's from anaerobic bacteria including *Methanosarcina* are inactivated by traces of oxygen [70,86]. The properties of this oxygen stable CDH are described in chapter 6. Since the enzyme contained Ni, Fe and acid-labile sulfur, the presence of paramagnetic centers were investigated by EPR spectroscopy. The actual acetyl-CoA cleavage function of the enzyme is difficult to demonstrate, since the functional acceptors of the cleavage products have to be available [87,88]. As an alternative for the cleavage function, the exchange reaction between the carboxyl-group of acetyl-CoA and CO can be studied in absence of a functional methyl-acceptor [87-90]. The acetyl-CoA/CO exchange activity of the CDH and the paramagnetic centers of the anaerobically purified CDH are presented in chapter 7. In order to characterize the

CH₃-COOH

$CH_3\text{-}COOH$

2 Pi + 2 ADP → ATP — HSCoA

ATP

7   6   1

PPi + AMP

$CH_3\text{-}CO\text{-}SCoA$

2a

$[CH_3\text{-}]$ $[CO]_{enz}$

3   HSCoM

$CH_3\text{-}SCoM$ 5   2b

HS-HTP E_{ox}

4

CoM-S-S-HTP E_{red}

CH_4 CO_2

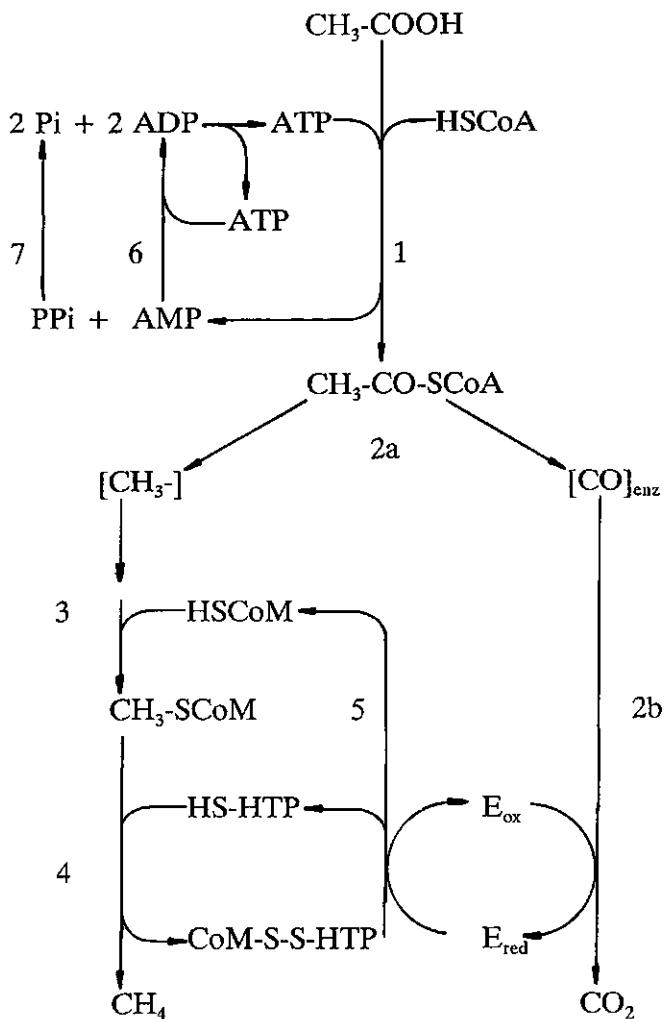**Fig. 3 :** General scheme for acetate degradation by *Methanothrix soehngenii*, based on the information available up to now.

1 = Acetyl-CoA synthetase
2a = Carbon monoxide dehydrogenase (cleavage reaction)
2b = Carbon monoxide dehydrogenase (oxidation reaction)
3 = Methyltranferases
4 = Methyl-coenzyme M reductase
5 = Heterodisulfide reductase
6 = Adenylate kinase
7 = Inorganic pyrophosphatase

possible Ni site and some unusual clusters in the CDH, the enzyme was isolated from *Methanothrix* grown in the presence of ^{61}Ni. The EPR properties of these unusual clusters are presented in chapter 8.

The methyl-group of acetyl-CoA is ultimately transferred to coenzyme M by several methyltransferases [64,91-95]. In *Methanosarcina* tetrahydromethanopterin and corrinoid containing proteins are involved in this transfer [91-95]. For *Methanothrix* this transfer proces is not yet studied, but the presence of both tetrahydromethanopterin and corrinoids in *Methanothrix* indicates that similar processes could be envisaged in this organism [96,97]. The last step in methanogenesis is reduction of methyl-coenzyme M to methane [98]. This reaction is catalyzed by the methyl-CoM reductase. In chapter 9 of this thesis some of the properties of the methyl-CoM reductase from *Methanothrix* are presented and compared with data from other methyl-CoM reductases.

The direct electron donor for methyl-CoM reduction has been shown to be 7-mercapto-heptanoyl-threoninephosphate (HS-HTP) [99]. HS-HTP and HS-CoM are regenerated by the reduction of the mixed heterodisulfide (CoM-S-S-HTP, HDS). This reaction is catalyzed by the heterodisulfide reductase (HDR) [100, 101]. HDR activity was also observed in cell extracts of *Methanothrix* [102]. The electrons necessary for reduction of HDS are probably derived from the oxidation of the CO-group by the CDH [64]. The exact mechanism and electron carriers involved in this proces in *Methanothrix* are not known. In *Methanosarcina* evidence was presented that a ferredoxin is involved in the transfer of electrons from the CDH to a membrane bound hydrogenase (electron transport chain) [103-105]. The coupling of electron flow to heterodisulfide reductase was studied in membrane vesicles of a sarcina like methanebacterium Göl [106-

110]. For this bacterium it was demonstrated that proton extrusion during HDS reduction could be used to drive ATP synthesis [106]. A similar mechanism may operate in *Methanothrix*, where 20 % of the HDR is membrane associated [102].

Molecular biology of *Methanothrix*

Most of our knowledge about the molecular biology of *Methanothrix* is obtained from studies by Eggen et al., who recently reviewed their work [111-113]. Their results on the genes encoding for acetyl-CoA synthetase and carbon monoxide dehydrogenase are summarized here.

The gene encoding for ACS was isolated from a genomic library in *E. coli* using polyclonal antibodies raised against the purified ACS. After the gene was introduced in *E. coli* a major immunoreactive polypeptide was produced, which made up to 5 % of the cellular protein with a slightly smaller molecular mass than ACS purified from *Methanothrix*. Despite the difference in molecular mass, ACS activity could be detected in cell free extracts of *E. coli*, with even higher specific activities (11.2 μmol.min^{-1}.mg^{-1}) than measured in *Methanothrix* extracts. This indicated that functionally active ACS is efficiently produced by *E. coli*. Data base searches showed that the deduced amino acid sequence of *Methanothrix* ACS is homologous to that of proteins with similar functions found both in bacterial and eucaryal species. In addition, 2 putative ATP binding sequences could be deduced.

The *Methanothrix* cdhA and cdhB genes that code for the large and the small subunit of CDH respectively, were also isolated from a genomic library using polyclonal antibodies raised against purified CDH. Subcloning of these genes into *E. coli* resulted in the production of two immunoreactive polypeptides, which corresponded in size to the

11

purified CDH subunits. The CDH oxido-reductase activity could not be observed in extracts of *E. coli* containing the cdh genes. The *Methanothrix* cdh genes are organized in an operon-like structure with the order cdhA - cdhB. Database searches showed that cdhA contained a strech of 110 amino acids, with 24 % identity to acyl-CoA oxidase from *C. tropicalis*. This is compatible with the acetyl-CoA cleavage function of the CDH. Another region consisting of 64 residues could be identified as a ferredoxin domain, similar with archaeal type ferredoxins. This confirms the functions of the CDH as an electron carrier. The ferredoxin domain contained 8 cysteine residues per cdhA molecule which could bind two [4Fe-4S] clusters. In the NH_2 - terminal region of the cdhA another possible fixation site of several cysteine residues for a iron-sulfur cluster was found.

Outline of this thesis

Since *Methanothrix* was difficult to cultivate, most research on acetoclastic methanogenesis was done with *Methanosarcina*. The aim of this thesis was to extend the knowledge of the acetate metabolism in *Methanothrix* and to compare the results with the knowledge about *Methanosarcina*.

Chapter 2 provides information on the enzyme systems which methanogenic bacteria, including *Methanosarcina* and *Methanothrix*, use for acetate activation. Also the minimum threshold concentrations for acetate utilization of several methane bacteria are presented. The purification and properties of the acetyl-CoA synthetase, the acetate activating enzyme of *Methanothrix*, are described in chapter 3. In chapter 4 the isolation and the characteristics of the inorganic pyrophosphatase purified from acetate grown *Methanothrix* are presented. Chapter 5 describes the interconversion of

adenine nucleotides during acetate degradation by *Methanothrix*.

The enzyme responsible for both the cleavage of acetyl-CoA (into a methyl and carbonyl moiety) and the oxidation of the carbonyl-group in *Methanothrix* is CO dehydrogenase. In chapter 6 the oxygen stable CO oxidation activity of the enzyme is described. The paramagnetic centers and the acetyl-CoA/CO exchange activity of the enzyme are discussed in chapter 7. A more detailed description of a high spin system in the CDH is presented in chapter 8. In chapter 9 some of the properties of the methyl-CoM reductase, which catalyzes the common termimal step in all methanogenic bacteria, are presented. Finally in chapter 10 some aspects of the preceeding chapters are discussed and summarized.

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Chapter 2

Acetate threshold values and acetate activating enzymes
in methanogenic bacteria.

Acetate threshold values and acetate activating enzymes in methanogenic bacteria

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1. SUMMARY

The minimum threshold concentrations of acetate utilization and the enzymes responsible for acetate activation of several methanogenic bacteria were investigated and compared with literature data. The minimum acetate concentrations reached by hydrogenotrophic methane bacteria, which require acetate as carbon source, were between 0.4 and 0.6 mM. The acetoclastic *Methanosarcina* achieves acetate concentrations between 0.2 and 1.2 mM and *Methanothrix* between 7 and 70 μM. For the activation of acetate most of the hydrogenotrophic methane bacteria investigated use an acetyl-CoA synthetase with a relatively low K_m (40–90 μM) for acetate. Although the affinity for acetate was high, the hydrogenotrophic methane bacteria were not able to remove acetate to lower concentrations than the acetoclastic methane bacteria, neither in pure cultures nor in anaerobic granular sludge samples. Based on these observations, it is not likely that hydrogenotrophic methanogens compete strongly for acetate with the acetoclastic methane bacteria.

2. INTRODUCTION

The conversion of H_2/CO_2 or acetate by methanogens is the terminal step in the degradation of organic matter under methanogenic conditions [1]. Only two genera of methanogenic bacteria, *Methanosarcina* and *Methanotrix*, are described which are capable of metabolizing acetate to methane. However, many hydrogenotrophic methane bacteria require acetate as a carbon source [2,3]. Acetate is assimilated via acetyl-CoA, which is the central metabolite for cell synthesis [4]. Autotrophic methanogens synthesize acetyl-CoA from one-carbon substrates, via the acetyl-CoA pathway [5]. Methanogenic bacteria, which require acetate for cell synthesis, lack this ability. Therefore, they must posses a similar mechanism of acetyl-CoA synthesis as the acetoclastic methanogens which activate acetate via an acetate kinase or an acetyl-CoA synthetase [6,7]. Since both hydrogenotrophic and acetoclastic methanogens use acetate for growth, competi-

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tion for this substrate could occur in ecosystems where both types are present [8].

The aim of the present study was to examine the capability of hydrogenotrophic methanogens to use acetate and to investigate their acetate activating enzymes. The threshold values for acetate and the enzyme activities were compared with the data obtained for the acetoclastic methane bacteria. Further, the contribution of the hydro-genotrophic methane bacteria to the removal of acetate in methanogenic granular sludge was studied.

3. MATERIAL AND METHODS

3.1. Chemicals and gases

All chemicals were at least of reagent grade and were obtained from Merck (Darmstadt, F.R.G.). Biochemicals were from Boehringer Mannheim (Almere, The Netherlands). Gases and gas mixtures were supplied by Hoekloos (Schiedam, The Netherlands). Platinum catalyst was a gift of BASF (Arnhem, The Netherlands). [2-^{14}C] sodium acetate (58 mCi/mmol) was purchased from Amersham (Houten, The Netherlands). Aqualuma scintillation cocktail was obtained from Lumac (Schaesberg, The Netherlands).

3.2. Bacterial strains and culture media

Methanobacterium thermoautotrophicum strain Marburg (DSM 2133), *Methanobrevibacter arboriphilus* strain AZ (DSM 744), and *Methanospirillum hungatei* strains JF-1 (DSM 864) and GP-1 (DSM 1101) were from the German Culture Collection (Braunschweig, F.R.G.). *Methanosarcina barkeri* strain Fusaro (DSM 804) was a gift of Dr. C. van der Drift (Nijmegen, The Netherlands) and *Methanothrix soehngenii* strain Opfikon (DSM 2139) was from our own collection. All strains, except *M. barkeri*, were cultivated on a mineral medium described by Huser et al. [9]. For *M. thermoautotrophicum* and *M. arboriphilus* 0.2 g of yeast extract and 0.5 g of cysteine-HCl were added per liter, and for *M. hungatei* JF-1 and GP-1 0.1 g of yeast extract, 0.1 g trypticase and 0.5 g of cysteine-HCl were added per liter medium. *M.*

barkeri was grown in an imidazole-buffered medium [10].

The bacteria were grown in 1-liter serum vials containing 300 ml of medium, supplemented with 1.5 or 20 mmol of sodium acetate per liter. The gas phase was either H_2/CO_2 (8 : 2) or N_2/CO_2 (8 : 2). Growth was monitored by measuring methane production and acetate consumption.

Methanogenic granular sludge was obtained from the Centrale Suiker Maatschappij (CSM) sugar factory at Breda, The Netherlands [11]. The sludge contained $2.1 \cdot 10^{12}$ microorganisms per ml. *Methanothrix* constituted about 20%, *Methanosarcina* about 10% and the hydrogenotrophic methanogens about 15% of the total bacterial population. The organic content expressed as VSS (Volatile Suspended Solids) was 0.7 g per g dry weight.

3.3. Analytical procedures

Methane was measured with a Packard-Becker 417 gaschromatograph equipped with a molecular sieve column (60–80 mesh). The carrier gas was Argon at a flow rate of 20 ml min^{-1}. Acetate was determined either by gaschromatography or by HPLC. A Varian 2400 gaschromatograph equipped with a chromosorb 101 column (80–100 mesh) and a FID detector was used at 150°C. Carrier gas was N_2 saturated with formic acid at a flow rate of 30 ml min^{-1}. Prior to injection the samples were acidified with amberlite anion exchange resin (H^+). The detection limit of acetate by gaschromatography was about 10 μM. A Chrompack organic acids column (Chrompack, Middelburg, The Netherlands) run at 60°C was used in the HPLC analysis. Acetate was determined by a 2142 refractive index detector (LKB, Woerden, The Netherlands). The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}.

3.4. Preparations of cell extract

All operations were performed at room temperature (19°C) under strictly anaerobic conditions in an anaerobic chamber with N_2/H_2 (95 : 5) gas phase; traces of oxygen were removed by a platinum catalyst. Cells (300 ml cultures) were harvested at the late log phase by centrifugation at $20\,000 \times g$ for 30 min, washed twice in 50 mM

Tris-HCl pH 7.5, 5 mM MgCl$_2$ and 1 mM DTT, and resuspended in 5 ml of the same buffer. Cell extracts were prepared by passing the cell suspensions through a French pressure cell at 130 MPa. The cell debris was removed by centrifugation at 8 000 × g for 15 min. The protein content was determined according to Bradford [12].

3.5. Enzyme activity determinations

Acetate kinase and Acetyl-CoA synthetase activities were determined as described previously [7].

3.6. [^{14}C] acetate measurements

Since the detection of acetate concentrations below 10 μM was not accurate by gaschromatography, acetate removal by *Methanothrix* and methanogenic granular sludge was measured radiometrically with [2-^{14}C] sodium acetate (58 mCi/mmol) as substrate. Concentrated cell suspensions of *Methanothrix* or washed sludge were incubated at 37°C in a mineral medium under an atmosphere of N$_2$/CO$_2$ (8 : 2) [7]. The initial acetate concentration was 5 mM. 20 μCi of [2-^{14}C]sodium acetate were added when the acetate

concentration had decreased to 50 μM. At appropriate time intervals, 1-ml samples were removed and centrifuged at 10 000 × g. The supernatant of representative samples was separated on HPLC after 10 mM of carrier acetate was added. The acetate fractions were pooled and the radioactivity was quantified by counting 100-μl samples in 4 ml of aqualuma scintillation cocktail in a LKB Wallac scintillation counter (Pharmacia/LKB, Woerden, The Netherlands).

When the radioactivity did not decrease any further, 50 μM of non-labelled acetate was added, and radioactivity was monitored to confirm that the decline in radioactivity was caused by acetate consumption.

4. RESULTS AND DISCUSSION

4.1. Acetate threshold values

The acetate thresholds of several methanogenic bacteria are summarized in Table 1. The thresholds obtained for the hydrogenotrophic methane bacteria under non-energy-limiting conditions were between 0.4 and 0.6 mM. *Methanobrevibacter*

Table 1

Lowest measured concentrations of acetate in pure cultures of methanogenic bacteria

Species	Strain	Initial concentration (mmol/l)	End concentration (μmol/l)	References
Methanothrix	Opfikon	20	≤ 10	This study
soehngenii	Opfikon	2	≤ 10	This study
	Opfikon	4.6	7	This study [a]
	Sp.	3.5	69	13
	CALS-1	1.0	12	14
Methanosarcina	Fusaro	20	244	This study
barkeri	Fusaro	40	200	9
	CALS	10	190	14
	227	10	1 180	13
	mazei	10	397	13
Methanobacterium	Marburg	1.3	345	This study
thermoautotrophicum		5	500	15
Methanospirillum	GP-1	1.2	560	This study
hungatei	JF-1	1.4	590	This study
Methanobrevibacter				
arboriphilus	AZ	1.4	1 530	This study [b]

[a] Determined radiometrically.

[b] Strain AZ is able to convert cysteine to acetate and propionate.

Table 2

Acetate activating enzymes in methanogens

Species	Strain	Enzyme [a]	Activity (nmol min^{-1} mg^{-1} protein)	K_m (μM)	References
Methanothrix					
soehngenii	Opfikon	ACS	2 700	860	This study
Methanosarcina	MS	AK	560	3 000	1
barkeri	Fusaro thermo-	AK	9 000	7 000	17
	phila	AK	6 400	22 000	6
	227	AK	319	N.D. [b]	18
Methanobacterium					
thermoautotrophicum	Marburg	ACS	60	40	15
Methanospirillum	GP-1	ACS	97	78	This study
hungatei	JF-1	ACS	44	90	This study
Methanococcus					
marispaludis		ACS	425	90	19
Methanococcus	A3	ACS	5	N.D.	19
		AK	757	250	19
Methanobrevibacter arboriphilus	AZ	AK	264	280	This study

[a] ACS, Acetyl-CoA synthetase; AK, Acetate kinase.

[b] N.D. = not determined.

arboriphilus did not use any acetate. Instead, it produced some acetate and propionate from cysteine. For *Methanosarcina barkeri* strain Fusaro a threshold value of 0.244 mM was found, which was in the same range (0.2 mM to 1.2 mM) as observed by other authors [10,13,14]. *Methanothrix* reached the lowest acetate concentration (< 10 μM). This is in good agreement with the data reported [13,14]. The threshold value for *Methanothrix* was also determined radiometrically in cell suspensions. A representative acetate degradation curve is depicted in Fig. 1. After a brief lag, acetate was utilized at a rate of 60 nmol min^{-1} mg^{-1} of protein. The acetate consumption was linear until acetate concentration reached about 0.2 mM. Subsequently the rate levelled off very rapidly, attaining a threshold value of 7 ± 2 μM. Hang Min and Zinder observed similar curves for the acetate consumption by *Methanothrix* CALS-1 and *Methanosarcina* CALS-1 [14]. Their data as well as ours could not be to fitted to Michaelis-Menten kinetics, unless a high degree of cooperativity was invoked. The rapid decrease of the acetate consumption rate in the threshold range indicates that ATP generation in this range limits substrate activation or substrate transport. This is in good agreement with the weak sigmodial kinetics of the purified acetyl-CoA synthetase of
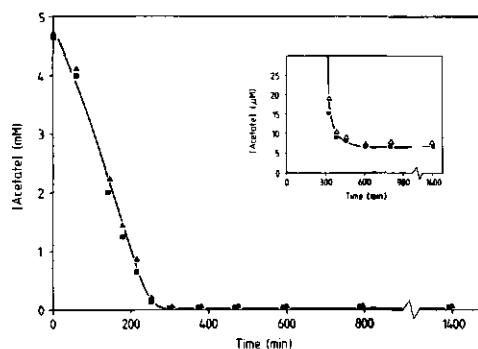

Fig. 1. Acetate utilization by washed and concentrated cell suspensions of *Methanothrix soehngenii* strain Opfikon. Two different 500-ml cultures (■, △) were washed and each resuspended in 40 ml medium (400 mg protein per liter). Acetate was added to an initial concentration of 5 mM. At appropriate time intervals 1-ml samples were removed and analysed for acetate by HPLC. When the acetate concentration had decreased to 50 μM, 20 μCi of [2-^{14}C] sodium acetate were added and the acetate concentration was determined radiometrically.

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Methanothrix soehngenii which are observed with varying amounts of ATP. This enzyme, however, followed simple Michaelis-Menten kinetics with varying concentrations of acetate at high concentrations of ATP [7].

4.2. Acetate activating enzymes

All the investigated methanogenic bacteria were able to activate acetate to acetyl-CoA. The two enzyme systems for acetate activation, acetate kinase and acetyl-CoA synthetase, were found in both hydrogenotrophic and acetoclastic methanogens (Table 2). The difference in K_m value for acetate of the acetate kinase of *Methanosarcina* and of the acetyl-CoA synthetase of *Methanothrix* are consistent with the general model by which *Methanosarcina* dominates in environments with high acetate concentrations while low acetate concentrations favour *Methanothrix* [7,14]. *Methanobacterium thermoautotrophicum*, *Methanospirillum hungatei* and *Methanococcus marispaludis* had an acetyl-CoA synthetase with a very low K_m value (40–90 μM) for acetate as compared to the K_m (860–22 000 μM) for the acetate-activating enzymes of *Methanothrix* and *Methanosarcina*. Although the K_m of the acetate activating enzymes of the hydrogenotrophic methanogens was very low, these bacteria in pure culture were not able to remove acetate to lower concentrations than *Methanothrix* did (Table 1).

4.3. Acetate removal in methanogenic granular sludge

The contribution of the hydrogenotrophic methane bacteria to acetate utilization was studied in methanogenic granular sludge where both types of bacteria were present. Acetate degradation in the sludge is depicted in Fig. 2. The acetate concentration decreased rapidly and a threshold value of 4.1 μM was obtained. This is in the same range (2.7–4.5 μM) as observed for lake sediments [20]. When the threshold was reached, the gas phase was changed to H_2/CO_2 (8:2) and acetate was monitored further (Fig. 2). No significant decrease in acetate concentrations was found thereafter, indicating that the hydrogenotrophic methane bacteria were not able to use acetate at very low concentrations. Based on these observations it is
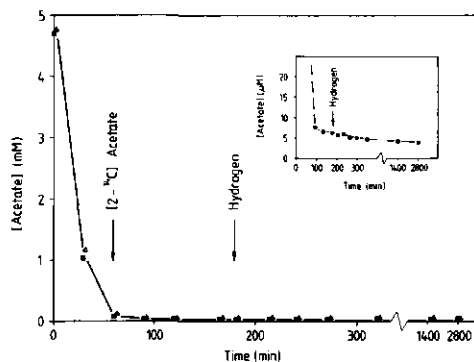

Fig. 2. Acetate utilization and influence of hydrogen on acetate degradation in methanogenic granular sludge. Two portions of 25 ml methanogenic granular sludge (■, △) were washed and resuspended in 25 ml of medium. Acetate was added to an initial concentration of 5 mM. At appropriate time intervals 1-ml samples were removed and analysed for acetate by gas chromatography. When the acetate concentration had decreased to 50 μM, 20 μCi of [2-^{14}C] sodium acetate were added and the acetate concentration was determined radiometrically. When the acetate concentration did not decrease further, the gas phase was changed from N_2/CO_2 (8:2) to H_2/CO_2 (8:2) and acetate was monitored.

not likely that the hydrogenotrophic methane bacteria significantly compete with the acetoclastic methanogens for acetate in natural environments.

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Chapter 3

Isolation and characterization of acetyl-coenzyme A synthetase
from *Methanothrix soehngenii*

Mike Jetten, Alfons Stams and Alexander Zehnder (1989)
J. Bacteriol. 171, 5430-5435.

Isolation and Characterization of Acetyl-Coenzyme A Synthetase from *Methanothrix soehngenii*

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In *Methanothrix soehngenii*, acetate is activated to acetyl-coenzyme A (acetyl-CoA) by an acetyl-CoA synthetase. Cell extracts contained high activities of adenylate kinase and pyrophosphatase, but no activities of a pyrophosphate:AMP and pyrophosphate:ADP phosphotransferase, indicating that the activation of 1 acetate in *Methanothrix* requires 2 ATP. Acetyl-CoA synthetase was purified 22-fold in four steps to apparent homogeneity. The native molecular mass of the enzyme from *M. soehngenii* estimated by gel filtration was 148 kilodaltons (kDa). The enzyme was composed of two subunits with a molecular mass of 73 kDa in an α_2 oligomeric structure. The acetyl-CoA synthetase constituted up to 4% of the soluble cell protein. At the optimum pH of 8.5, the V_{max} was 55 μmol of acetyl-CoA formed per min per mg of protein. Analysis of enzyme kinetic properties revealed a K_m of 0.86 mM for acetate and 48 μM for coenzyme A. With varying amounts of ATP, weak sigmoidal kinetic was observed. The Hill plot gave a slope of 1.58 ± 0.12, suggesting two interacting substrate sites for the ATP. The kinetic properties of the acetyl-CoA synthetase can explain the high affinity for acetate of *Methanothrix soehngenii*.

The terminal step in the breakdown of organic polymers under methanogenic conditions is the conversion of H_2/CO_2 or acetate by methanogens (9). The most abundant methanogenic substrate under these conditions is acetate (9, 33). Only two genera of methanogenic bacteria, *Methanosarcina* and *Methanothrix*, are capable of metabolizing acetate to methane. *Methanosarcina* spp., the most versatile methane bacteria, can use several compounds (H_2/CO_2, methanol, methylamines, and acetate) as growth substrates (11, 15, 18, 32). Its affinity for acetate is rather low (K_s, 5 mM), the growth yield is 2.1 g (dry weight) per mol of acetate, and the doubling time is 2 days (11, 32). *Methanothrix* can use acetate as a sole growth substrate. The growth yield (1.4 g/mol of acetate) and the growth rate (doubling time, 7 days) are low, but its affinity for acetate is high (K_s, 0.5 mM) (10, 40).

The pathway for acetate degradation involves transfer of the methyl group of acetate to coenzyme M, forming methyl-coenzyme M (methyl-CoM), which is then reductively demethylated to methane (6, 7, 19, 21, 22, 24, 37). The actual cleavage of the carbon-carbon bond in acetate is proposed to be catalyzed by carbon monoxide dehydrogenase (1, 5, 8, 28). High activities of carbon monoxide dehydrogenase were found in both *Methanosarcina* and *Methanothrix* spp. grown on acetate (8, 12, 16, 17, 35). Prior to the cleavage of the carbon-carbon bond, acetate is proposed to be activated to acetyl-coenzyme A (acetyl-CoA) (1, 6, 34). Different mechanisms of acetate activation were found in the two acetoclastic methanogens. For *Methanosarcina* spp., high activities of acetate kinase (EC 2.7.2.1) and phosphate acetyltransferase (EC 2.3.1.8) were reported (1, 6, 34, 36). In *Methanothrix* spp., only an acetyl-CoA synthetase (acetate:CoA ligase [AMP forming], EC 6.2.1.1) was present (16, 26). Recently, the purification and properties of the acetate kinase of *Methanosarcina thermophila* were described (1). This report summarizes the purification and characterization of the acetyl-CoA synthetase from *Methanothrix soehngenii*

and a comparison is made between the acetate-activating systems of *Methanosarcina* and *Methanothrix* spp.

MATERIALS AND METHODS

Organism and cultivation. *Methanothrix soehngenii* (DMS 2139) was the Opfikon strain isolated by Huser et al. (10). The organism was mass cultured on 80 mM sodium acetate in 25-liter carboys containing 20 liters of the medium described previously (12). Cultures were incubated without stirring at 35°C in the dark under an 80% N_2–20% CO_2 gas phase. Cells were harvested at the late log phase by continuous centrifugation (Carl Padberg Zentrifugenbau GmbH, Lahr/Schwarzwald, Federal Republic of Germany), washed in 50 mM Tris hydrochloride (Tris-HCl) (pH 8.0), and stored under N_2 at −20°C.

Chemicals. All chemicals were at least of analytical grade. Acetyl-CoA and P_1,P_5-di(adenosine-5')-pentaphosphate were purchased from Sigma Chemical Co. (Amsterdam). All other biochemicals were obtained from Boehringer Mannheim (Almere, The Netherlands). Sodium dodecyl sulfate (SDS) and acrylamide were from Bio-Rad Laboratories (Utrecht, The Netherlands). Gases were purchased from Hoekloos (Schiedam, The Netherlands). Platina catalyst was a gift of BASF (Arnhem, The Netherlands). Mono-Q HR 5/5, Q-Sepharose, Phenyl Superose HR 5/5, Superose 6 HR 10/30, and molecular mass standards for gel filtration and polyacrylamide gel electrophoresis (PAGE) were obtained from Pharmacia Fine Chemicals (Woerden, The Netherlands).

Analytical methods. Protein was determined with Coomassie brilliant blue G250 as described by Bradford (2). Bovine serum albumin was used as the standard. The purity of the enzyme after various chromatographic steps was determined by SDS-PAGE following the method of Laemmli (20). Molecular mass standards were α-lactalbumin, 14.4 kilodaltons (kDa); trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 43 kDa; bovine serum albumin, 67 kDa; and phosphorylase B, 94 kDa. Gels were stained with Coomassie brilliant blue R250. Native enzyme molecular mass was determined on Superose 6 HR 10/30

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equilibrated with 50 mM Tris-HCl (pH 8.0) with thyroglobulin (669,000 Da), ferritin (445,000 Da), catalase (232,000 Da), and aldolase (158,000 Da) as standards.

Enzyme purification. The purification of the acetyl-CoA synthetase was regularly performed within 1 day. Unless indicated otherwise, all procedures were carried out aerobically at room temperature ($\pm 19°C$). A frozen cell paste was thawed, diluted with 50 mM Tris-HCl (pH 8.0) in a 1:3 ratio, disrupted by passing through a French pressure cell at 135 MPa, and centrifuged for 30 min at $30,000 \times g$. The supernatant contained about 20 mg of protein per ml and is referred to as the crude extract. Membrane fractions were prepared by centrifugation of the crude extract at $110,000 \times g$ for 75 min. The crude extract (25 ml) was applied to a column (3.3 by 10 cm) of Q-Sepharose (fast flow) equilibrated with 50 mM Tris-HCl (pH 8.0) (buffer A). After the column was washed with 40 ml of buffer A, the adsorbed protein was eluted in a 500-ml linear gradient of 1 M NaCl in buffer A. The flow rate was 4 ml/min. Fractions (10 ml) were collected and analyzed for acetyl-CoA synthetase activity. The acetyl-CoA synthetase eluted at 0.15 M NaCl. Fractions with activities higher than 2 U/mg were pooled and desalted in an Amicon ultrafiltration cell (Grace, Rotterdam, The Netherlands) with a PM 30 filter. The remaining steps in the purification were performed with a high-resolution fast protein liquid chromatography (FPLC) system (Pharmacia/LKB, Woerden, The Netherlands) equipped with a model 2152 LC controller. Repetitively, four samples of 5 ml of the concentrated desalted enzyme solution were injected onto a Mono-Q HR 5/5 anion-exchange column equilibrated with Tris-HCl, pH 9.0. A 12-ml linear gradient from 0 to 0.4 M of NaCl in Tris-HCl, pH 9.0, was applied at a flow rate of 1.0 ml/min. Fractions with acetyl-CoA synthetase activity were concentrated to 2.0 ml in a Centricon PM 30 (Grace, Rotterdam, The Netherlands). The enzyme solutions of four runs were combined and mixed in a 1:1 ratio with 2 M $(NH_4)_2SO_4$ in buffer A and applied to a Phenyl Superose HR 5/5 column. A 20-ml linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in buffer A was applied at a flow rate of 0.5 ml/min. Fractions with acetyl-CoA synthase activity were concentrated to 2.0 ml in a Centricon PM 30. The concentrated enzyme solution was injected on a Superose 6 HR 10/30 gel filter equilibrated with buffer A. The column was developed at a flow rate of 0.5 ml/min. Purified acetyl-CoA synthetase was collected, concentrated in Centricon PM 30, frozen in liquid N_2, and stored at $-80°C$ until use.

Assays. Acetyl-CoA synthetase (EC 6.2.1.1) was assayed either by following the formation of acetyl-CoA as hydroxamate from acetate, HSCoA, and ATP or by coupling the acetyl-CoA synthetase reaction with adenylate kinase, pyruvate kinase, and lactate dehydrogenase (25, 39). In the first assay, the standard reaction mixture included the following compounds (in micromoles per milliliter): ATP, 2; sodium acetate, 10; $MgCl_2$, 2; glutathione, 2; Tris-HCl (pH 8.5), 100; neutralized NH_2OH, 600; coenzyme A, 0.2; and enzyme. Acetyl-CoA was determined as the hydroxamate by the method of Rose et al. (27). For the second assay, the reaction mixture contained (in micromoles per milliliter): Tricine-KOH (pH 8.5), 100; $MgCl_2$ 4; phosphoenolpyruvate, 2; NADH, 0.4; ATP, 2; sodium acetate, 10; coenzyme A, 0.2; glutathione, 2.0; plus adenylate kinase, 1 U; pyruvate kinase, 0.8 U; lactate dehydrogenase, 3 U; and enzyme. The rate of NADH oxidation was followed continuously at 340 nm in an LKB/Biochrom Ultrospec K spectrophotometer. All incubations were done at 35°C. One unit of enzyme is

defined as the amount which catalyzes the formation of 1 μmol of acetyl-CoA per min.

Acetate kinase (EC 2.7.2.1) and phosphate acetyltransferase (EC 2.3.1.8) concentrations were determined as described by Aceti and Ferry (1).

Adenylate kinase (EC 2.7.4.3) was measured photometrically by following the formation of ADP from AMP and ATP at 340 nm by coupling the reaction to the oxidation of NADH via pyruvate kinase and lactate dehydrogenase or by following the formation of ATP from ADP at 340 nm by coupling the reaction to the reduction of $NADP^+$ via hexokinase and glucose-6-phosphate dehydrogenase (25). The reaction mixture for the formation of ADP contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$ 4; phosphoenolpyruvate, 2; NADH, 0.4; ATP, 2; AMP, 2; glutathione, 2.0; plus pyruvate kinase, 0.8 U; and lactate dehydrogenase, 3 U. The reaction mixture for the formation of ATP contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$, 4; ADP, 4; glutathione, 2.0; $NADP^+$, 0.4; D-glucose, 100; plus glucose-6-phosphate dehydrogenase, 0.7 U; and hexokinase, 0.7 U.

Inorganic pyrophosphatase (EC 3.6.1.1) was measured by following the formation of P_i (14). The reaction mixture contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$, 4; sodium pyrophosphate, 5; and glutathione, 2. Samples (200 μl) were taken at 1-min time intervals and added to 100 μl of 5 M H_2SO_4 to stop the reaction. The precipitated protein was removed by centrifugation, and the phosphate content was determined in the supernatant by the modified method of Fiske-SubbaRow as described by Josse (14).

Pyrophosphate:AMP phosphotransferase was measured by following the formation of ADP from AMP and PP_i by coupling the reaction to the reduction of $NADP^+$ via adenylate kinase, hexokinase, and glucose-6-phosphate dehydrogenase (38). The reaction mixture contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$, 4; PP_i, 20; $NADP^+$, 0.4; AMP, 4; D-glucose, 100; glutathione, 2.0; plus adenylate kinase, 1 U; hexokinase, 0.7 U; and glucose-6-phosphate dehydrogenase, 0.7 U.

Pyrophosphate:ADP phosphotransferase was measured by following the formation of ATP from ADP and PP_i by coupling the reaction to the reduction of $NADP^+$ via hexokinase and glucose-6-phosphate dehydrogenase. Adenylate kinase was inhibited by Ap_5A [P_1,P_5-di(adenosine-5')-pentaphosphate]. The reaction mixture contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$, 4; PP_i, 20; $NADP^+$, 0.4; AMP, 4; D-glucose, 100; glutathione, 2.0; Ap_5A, 0.2; plus hexokinase, 0.7 U; and glucose-6-phosphate dehydrogenase, 0.7 U (25).

ATPase (EC 3.6.1.3) content was determined by following formation of P_i from ATP. The reaction mixture contained (in micromoles per milliliter): Tricine-KOH (pH 8.2), 100; $MgCl_2$, 4; and sodium ATP, 5. Samples (200 μl) were taken at 1-min time intervals. The reaction was stopped by the addition of 100 μl of 5 M H_2SO_4. The precipitated protein was removed by centrifugation, and phosphate content was determined as described by Skrabanja et al. (31).

In all assays, an appropriate amount of cell extract was used. The reactions were started by the addition of cell extract or substrate.

The assays were performed under both strict anaerobic and aerobic conditions. Since no difference in activity was found under either condition, assays were routinely performed aerobically.

Kinetic analysis. The kinetic parameters of acetyl-CoA

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TABLE 1. Enzyme activities in cell extract of
Methanothrix soehngenii[a]

Enzyme	EC no.	Sp act (μmol/min per mg)
Acetyl-CoA synthetase	6.2.1.1	2.7 ± 0.6
Adenylate kinase	2.7.4.3	4.2 ± 0.7
Pyrophosphatase	3.6.1.1	0.92 ± 0.1
ATPase	3.6.1.3	0.041 ± 0.005
Acetate kinase	2.7.2.1	ND[b]
Phosphate acyltransferase	2.3.1.8	ND
PP$_i$:AMP phosphotransferase		ND
PP$_i$:ADP phosphotransferase		ND

[a] The values represent mean activities of at least four extract preparations. Enzymatic activity for each extract was the average of five determinations.
[b] ND, Not detected.

synthetase were determined at 35°C and pH 8.5. The continuous assay was used for the determination of the K_m and V_{max} for acetate and coenzyme A. The K_m for ATP was determined with the discontinuous assay. The inhibitory effects of AMP and PP$_i$ on the acetyl-CoA synthetase were tested with the discontinuous assay at different AMP, PP$_i$, and ATP concentrations.

RESULTS

Acetate activation. Activities of enzymes possibly involved in the activation of acetate in *Methanothrix soehngenii* are summarized in Table 1. None of the enzyme activities was influenced by oxygen. A high activity of an acetyl-CoA synthetase and no acetate kinase activity were detected in cell extracts. Enzymes necessary for the conversion of AMP and PP$_i$, which are formed in the acetyl-CoA synthetase reaction, were also present in high levels. This confirms earlier findings made by Kohler and Zehnder (16). After ultracentrifugation, these enzymes were found in the soluble fraction, whereas the control enzyme ATPase was completely recovered in the particulate fraction. Since the energy in the PP$_i$ represents metabolically useful energy, cell extracts were tested for the most obvious PP$_i$-dependent enzymes. No PP$_i$:AMP or PP$_i$:ADP phosphotransferase activities were found.

Enzyme purification. The acetate-activating enzymes in *Methanosarcina* and *Methanothrix* spp. were reported to be insensitive to molecular oxygen (1, 15, 16). The purification of the acetyl-CoA synthetase therefore required no strict anaerobic conditions. The purification was carried out at room temperature and generally took only 1 day. In four steps, a 22-fold-purified enzyme was obtained (Table 2). Q-Sepharose chromatography proved to be an effective first step in the purification of the acetyl-CoA synthetase, since the enzyme was one of the first proteins to elute from the

TABLE 2. Purification of acetyl-CoA synthetase
of *Methanothrix soehngenii*

Step	Protein (mg)	Activity (U)	Sp act[a] (U/mg)	Purification (fold)	Yield (%)
Crude extract	400	880	2.2	1	100
Q-Sepharose	40	392	9.8	5	45
Mono-Q	16	272	17.2	8	30
Phenyl-Superose	8	192	24.1	11	22
Superose	1	50	48.3	22	6

[a] Micromoles of acetyl-CoA formed per minute per milligram of protein.
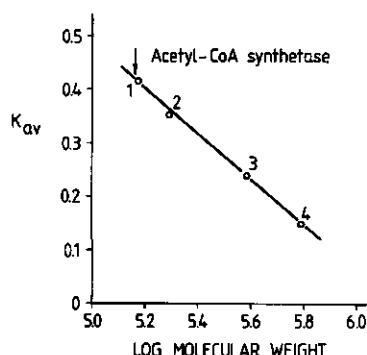

FIG. 1. Molecular mass estimation of the native acetyl-CoA synthetase on Superose 6 HR 10/30. Standards were aldolase (158 kDa, 1); catalase (232 kDa, 2); ferritin (446 kDa, 3); and thyroglobulin (669 kDa, 4). The position of acetyl-CoA synthetase is indicated by the arrow.

column after application of the NaCl gradient. After concentration and desalting, the enzyme preparation was injected onto an FPLC system and purified to homogeneity. The loss in total activity as given in Table 2 mainly occurred because only those fractions with high specific activities were pooled in each purification step.

Characterization of the purified enzyme. The molecular mass of the native acetyl-CoA synthetase was estimated by gel filtration on Superose 6 HR 10/30 and appeared to be 148 kDa, compared with standards of known molecular mass (Fig. 1). SDS-PAGE of the purified enzyme revealed one subunit with relative molecular mass equal to 73 kDa, which suggests an α_2 subunit stoichiometry for the native enzyme (Fig. 2). The activity of the purified enzyme did not decrease significantly in the presence of air. The enzyme could be stored at −20 or −70°C without any loss of activity for at least 2 months. When the enzyme was kept at 4°C, aerobically or anaerobically, 50% of the activity was lost within 72 h.

Substrate specificity. In addition to acetate, a coenzyme A-dependent activation of some other organic acids was catalyzed to some extent by the purified enzyme (Table 3).

Kinetic properties. The reaction rate at different acetate and coenzyme A concentrations followed Michaelis-Menten kinetics. Half-maximal rates were obtained at 0.86 mM acetate and at 48 μM coenzyme A. The V_{max}, at the optimal pH of 8.5 (100 mM Tricine-KOH) and at 35°C, was 55 μmol of acetyl-CoA formed per min per mg of protein. With ATP, however, a weak sigmoidal velocity curve was found. The concentration of ATP which gave half-maximal rates was obtained from the double-reciprocal plot and appeared to be 1 mM. These data suggest cooperative binding of ATP, as reported for the acetate kinase of *Clostridium thermoaceticum* (29). A Hill plot of the data resulted in a Hill coefficient of 1.58 ± 0.12, suggesting two interacting substrate sites (Fig. 3) (29).

Inhibition studies. Acetyl-CoA synthetase was inhibited by the end products AMP and PP$_i$. When the activities at various concentrations of AMP, PP$_i$, and ATP were plotted by the method of Dixon (4), a K_i of 4 and 6.5 mM was determined for AMP and PP$_i$, respectively (Fig. 4A and B).

FIG. 2. SDS-PAGE of the different steps in the purification procedure. Lane 1, Molecular mass markers; lane 2, crude extract (80 μg); lane 3, pooled fractions from phenyl-Superose (30 μg); lane 4, pooled fractions from Superose 6 HR 10/30 (50 μg).

DISCUSSION

Methanosarcina and *Methanothrix* spp. have different enzyme systems for the activation of acetate. An acetate kinase and a phosphate acyltransferase convert acetate to acetyl-CoA in *Methanosarcina* spp., whereas this conversion is catalyzed by an acetyl-CoA synthetase in *Methanothrix* spp. (1, 16). The acetate kinase of *Methanosarcina thermophila* and the acetyl-CoA synthetase of *Methanothrix* spp. have now been purified, and their properties can be compared. Acetyl-CoA synthetase is an abundant protein of *Methanothrix soehngenii*. From the increase in specific activity upon purification and from the 6% recovery, it can be calculated that up to 4% of the soluble cell protein of *Methanothrix soehngenii* is acetyl-CoA synthetase. This level is somewhat higher than the 1% acetate kinase that can be calculated for *Methanosarcina thermophila* (1). The acetyl-CoA synthetase has a homodimeric subunit composition similar to that of the acetate kinase of *Methanosarcina*,

TABLE 3. Substrate specificity of the purified acetyl-CoA synthetase

Substrate[a]	Relative activity[b] (%)
Acetate	100
Propionate	5
Butyrate	1.6
Benzoate	0.6
Valerate	0.3
Formate	0
Succinate	0

[a] 10 mM sodium salt was used in the assay.
[b] Relative to activity with acetate (100%; 54 μmol of acetyl-CoA formed per min per mg of protein).
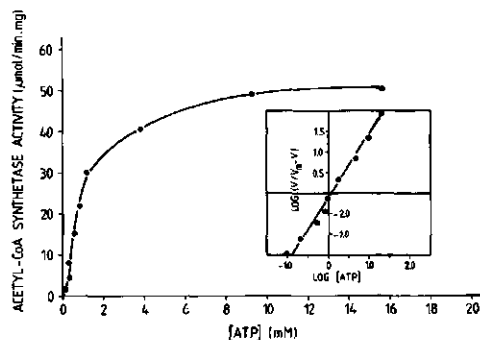

FIG. 3. Relationship between ATP concentration and activity of the purified acetyl-CoA synthetase. Conditions and calculations are described in Materials and Methods. Inset: Hill plot of the same data.

but the size of the subunits is somewhat larger, 73 versus 58 kDa (1). Both enzymes exhibit the same temperature stability and are not sensitive to oxygen (1). Both enzymes are capable of activating some other fatty acids, like propionate (1). The rate of these conversions, however, is very low. The
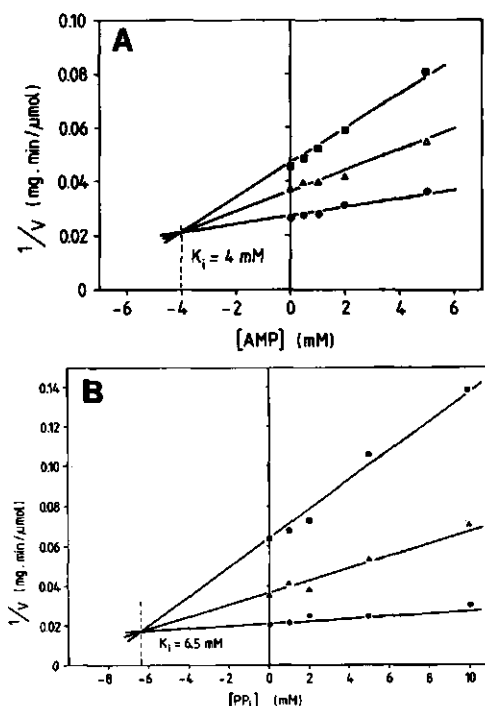

FIG. 4. Inhibition of acetyl-CoA synthetase by AMP (A) and PP$_i$ (B). The data are plotted by the method of Dixon (4). ATP concentration: ●, 1 mM; △, 2 mM; ■, 5 mM.

major differences between the enzymes are the kinetic properties. The acetate kinase of *Methanosarcina thermophila* has a high V_{max} (660 U/mg), but a low affinity for acetate (K_m, 22 mM) (1). The acetyl-CoA synthetase of *Methanothrix soehngenii* has a high affinity for acetate (K_m, 0.8 mM), but has a lower V_{max} (55 U/mg). The acetate kinase exhibits normal Michaelis-Menten kinetics towards acetate and ATP. The acetyl-CoA synthetase, however, shows a weak sigmoidal velocity curve with varying amounts of ATP, which indicates the cooperative binding of ATP to the enzyme. The Hill plot gave a slope of 1.58, which suggests two interacting binding sites for ATP. This might enable the cell to regulate the acetate activation (30).

The differences in V_{max} and K_m value for acetate of the two acetate activating enzymes may explain the differences in the physiological properties and the ecological distribution of the two types of acetoclastic methanogens in nature. *Methanosarcina* spp., which have a high maximal specific growth rate and a low affinity for acetate, are dominant in environments with high acetate concentrations, whereas *Methanothrix* spp., which have the reverse properties, are most abundant in environments with low acetate concentrations (9, 33). It cannot, however, be excluded that the favorable surface-volume ratio of *Methanothrix* spp. and differences in acetate uptake systems are additional factors of importance in the affinity for acetate (23). The two sets of acetate-activating enzymes were also found in *Escherichia coli* and function at different acetate concentration. At high acetate concentrations, acetate is activated with an acetate kinase-phosphate acyltransferase system, whereas studies with mutants showed that at low acetate concentrations, an acetyl-CoA synthetase activity is displayed (3, 36).

The presence of the acetyl-CoA synthetase-adenylate kinase-pyrophosphatase system in *Methanothrix soehngenii* implies that 2 ATP molecules are required for the activation of 1 molecule of acetate. No enzymes were detected which make use of the energy present in the PP_i bond. It is possible that these enzymes were inactivated or that the hydrolysis of PP_i is just needed to pull the activation process at low acetate concentrations. It is rather intriguing how *Methanothrix* is able to generate metabolic energy for growth, especially because it was postulated that the acetoclastic cleavage only yields 1 ATP (6, 36, 37). It has to be ensured, therefore, that the transfer of electrons formed in the oxidation of the enzyme-bound carbonyl moiety to the methyl-CoM reductase forms an electrochemical gradient which is high enough to enable the synthesis of more than 2 ATP. Future research is concentrated on the elucidation of the electron transfer processes in *Methanothrix soehngenii*.

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Chapter 4

A fluoride-insensitive inorganic pyrophosphatase isolated
from *Methanothrix soehngenii*

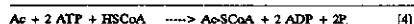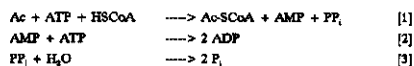Mike Jetten, Tineke Fluit, Alfons Stams and Alexander Zehnder (1991)
J. Bacteriol. submitted.

A fluoride-insensitive inorganic pyrophosphatase
isolated from *Methanothrix soehngenii*

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An inorganic pyrophosphatase [E.C. 3.6.1.1] was isolated from *Methanothrix soehngenii*. In three steps the enzyme was purified 400-fold to apparent homogeneity. The molecular mass estimated by gelfiltration was 139 ± 7 kDa . SDS-PAGE indicated that the enzyme is composed of subunits with molecular masses of 35 and 33 kDa in an $\alpha_2\beta_2$ oligomeric structure. The enzyme catalyzed the hydrolysis of inorganic pyrophosphate, tri- and tetrapolyphosphate, but no activity was observed with a variety of other phosphate esters. The cation Mg^{2+} was required for activity. With Mn^{2+} and Co^{2+} (1 mM) activities were 160 % and 24 % of the activity with 1 mM Mg^{2+} at 1 mM PP_i , respectively. The pH optimum was 8 at 1 mM PP_i and 5 mM Mg^{2+}. The enzyme was heat-stable, insensitive to molecular oxygen and not inhibited by fluoride. Analysis of the kinetic properties revealed an apparent K_m for PP_i of 0.1 mM in the presence of 5 mM Mg^{2+}. The V_{max} was 590 μmol of pyrophosphate hydrolyzed per min per mg protein, which corresponds to a K_{cat} of 1400 per second. The enzyme was found in the soluble enzyme fraction after ultracentrifugation, when cells were disrupted by French Press. Upto 5 % of the pyrophosphatase was associated with the membrane fraction, when gentle lysis procedures were applied.

Methanothrix soehngenii is an anaerobic archaebacterium, which solely uses acetate as carbon and energy source (7,29). The growth yield (1.4 g/mol acetate) and the growth rate (average doubling time of 7 days) are low, but the affinity (K_s = 0.5 mM) for acetate of *M. soehngenii* is high (7,11). In *M. soehngenii* acetate is activated to acetyl-CoA by acetyl-CoA synthetase [1] (9). The formed AMP is converted to ADP by adenylate kinase [2] and pyrophosphate is hydrolyzed by pyrophosphatase [3] (9). The sum of these reactions [4] indicates that acetate in *M. soehngenii* is activated to acetyl-CoA at the expense of two energy rich phosphate-bonds (9).

Ac + ATP + HSCoA	----> Ac-SCoA + AMP + PP$_i$	[1]
AMP + ATP	----> 2 ADP	[2]
PP$_i$ + H$_2$O	----> 2 P$_i$	[3]
Ac + 2 ATP + HSCoA	----> Ac-SCoA + 2 ADP + 2P$_i$	[4]

Because conversion of acetate to methane is supposed to yield only one ATP, it is not likely that *Methanothrix* will just hydrolyze pyrophosphate (24). For a favourable energy balance the energy from the pyrophosphate bond has to be conserved. Therefore, the inorganic pyrophosphatase of *M. soehngenii* was investigated.

Enzymes relatively specific to the hydrolysis of inorganic pyrophosphate are widely distributed in nature. Inorganic pyrophosphatases have been purified from heterotrophic, chemolithoautotrophic, sulfate reducing and phototrophic bacteria and from yeasts (6,12-16,18,19,21,25-27). The isolation and characterization of this enzyme from an archaebacterium is not yet reported. The purification and some of the properties of inorganic pyrophosphatase from *M. soehngenii* are described here.

MATERIALS AND METHODS

Organism and cultivation. *Methanothrix soehngenii* was the Opfikon strain isolated by

TABLE 1: Purification of pyrophosphatase from *Methanothrix soehngenii*.

step	protein	units	Sp. Act. [a]	purification	recovery
	mg		U/mg	fold	%
Crude extract	224	291	1,3	1	100
Q-sepharose	2,5	292	117	90	100
Hydroxylapatite	0,8	180	225	173	62
Phenyl-superose	0,3	155	515	396	53

[a] micromoles of pyrophosphate hydrolyzed per minute per mg protein

TABLE 2 : Inhibition of pyrophosphatase

Inhibitor	Relative activity [a]
none	100 [b]
KF	82
p-Cl-Hg-benzoate	82
Titanium citrate	78
NaN_3	74
Sodium dithionite	71
Iodoacetamide	70
KCN	60
ATP	38
ATP + 10 mM Mg^{2+}	87
EDTA	35
EDTA + 10 mM Mg^{2+}	83

[a] Pyrophosphatase (10 μg) was preincubated 30 min at 35°C in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0 and 5 mM inhibitor. Thereafter 1 mM pyrophosphate was added and the activity determined as described in Material and Methods.

[b] 100 % activity is 278 μmol PP_i hydrolyzed per min per mg protein.

Huser et al. and provided by A.J.B. Zehnder (7,29). The organism was mass cultured on 80 mM sodium acetate in 25 l carboys with a sterile gas outlet, containing 20 l of the medium as described previously (7). Cultures were incubated without stirring at 35 °C in the dark under a N_2/CO_2 (80/20 %) gas phase. Cells were harvested after 6 weeks at the late log phase by continuous centrifugation (Heraeus Sepatech, Osterode, FRG), washed in 50 mM anaerobic Tris-HCl pH 8.0, frozen in liquid nitrogen and stored at -20 °C under N_2/H_2 (96:4). The yield was about 1 g wet weight per liter.

Chemicals. All chemicals were at least of analytical grade. Tris(hydroxymethyl)amino-methane, tri- and tetrapolyphosphate were purchased from Sigma (Amsterdam). Tetrasodium pyrophosphate was from Janssen (Beerse, Belgium). All other chemiclas were from Merck (Darmstadt, FRG). Biochemicals were obtained from Boehringer Mannheim (Almere, the Netherlands). Sodium dodecylsulfate, acrylamide and hydroxylapatite were from Biorad (Utrecht, the Netherlands). Q-Sepharose, Phenyl Superose HR 5/5, Superose 6 HR 10/30 and molecular mass standards for gelfiltration and SDS-PAGE were obtained from Pharmacia Fine Chemicals (Woerden, the Netherlands). Ti(III)citrate (100 mM) was prepared by adding 5 ml 1 M Ti(III)chloride to 40 ml of anaerobic 0.2 M sodium citrate solution, pH was adjusted with 2 M Tris to 7.5.

Analytical methods. Protein was estimated with Coomassie brillant blue G250 as described by Bradford (4). Bovine serum albumin was used as standard. The purity of the enzyme after various chromatographic steps was determined by SDS-PAGE according to the method of Laemmli (17). The following molecular mass standards were used : trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 43 kDa; bovine serum albumine, 67 kDa; and phosphorylase b, 94 kDa. Gels were silver stained as described by Wray

et al. (28). Native PAGE and activity staining of the pyrophosphatase were performed as described (2). Native enzyme molecular mass was determined on Superose 6 HR 10/30 equilibrated with 50 mM Tris-HCl pH 8.0 containing 150 mM NaCl, using catalase (232), aldolase (158) and bovine serum albumine (dimer 134 and monomer 67 kDa) as standards. The column was developed at a flow rate of 0.3 ml/min.

Enzyme purification. The purification of the pyrophosphatase was regularly performed within one day. Unless indicated otherwise all procedures were carried out aerobically at room temperature ($\pm$ 19 °C). A frozen cell paste was thawed, diluted with 50 mM Tris-HCl pH 8.0 (Buffer A) in a 1:3 ratio, sonified 5 times 30 seconds and disrupted by passing twice through a French pressure cell at 135 MPa and centrifuged 10 min at 10,000 x g. In this way 90-95 % of the cells were disrupted. The supernatant was centrifuged 150 min at 110,000 x g. The supernatant after ultracentrifugation contained about 10 mg of protein per ml and is referred to as crude extract. When membrane association was studied, cells were lyzed more gentle. One gram of freshly haversted cells were resuspended in 10 ml 20 mM Tricine-KOH pH 7.5, 10 mM $MgSO_4$ and 0.4 M sucrose (buffer B). The suspension was sonified 20 times one second to break the long filaments. The homogeneous suspension was centrifuged 20 min 15,000 x g and the pellet was resuspended in 20 ml of buffer B, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lyzed by passage through a French pressure cell at 65 Mpa and thereafter centrifuged 15 min at 12,000 x g to remove unbroken cells. This gentle method disrupted 40-50 % of the cells. The supernatant was ultracentrifuged one hour at 300,000 x g and the pellet was washed three times with buffer B, containing 0.5 mM PMSF. The obtained membrane fraction was assayed immediately for pyrophosphatase, acetyl-
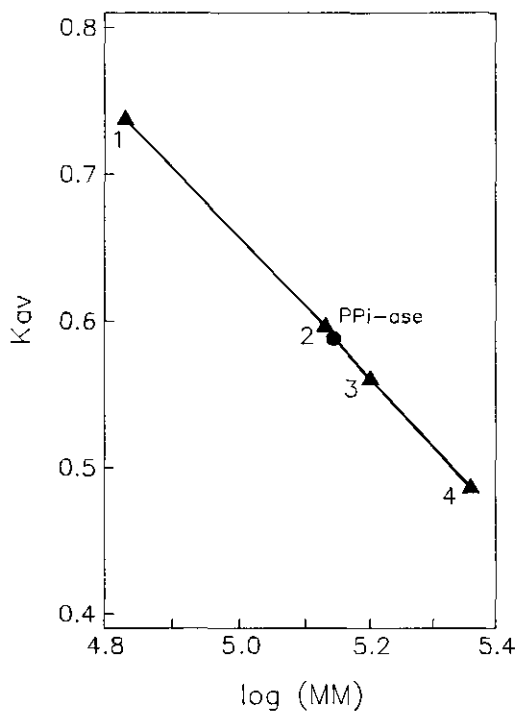
Fig. 1. Molecular mass determination of purified pyrophosphatase on Superose 6 HR 10/30.

Standards (▲) were Bovine Serum Albumine (Monomer, 67 kDa, [1] and Dimer, 134 kDa, [2]), Aldolase (152 kDa, [3]) and Catalase (232 kDa, [4]). The purified pyrophosphatase is marked by ●
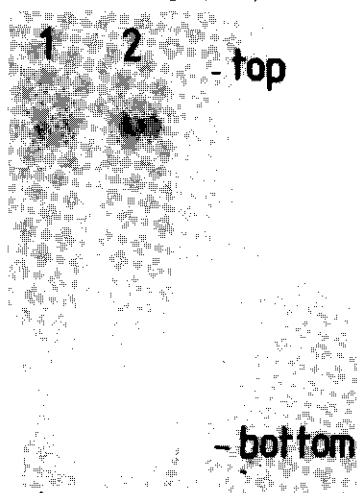
Fig. 2. Native PAGE of purified pyrophosphatase

Lane 1: 10 μg pyrophospatase stained with Coomassie R 250.

Lane 2: 0.5 μg pyrophosphatase stained for accumulated phosphate after 1 min incubation in the standard assay mixture.
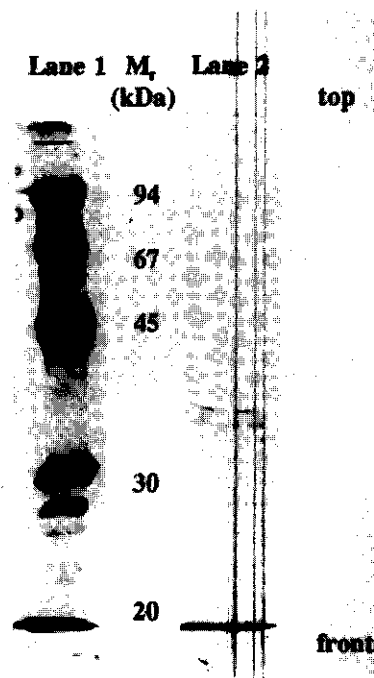
Fig. 3. SDS-PAGE of purified pyrophosphatase.

Lane 1: Molecular mass markers.
Lane 2: 5 μg pyrophosphatase.

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coenzyme A synthetase and ATP-ase activity in the standard assays.

The crude extract (20 ml) was applied to a column (3.3 by 10 cm) of Q-Sepharose (fast flow) equilibrated with buffer A. The column was washed with 150 ml of buffer A. The inorganic pyrophosphatase was collected just after the void volume. The rest of the adsorbed protein was washed from the column in a 200 ml linear gradient of 1 M NaCl in buffer A. The flow rate was 3 ml/min. Fractions (12 ml) were collected and analysed for pyrophosphatase activity. Fractions with pyrophosphatase activity were pooled and concentrated in an Amicon ultrafiltration cell (Grace, Rotterdam, the Netherlands) with a PM 30 filter. The concentrated enzyme solution was applied to a hydroxylapatite column (1 by 6 cm) equilibrated with buffer A. The adsorbed protein was eluted from the column in a 120 ml linear gradient of 1.7 M $(NH_4)_2SO_4$ in buffer A. Fractions of 6 ml were collected at a flow rate of 2 ml/min. The inorganic pyrophosphatase was eluted at 0.7 M $(NH_4)_2SO_4$. Fractions with pyrophosphatase activity were pooled and concentrated in an Amicon ultrafiltration cell with a PM 30 filter.

The remaining step in the purification was performed with a high resolution fast protein liquid chromatography (FPLC) system (Pharmacia / LKB, Woerden, the Netherlands) equipped with a model 2152 LC controller. The enzyme solution was mixed in a 1:1 ratio with 4 M $(NH_4)_2SO_4$ in buffer A and applied to a Phenyl Superose HR 5/5 column. The column was washed with 5 ml of 2 M $(NH_4)_2SO_4$ in buffer A at a flow rate of 0.5 ml/min and then a 15 ml gradient from 2 M to 0 M $(NH_4)_2SO_4$ in buffer A was applied. Pyrophosphatase was collected at 0.05 M $(NH_4)_2SO_4$. Fractions with pyrophosphatase activity were concentrated to 0.3 ml in a Centricon PM 30 and frozen in liquid nitrogen and stored at -80 °C until use.

Assay. Inorganic pyrophosphatase (E.C. 3.6.1.1) was measured by following the formation of inorganic phosphate. The reaction mixture contained (in μmoles per ml) : Tris-HCl (pH 8.0), 40 ; $MgCl_2$, 5 and sodium pyrophosphate, 1 . Samples of 100 μl were taken in 0.5 min time intervals and added to 2 ml of stop reagent (4 mM ammoniumheptamolybdate, 0.7 mM potassium antimony(III)oxide tartrate and 27 mM L-ascorbic acid in 1 M H_2SO_4). Phosphate was determined as a reduced molybdenium blue at 690 nm after the addition of 8 ml of distilled water to the stop reagent.

ATP-ase (E.C. 3.6.1.3) was measured by following the formation of inorganic phosphate from ATP. The standard reaction mixture was the same as for the pyrophosphatase assay, except that PP_i was replaced by 2 mM of ATP. Acetyl-coenzyme A synthetase was assayed as described (9).

In all assays an appropriate amount of enzyme was used. The reactions were started by the addition of enzyme or substrate. The assays were performed under both strict anaerobic (flushed assay mixtures, 0.1 mM Ti(III)citrate, N_2 as gas phase) and aerobic conditions. Since no difference in activity was found with the purified enzyme under either condition, assays were routinely performed aerobically.

The heat stability was investigated by heating samples of 1 ml pyrophosphatase (10 μg protein) in 50 mM Tris-HCl pH 8.0 and 10 mM $MgCl_2$ in eppendorf cups for 10 min at several temperatures. After cooling to 35 °C, 1 mM of pyrophosphate was added and the activity determined with the standard assay.

The substrate specificity of the enzyme was determined by replacing 1 mM pyrophosphate in the standard assay for 1 mM of various other phosphate-esters. Several compounds were tested for their ability to inhibit pyrophosphatase. The enzyme was preincubated 30 min at 35 °C with 5 mM inhibitor in 50 mM Tris-HCl pH 8.0, 5 mM Mg^{2+}. Thereafter 1 mM PP_i was added and
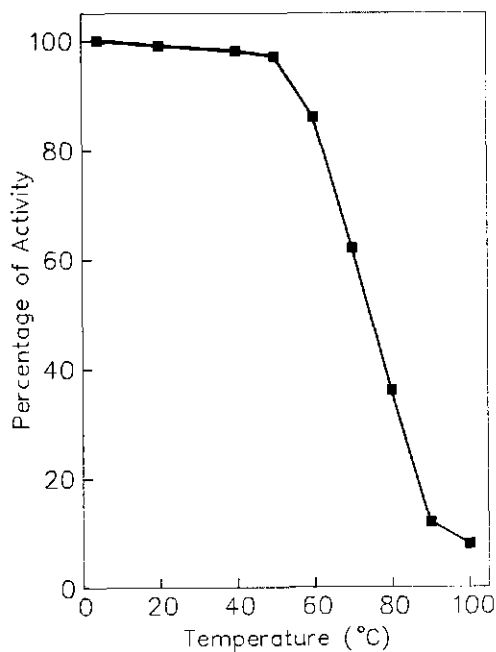
Fig. 4. Heat stability of the pyrophosphatase.

Samples of 1 ml pyrophosphatase (10 μg protein) were heated for 10 min at the temperatures indicated. After cooling to 35 °C, the activity was determined as described in material and methods.
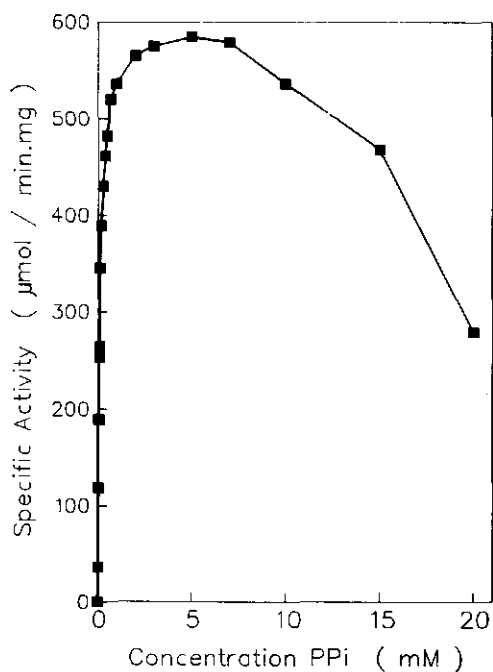
Fig. 5. Relation between pyrophosphate concentration and specific activity of pyrophosphatase.

Samples of 1 ml pyrophosphatase (5 μg protein) in 50 mM Tris-HCl, pH 8.0, and 5 mM MgCl₂ were incubated in eppendorf cups for 10 min at 35 °C. Then the indicated amounts of pyrophosphate were added and the activity determined as described in material and methods.

the activity determined with the standard assay.

RESULTS

Localization. When the bacterial cells were rigorously disrupted by prolonged sonification and two passages through a French pressure cell at 135 MPa, the pyrophosphatase was found in the soluble fraction after ultracentrifugation. However, when gentle lysis conditions were applied in the presence of 0.5 mM of the protease inhibitor Phenylmethylsulfonyl fluoride (PMSF), upto 5 % of the pyrophosphatase activity was associated with the membrane fraction, while the acetyl-coenzyme A synthetase was exclusively found in the cytoplasmic fraction. The control enzyme ATPase was completely recovered in the membrane fraction.

Enzyme purification. The purification of pyrophosphatase required no strict anaerobic conditions. The purification was carried out at room temperature. In three steps a 400 fold purified enzyme was obtained (Table 1). Q-Sepharose chromatography proved to be an effective first step in the purification of pyrophosphatase, since the enzyme was one of the first proteins to elute from the column after the void volume. Hydroxylapatite was used to separate pyrophosphatase from interfering acetyl-CoA synthetase. Phenyl-superose removed the last contaminating proteins from the pyrophosphatase.

Characterization of the purified enzyme. The molecular mass of the native pyrophosphatase was estimated by gelfiltration on Superose 6 HR 10/30 and appeared to be 139 ± 7 kDa, when compared with standards of known molecular mass (Figure 1). Native PAGE revealed one band when stained with coomassie brillant blue R 250. When the native gels were incubated 1-2 min in pyrophosphatase assay mixture and stained with ammonium-heptamolybdate and methylgreen in 1 M

H_2SO_4, this band showed a strong accumulation of released phosphate (Figure 2). Sodium dodecyl sulfate gel electrophoresis of the purified enzyme revealed two subunits with relative molecular mass equal to 35 and 33 kDa of equal intesity, which suggests an $\alpha_2\beta_2$ subunit stoichiometry for the native enzyme (Figure 3).

Stability. The activity of the purified enzyme did not decrease significantly in the presence of air. The enzyme could be stored at +4°C for at least one week without appreciable loss of activity . When the enzyme was kept at -20 °C, aerobically or anaerobically, 50 % of the activity was lost within 24 hours. However, when the enzyme was frozen in liquid nitrogen, it could be stored for at least two months at -80 °C without loss of activity. The enzyme was relatively thermostable in the presence of 0.01 M Mg^{2+}. Heating for 10 min at 60 °C resulted only in a loss of activity of 15 %. Complete inactivation occurred after heating at 90 °C (Figure 4).

Substrate specificity. In addition to pyrophosphate, a Mg^{2+} dependent hydrolysis of tri- and tetrapolyphosphates was catalysed by the purified enzyme at 44 % and 8 % of the PP_i hydrolysis rate, respectively. Organic phosphates at 5 mM (ATP, ADP, AMP, Phosphoenol pyruvate, Thiamine pyrophosphate, Acetylphosphate, Glucose-1-phosphate, Glucose-6-phosphate and Paranitrophenyl phosphate) were not hydrolyzed by the enzyme. Mn^{2+} and Co^{2+} could replace Mg^{2+} as cation . With these cations 160 % and 24 % of the activity of Mg^{2+} at 1 mM was found, respectively. At concentrations higher than 1 mM Mn^{2+} and Co^{2+} precipitation of pyrophosphate occurred. Ca^{2+} did not promote the pyrophosphatase activity.

Kinetic properties. The reaction rate at different pyrophosphate and Mg^{2+} concentrations did not follow Michaelis-Menten kinetics. Half maximal rates were obtained at 0.1 mM PPi at 5 mM Mg^{2+}. High concentrations of pyrophosphate inhibited the

pyrophosphatase (Figure 5). The optimal Mg^{2+} to pyrophosphatse ratio was 5. The V_{max} at the optimal pH of 8 (50 mM Tris-HCl) and at 35 °C, was 590 μmoles of pyrophosphate hydrolyzed per min per mg protein, which corresponded to a K_{cat} of 1400 per second.

Inhibition studies. Pyrophosphatase was inhibited for more than 40 % by KCN , ATP and EDTA (table 2). ATP and EDTA inhibited the enzyme, probably by competition for magnesium. The inhibition could be reversed by addition of extra (10 mM) Mg^{2+}. The enzyme was not inhibited by fluoride, which is an potent inhibitor of the pyrophosphatases of *E.coli* (90 %, 1 mM), yeast (50 %, 6.7 μM) and *Thiobacillus* (89 %, 0.1 mM) (13,20,21, 24). Reducing agents (titanium citrate, sodium dithionite) stimulated the activity of pyrophosphatase in crude extract 2 to 5 fold (data not shown). The purified enzyme, however, was not stimulated by reducing agents, it was slightly inhibited (Table 2).

DISCUSSION

Together with the acetyl-CoA synthetase and adenylate kinase, the inorganic pyrophosphatase plays an important role in the acetate activation of *M.soehngenii* (9). From the increase in specific activity upon purification and from the 50 % recovery, it can be calculated that about 0.2 % of the soluble cell protein of *M. soehngenii* is pyrophosphatase. This is somewhat lower than the value of other important proteins involved in the acetate metabolism of *M. soehngenii* (8-10).

The purified pyrophosphatase of the archaebacterium *Methanothrix soehngenii* shows some marked differences with enzymes isolated from eubacteria and eukaryotes. The apparent molecular mass of the enzyme was estimated to be 139 kDa. This value is different from molecular masses reported for pyrophosphatases from *E. coli* (120 kDa),

Thiobacillus (80 kDa) *Rhodosprillum* (100 kDa), *Streptococcus* (128 kDa), *Bacillus* (122 kDa), *Desulfovibrio* (42 kDa) and yeast (60 kDa) (6,14-16,19,25-27). Electrophoresis on SDS-PAGE indicated that inorganic pyrophosphatase from *Methanothrix* is composed of subunits with molecular masses of 35 and 33 kDa arranged in $\alpha_2\beta_2$ oligomeric structure. This subunit structure is different from those described for other microorganisms. The yeast enzyme consists of two identical subunits of 30 kDa, the *Bacillus* enzyme of two subunits of 70 kDa, while the *Desulfovibrio* enzyme consisted of only one subunit of 42 kDa (6,16, 26). The enzyme from *Thiobacillus* was composed of 4 identical subunits of 20 kDa, and the *Streptococcus* enzyme of four subunits of 32.5 kDa (19,25). The enzyme of *E. coli* has six identical subunits with molecular mass of 20 kDa (14,27)

The enzyme required magnesium for full activity; manganese and cobalt could replace magnesium. The effectiveness of manganese and cobalt as cation for the hydrolysis of pyrophosphate is limited to a narrow range of concentration of these ions. Manganese- or cobalt- pyrophosphate salts are poorly soluble as compared to magnesium pyrophosphate (16). A similar cation specificity is reported for other pyrophosphatases, although the stimulation by manganese is relatively high for the enzyme in *Methanothrix* (14,18,25).

The relationship of magnesium and pyrophosphate concentration relative to the pyrophosphatase activity was studied extensively with the *E.coli* and yeast enzyme (1,13,18,23). As the actual subtrate a stoichiometric complex, $MgPP_i$, is proposed (13,18). For the *Methanothrix* enzyme a magnesium to pyrophosphate ratio of 5 was found to be optimal. As observed for the enzymes of *E.coli* and yeast, high concentrations of free pyrophosphate inhibited the pyrophosphatase of *Methanothrix* (18).

The enzyme of *Methanothrix* was not inhibited by fluoride, which is an potent inhibitor of the

pyrophosphatases of *E.coli* (90 %, 1 mM), yeast (50 %, 6.7 μM) and Thiobacillus (89 %, 0.1 mM) (14,22,25). Sofar only the enzyme of *Streptococcus* is reported not to be inhibited by fluoride (19). However, this enzyme exists in two conformation, with different activity and stability. The pyrophoshatase found in cell extracts of *Ureoplasma* was also not inhibited by fluoride (5). The inhibition by fluoride is extensively studied with the yeast enzyme (22,23). It was proposed that fluoride strenghtens the binding of the enzyme to the magnesium pyrophosphate complex. The fluoride insensitivity of the *Methanothrix* enzyme could indicate that this enzyme has an altered active site in which fluoride is not able to covalently bind the magnesium pyrophosphate to the magnesium enzym complex. As decribed for several other pyrophosphatases, the enzyme of *Methanothrix* was also not inhibited by sulfhydryl-reagents, which indicates that cysteine residues do not play an important role in the PP_i hydrolysis (14,18, 19,25).

As reported for many pyrophosphatases of gram-negative bacteria, the enzyme of *Methanothrix* is also remarkably heat-resistent in the presence of magnesium (3,18,25). The enzyme was highly specific for pyrophosphate, although it hydrolyzed tri- and tetrapolyphosphate to some extent. No other phosphate esters were hydrolyzed by the enzyme. This reflects the high specificity reported for other pyrophophatases (12,14,19,25).

When rather rigorous methods were used to disrupt the cells, 99 % of the enzyme activity of the cell extract was present in the 110,000 g supernatant, which suggests that this enzyme is present in the cell cytoplasm, like enzymes of *E.coli*, *Bacillus*, *Streptococcus*, *Thiobacillus* and yeast (6,14,15,19,25). However, when gentle lysis procedures in the presence of the serine protease inhibitor, Phenylmethylsulfonyl fluoride (PMSF), were applied up to 5 % of the pyrophosphatase activity was associated with the membrane fraction. This mem-

brane association could indicate that hydrolysis of the pyrophosphate, which is formed during acetate activation, is not solely used to displace the equilibrium of the acetate activation. In the chromatophores of photothrophic bacteria a proton translocating pyrophosphatase has been observed (15,20,21). The main task of this integral-membrane enzyme is to maintain a substantial protonmotive force under circumstances of low energy (20). A similar function of the membrane associated pyrophosphatase of *Methanothrix* could be envisaged. Future research therefore will deal with the study of the energy in- and output of *Methanothrix* during acetate fermentation.

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Chapter 5

Adenine Nucleotide Content and Energy Charge of
Methanothrix soehngenii during Acetate Degradation

Adenine Nucleotide Content and Energy Charge of *Methanothrix soehngenii* during Acetate Degradation.

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1. Summary

The interconversion of adenine nucleotides during acetate fermentation was investigated with concentrated cell suspensions of *Methanothrix soehngenii*. Starved cells contained high levels of AMP (2.2 nmol/mg protein), but had hardly any ADP or ATP. The Energy Charge (EC) of these cells was 0.1. Immediately after the addition of the substrate acetate, the level of ATP increased, reaching a maximum of 1.4 nmol/mg protein, corresponding to an EC of 0.7 when half of the acetate was consumed. Once the acetate was depleted, the ATP concentration decreased to its original level of 0.1 nmol/mg protein. Since *M. soehngenii* contained relatively high amounts of AMP, the luciferase system for the determination of ATP gave not always satisfactory results. Therefore a reliable method based on the separation of adenine nucleotides by anion exchange HPLC was used.

2. Introduction

Methanothrix soehngenii is an anaerobic archaebacterium, which is specialized in the use of acetate as sole energy source (1-3). The growth yield (1.4 g/mol acetate) and the growth rate (average doubling time of 7 days)

are low, but the affinity for acetate ($K_s = 0.5$ mM, threshold concentration $< 10\ \mu$M) of *M. soehngenii* is high (1-4). In *M. soehngenii* acetate is activated to acetyl-CoA by acetyl-CoA synthetase (5). The energy for this activation is supplied by hydrolysis of ATP to AMP and pyrophosphate (5). The formed AMP is converted to ADP by adenylate kinase at the expense of another ATP, and pyrophosphate is hydrolyzed by pyrophosphatase to two orthophosphate (5,6). The sum of these reactions indicates that acetate in *M. - soehngenii* is activated to acetyl-CoA at the expense of two energy rich phosphate-bonds (5-7). Because conversion of acetate to methane is supposed to yield only one ATP, it is difficult to concieve how *M. soehngenii* conserves energy for growth (7,8). The aim of the present study was to examine the size of the different adenine nucleotide pools in *M. soehngenii* during acetate fermentation.

3. Material and Methods

3.1 *Chemicals and gases*. All chemicals were at least of reagent grade and were obtained from Merck (Darmstadt, F.R.G.). Biochemicals and ATP bioluminescence CLS kit were from Boehringer Mannheim (Almere, NL). Gases and gas mixtures were supplied by Hoekloos (Schiedam, NL). Palladium catalyst
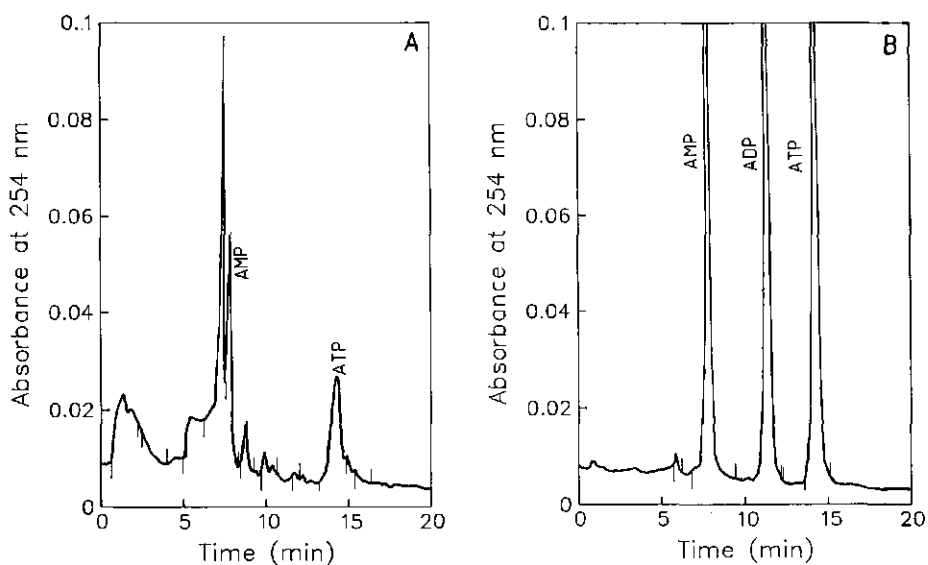
Fig. 1. HPLC chromatogram of perchloric extract of *Methanothrix soehngenii*.

(A) 0.5 ml cell suspension (1 mg protein) was extracted with perchloric acid and analyzed for adenine nucleotides on HPLC as described in Material and Methods.

(B) 0.5 ml adenine nucleotide mix (AMP, ADP and ATP, each 0.5 mM) was treated in the same way as a cell suspension and analyzed on HPLC.

was a gift of BASF (Arnhem, NL). Ti-(III)citrate (100 mM) was prepared by adding 5 ml 1 M Ti(III)chloride to 40 ml of an anaerobic 0.2 M sodium citrate solution, pH was adjusted with 2 M Tris to 7.5.

3.2 *Organism and cultivation. Methanothrix soehngenii* was the Opfikon strain isolated by Huser et al. and provided by A.J.B. Zehnder (3). *M. soehngenii* was cultivated in a mineral medium containing 40 mM $NaHCO_3$, 3 mM Na_2HPO_4, 3 mM KH_2PO_4, 5 mM NaCl, 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, 6 mM NH_4Cl, 1 mM Na_2S, vitamins and trace elements according to (3). The bacteria were grown in three liter serum bottles containing 2 l of medium, supplemented with 80 mmol of sodium acetate per liter. The gas phase was N_2/CO_2 (8:2) giving a final pH of 7.2. Growth was monitored by measuring methane production and acetate consumption.

3.3 *Preparations of cell suspensions.* All operations were performed at room temperature (19 °C) under strictly anaerobic conditions in a chamber with a N_2/H_2 (95:5) gas phase; traces of oxygen were removed by a palladium catalyst. Cells (2 l cultures) were harvested at the late log phase. Two days before harvesting, the serum bottles were turned upside down, so that the large cell-aggregates could settle just on top of the rubber septa. The aggregates were removed with a 50 ml syringe, centrifuged 15 min at 5000 x g, and washed once in medium without phosphate, vitamins and sodium sulfide, but reduced with 0.5 mM Ti(III)citrate. The cells were resuspended in 10 ml of the same medium, giving a concentration of 1-2 mg protein per ml.

3.4 *Adenine nucleotide extraction.* Concentrated cell suspensions of *M. soehngenii* were incubated at 37 °C under an atmosphere of N_2/CO_2 (8:2). Acetate was added to a concentration of 8 mM. Acetate comsumption and methane production were followed gas-

chromatographically as described in (4). At appropriate time intervals, 0.5-ml samples were removed and immediately added to 0.2 ml of ice-cold 3 M perchloric acid (9). The samples were kept on ice for 2 h. Then, the pH was brought to 6.5 by the addition of 0.2 ml 3 M KOH and 0.1 ml 0.1 M KH_2PO_4 buffer. The formed $KClO_4$ was removed by centrifugation and supernatants were kept on ice.

3.5 *Analytical procedures.* The adenine nucleotide content of the perchloric extracts was determined with a LKB-HPLC system (Pharmacia/LKB, Woerden, NL) equipped with two high performance (2150) pumps and a high pressure gradient mixer. The perchloric extract (20 μl) was applied to an ionoSpher A column of 20 by 0.3 cm (Chrompack, Middelburg, NL) equilibrated with 10 mM KH_2PO_4 pH 6.5 (flow rate 0.8 ml.min^{-1}). The adenine nucleotides were separated in a 16 ml linear gradient of 0.5 M NaCl in 10 mM KH_2PO_4 pH 6.5, collected in 1 ml fractions and detected at 254 nm. The nucleotides were quantified by relating peak areas to standard curves using a Shimadzu C-R5A integrator (Shimadzu Analytical Instruments, Kyoto, Japan). The adenine nucleotide content was linear with the peak areas between 2 and 2000 pmol of the respective nucleotides. The ATP content of representative samples was checked by the luciferin/luciferase method (9). Ten μl of perchloric extract or of collected HPLC fractions were transferred to 4 ml counting vials containing 0.7 ml assay buffer (20 mM glycylglycine, 5 mM sodium arsenate, and 4 mM $MgSO_4$, pH 7.9). The reaction was started by the addition of 20 μl of ATP bioluminescence CLS-kit (15 mg/ml). The vial was rapidly closed, shaken and introduced into the counting chamber of a LKB-Wallac scintillation counter. 10 s after the start of the reaction the light flashes were counted for 10 to 30 s. For calibration of the system samples were spiked with known concentrations of
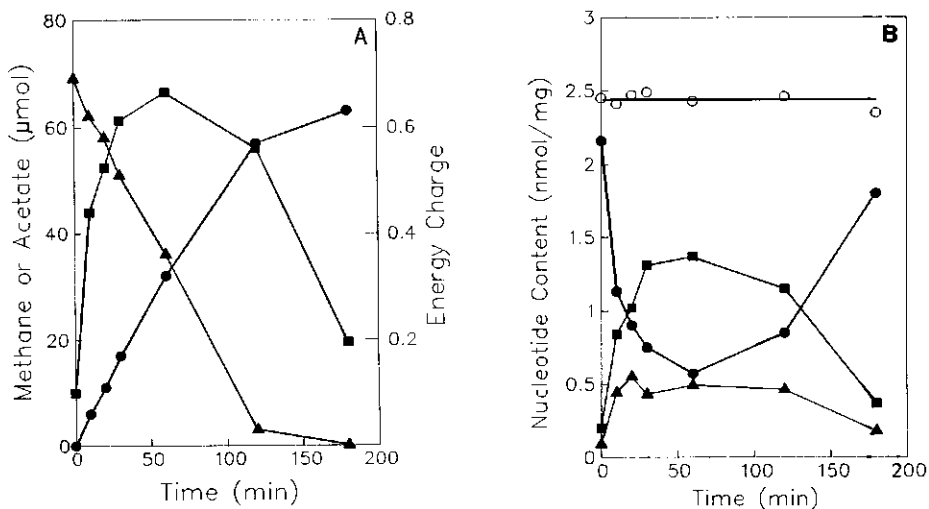
Fig. 2. Effect of the addition of acetate on the Energy Charge and adenine nucleotide content of *M. soehngenii*.

The cell suspension of 10 ml under N_2/CO_2 (8:2) had a protein content of 2 mg/ml. At time zero acetate was added to final concentration of 8 mM.

(A) Acetate (▲) and methane (●) were determined as described in Material and Methods. Energy charge (■) was calculated with the data from Fig 2B.

(B) AMP (●), ADP (▲) and ATP (■) were determined as described in Material and Methods. Total adenylates (O) are the sum of AMP, ADP and ATP.

ATP. Under the conditions employed the ATP content was linearly related to the counts between 0.5 and 10 pmol of ATP.

4. Results and Discussion

4.1 *Detection and quantification of adenine nucleotides*

The use of the luciferin/luciferase enzyme system for the detection of picomole amounts of ATP has been well established and documented (10). Initial attempts to determine the ATP content of perchloric extracts of *M. soehngenii* showed very small amounts of ATP. Because also the spiking with external ATP gave considerable underestimation of the ATP content in the samples, an alternative quantification method was used. Satisfactory and reproducible results were obtained when the different nucleotides were separated by anion exchange HPLC and quantified by relating peak areas. A representative chromatogram of an perchloric extract of *M. soehngenii* is shown in Fig. 1A. The chromatogram of a standard sample, containing 0.5 mM of different adenine nucleotides each in perchloric acid, treated in the same way as cell extracts is shown in Fig. 1B.

These chromatograms show that all three adenine nucleotides can be quantified in one determination by separation of perchloric acid extracts on HPLC. Difficult and often in-complete enzymatic conversion of AMP and ADP are circumvented by this method. Also the quenching effect of large amounts of AMP on the luciferase system are overcome. The detection method used is not as sensitive as the luciferase system. However, when the HPLC system is extended to semi-preparative columns on which larger (1-2 ml) samples can be applied the sensitivity of the method will be increased considerably.

4.2 *Energy Charge and adenine nucleotide content*

Washed and concentrated cell suspensions of *M. soehngenii* were incubated at 37 °C under N_2/CO_2 (8:2). Immediately after the addition of the substrate acetate, methane production and acetate consumption started (Fig. 2A). The rate of methane production and acetate consumption were about 40 $nmol.min^{-1}.mg^{-1}$ of protein. At appropriate time intervals samples were analyzed for adenine nucleotides (Fig. 2B). Before the addition of substrate, cells contained high levels of AMP (2.2 nmol/mg of protein) and low levels of ADP and ATP (0.1 nmol/mg of protein), resulting in a very low Energy Charge of 0.1 (Fig. 2 A and B). After the addition of acetate the levels of ATP and ADP started to increase and consequently AMP decreased. ADP reached a more or less constant level of 0.4 nmol/mg protein, while the ATP level was maximal (1.4 nmol/mg of protein) and AMP minimal (0.6 nmol/mg protein) when half of the acetate was consumed. The Energy Charge at that time was 0.7 (Fig. 2A). Once the acetate was depleted, the levels of ATP and ADP began to decrease until their original levels of about 0.2 nmol/mg of protein were reached, while the AMP level increased to 2 nmol/mg of protein. The Energy Charge dropped to 0.2.

The amount of adenine nucleotides (2.5 nmol/mg protein) in *M. soehngenii* is relatively low compared to the amounts found in the acetoclastic methane bacterium *Methano-sarcina barkeri* (10 nmol/mg protein) (9). The amounts found in (eu)bacteria vary between 6 and 14 nmol/mg protein (11). The low amount of adenine nucleotides in *M. soehn-genii* is not very surprising, since this organism has also the lowest levels of typical methano-genic cofactors (12).

The extremely low Energy Charge of 0.1 in starved cells of *M. soehngenii* resembles the low values of bacterial spores or of dry mature plant seeds (13). As observed for

germinating spores or seeds, the Energy Charge of *M. soehngenii* reaches more or less normal values after the addition of substrate (13). The maximum energy charge of 0.7, observed for *M. soehngenii* is low, since an Energy Charge between 0.85 and 0.9 is thought to be necessary for maintaining a metabolic stability (13). A mechanism by which a high Energy Charge is maintained by excretion or breakdown of AMP seems not to be present in *M. soehngenii* (13,14). However, a possible advantage of a low Energy Charge for this organism is that the free energy needed to form ATP out of ADP and P_i will be considerably lower than under conditions of high energy charge. Although the exact mechanism of energy generation for acetoclastic methanogens is not known, this paper shows that the breakdown of acetate by *M. soehngenii* yields enough energy to build up an ATP pool.

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Chapter 6

Purification and characterization of an oxygen stable
carbon monoxide dehydrogenase of *Methanothrix soehngenii*

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Purification and characterization
of an oxygen-stable carbon monoxide dehydrogenase of *Methanothrix soehngenii*

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Carbon monoxide dehydrogenase was purified to apparent homogeneity from *Methanothrix soehngenii*. In contrast with the carbon monoxide dehydrogenases from most other anaerobic bacteria, the purified enzyme of *Methanothrix soehngenii* was remarkably stable towards oxygen and it was only slightly inhibited by cyanide.

The native molecular mass of the carbon monoxide dehydrogenase of *Methanothrix soehngenii* determined by gel filtration was 190 kDa. The enzyme is composed of subunits with molecular mass of 79.4 kDa and 19.4 kDa in an $\alpha_2\beta_2$ oligomeric structure. The enzyme contains 1.9 ± 0.2 $(n = 3)$ mol Ni/mol and 19 ± 3 $(n = 3)$ mol Fe/mol and it constitutes 4% of the soluble cell protein. Analysis of enzyme kinetic properties revealed a K_m of 0.7 mM for CO and of 65 μM for methyl viologen. At the optimum pH of 9.0 the V_{max} was 140 μmol of CO oxidized min^{-1} mg protein^{-1}. The enzyme showed a high degree of thermostability.

Acetate is the most abundant methanogenic substrate in anaerobic ecosystems [1, 2]. The most important acetoclastic methanogens are *Methanosarcina* and *Methanothrix*. Due to its high affinity for acetate, *Methanothrix* is the predominant acetoclastic bacterium in applied methanogenic systems such as waste-water-treatment plants [2—4]. Because of its very low growth rate (doubling time is 5—14 days), it can only be isolated by continuous enrichment at low acetate concentrations [5, 6].

Methanogenesis from acetate proceeds via reaction (1).

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \Delta G'_o = -31 \text{ kJ/mol.} \quad (1)$$
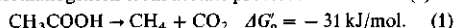
The methane originates predominantly from the methyl group and carbon dioxide from the carboxyl group of acetate [6, 7]. The enzymatic steps involved in this cleavage still have to be identified in detail. It was postulated that, in acetate-adapted *Methanosarcina*, CO dehydrogenase may participate in the cleavage of acetate [8], a sort of a reversed reaction of the CO-dehydrogenase-dependent acetate formation in acetogens, autotrophic methanogens and some sulfate-reducing bacteria [9, 10].

The CO dehydrogenases of the acetogenic bacteria *Clostridium* and *Acetobacterium* have been isolated and characterized in detail [10—13]. Recently the CO dehydrogenases of the methanogenic bacteria *Methanosarcina*, *Methanococcus* and *Methanobrevibacter* were purified and some of their properties were described [14—19].

The CO dehydrogenase of *Methanothrix* has not been isolated and characterized before. In contrast to the other CO dehydrogenases of anaerobic bacteria, its activity in cell extracts was reported to be stable towards molecular oxygen [20, 21]. The aerobic purification procedure, together with a number of physicochemical and catalytic properties of the highly purified CO dehydrogenase of *Methanothrix soehngenii*, are described here.

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Enzyme. Carbon monoxide dehydrogenase (EC 1.2.99.2).

MATERIALS AND METHODS

Materials

Tris, dithioerythritol and methyl viologen were obtained from Janssen Chimica (Beerse, Belgium). SDS and acrylamide were from Bio-Rad (Utrecht, NL). Gases were purchased from Hoekloos (Schiedam, NL). Platina catalyst was a gift of BASF (Arnhem, NL). Mono-Q HR 5/5, Q-Sepharose, Superose 6 H 10/30 and molecular mass standards for gel filtration and PAGE were obtained from Pharmacia Fine Chemicals (Woerden, NL). *Methanothrix soehngenii* (DSM 2139) was the Opfikon strain isolated by Huser et al. [5]. Coenzyme F_{420} isolated from *Methanobacterium thermoautotrophicum* was a generous gift of Dr B. Gruson and Dr Ph. Debeire (INRA, Villeneuve d'Asq, France).

Cultivation of Methanothrix

Methanothrix was mass cultured on 80 mM sodium acetate in 25-l carboys, containing 20 l of the phosphate/bicarbonate buffered medium described by Huser et al. [5]. Cultures were incubated without stirring at 35°C in the dark under an N_2/CO_2 (80/20%) gas phase. Cells were harvested at the late log phase by continuous centrifugation (Carl Padberg Zentrifugenbau GmbH, Lahr/Schwarzwald, FRG), washed in 50 mM Tris/HCl pH 8.0 and stored under N_2 at -20°C until use.

Preparation of cell extract and protein purification

The purification procedure was performed within one day. Unless indicated otherwise all procedures were carried out aerobically at room temperature (19°C). A frozen cell paste was thawed, diluted with 50 mM Tris/HCl pH 8.0 in a 1:3 ratio, disrupted by passing through a French pressure cell at 135 MPa and centrifuged 30 min at $35,000 \times g$. The supernatant contained 20 mg protein/ml and is referred to as crude extract.

The crude extract (10 ml) was applied to a column (3.3 by 10 cm) of Q-Sepharose (fast flow) equilibrated with 50 mM Tris/HCl pH 8.0 (buffer A). After the column was washed with 40 ml of buffer A, the adsorbed CO dehydrogenase was eluted at 0.23 M NaCl in a 180-ml discontinuous linear gradient of 1 M NaCl in buffer A. The flow rate was 4 ml/min. Individual fractions were collected and analysed for CO dehydrogenase activity. Fractions with activity higher than 5 U/mg were pooled and desalted in an Amicon ultrafiltration cell (Grace, Rotterdam, NL) with a PM10 filter. The remaining steps in the purification were performed with a high-resolution fast protein liquid chromatography (FPLC) system (Pharmacia/LKB, Woerden, NL) equipped with a model 2152 LC controller. A sample of 5 ml of the concentrated desalted enzyme solution was injected onto a Mono-Q HR 5/5 anion-exchange column equilibrated with buffer A. A linear gradient of $0-0.3$ M NaCl in buffer A was applied at a flow rate of 1.0 ml/min. Fractions with CO dehydrogenase activity were concentrated to 0.2 ml in a Centricon PM10 (Grace, Rotterdam, NL) and injected on a Superose 6 HR 10/30 gel filter equilibrated with buffer A. The column was developed at a flow rate of 0.5 ml/min. Purified CO dehydrogenase was collected, concentrated in Centricon PM10, frozen in liquid N_2 and stored at $-80\,^{\circ}$C until use.

Enzyme assay

The standard assay for CO dehydrogenase was performed by following the reduction of methyl viologen ($\varepsilon_{578\,nm} = 9.7\,mM^{-1}\cdot cm^{-1}$) with a Biochrom/LKB Ultrospec K spectrophotometer (LKB, Woerden, NL). The standard reaction mixture contained 50 mM Tris/HCl pH 8.9, 1 mM methyl viologen and 0.1 mM dithioerythritol. The reaction mixture was equilibrated with CO for the test assay and with N_2 for the control assay; 1 ml of the mixture was added to stoppered cuvettes (1.4 ml) filled with CO or N_2 respectively. The reaction was initiated by the addition of enzyme. NAD (20 μM), FAD (20 μM), FMN (20 μM) and coenzyme F_{420} were also tested as electron acceptors. The following absorption coefficients were used: NADH, $\varepsilon_{340\,nm} = 6.2\,mM^{-1}\cdot cm^{-1}$; FAD, $\varepsilon_{450\,nm} = 11.3\,mM^{-1}\cdot cm^{-1}$; FMN, $\varepsilon_{450\,nm} = 12.2\,mM^{-1}\cdot cm^{-1}$; coenzyme F_{420}, $\varepsilon_{420\,nm} = 41.4\,mM^{-1}\cdot cm^{-1}$. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of CO/min. The K_m of the purified CO dehydrogenase for CO was determined in cuvettes containing 1 mM methyl viologen. Different concentrations of CO were established by changing the partial pressure of CO in the gas phase. The concentration of CO in the liquid phase was calculated as described by Kohler et al. [20]. The K_m for methyl viologen was determined under 1 atm 100% CO. All assays were performed at 35$\,^{\circ}$C.

Electrophoresis and molecular mass determination

The purity of the enzyme after various chromatographic steps was determined electrophoretically following the method of Laemmli [22] by SDS/PAGE. Molecular mass standards (α-lactalbumin·14.4 kDa, trypsin inhibitor 20.1 kDa, carbonic anhydrase 30 kDa, ovalbumin 43 kDa, bovine serum albumin 67 kDa and phosphorylase b 94 kDa) were used to calibrate the SDS gels. Gels were stained with Coomassie brillant blue G250. Native enzyme molecular mass was determined on Superose 6 HR 10/30 equilibrated with buffer A: 200 μl purified CO dehydrogenase (1 mg/ml) or standards (thyroglobulin 669 kDa, ferritin 445 kDa, catalase 232 kDa

Table 1. *Purification of CO dehydrogenase of* Methanothrix soehngenii
One unit (U) is the amount catalyzing the oxidation of 1 μmol CO/min

Fraction	Volume	Protein	Activity	Specific activity	Purification	Yield
	ml	mg	U	U/mg	-fold	%
Crude extract	10	220	990	4.5		100
Q Sepharose	40	18	780	43.1	10	79
Mono Q	4	9	740	82.2	18	75
Superose 6	8	5	580	117	26	59

and aldolase 158 kDa) were injected onto the FPLC system and eluted with buffer A.

Determinations

Protein was determined with Coomassie brillant blue G250 by the method of Bradford [23]. Bovine serum albumin was used as standard. Metals were analysed with an atomic absorption spectrometer equipped with a graphite furnace atomizer HGA 76B (Perkin Elmer, Ridgefield, USA). Ultraviolet/visible spectra were recorded with a Beckman model 25 spectrophotometer (Beckman, Amsterdam, NL).

RESULTS

Purification

Initial experiments with cell-free extracts showed that CO dehydrogenase of *Methanothrix* was not inactivated by molecular oxygen. This property, which had already been observed by Kohler [21], allowed an aerobic purification of the CO dehydrogenase, in contrast to the purification of CO dehydrogenases of other anaerobic bacteria which required strict anaerobic conditions [10 – 19]. The purification was carried out at room temperature and generally took only one day. In three steps a 26-fold purified enzyme was obtained (Table 1).

The CO dehydrogenase could be precipitated with ammonium sulfate (47 – 54%), but due to an unsatisfactory enzyme recovery (less than 20%) this method was not used for primary purification. Q-Sepharose chromatography proved to be an effective first step for the isolation of the CO dehydrogenase. A good separation between the CO dehydrogenase and a broad protein band with a strong fluorescence at 420/430 nm (probably component C of the methyl-CoM reductase) was achieved, especially when a plateau of 0.23 M NaCl was introduced in the linear gradient. A representative elution pattern is depicted in Fig. 1. After concentrating and desalting, the enzyme preparation was injected onto a FPLC system and purified to homogeneity.

Composition and stability

The CO dehydrogenase was eluted from the Superose 6 HR 10/30 column at a volume indicating molecular mass of 190 kDa, as compared to standards of known molecular mass (Fig. 2). SDS/polyacrylamide gel electrophoresis of the purified CO dehydrogenase revealed two subunits with molecular mass equal to 79.4 and 19.4 kDa (Fig. 3), which suggests an $\alpha_2\beta_2$ subunit stoichiometry for the native enzyme.
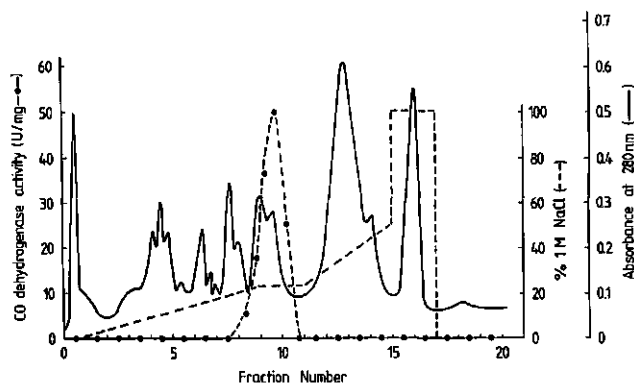
Fig. 1. *Elution pattern of a Q-Sepharose anion-exchange column (fast flow) of a cell extract from acetate-grown* Methanothrix. Cell extract (10 ml) containing 22 mg of protein/ml was applied to a the column (3.3 × 10 cm), equilibrated with 50 mM Tris/HCl pH 8.0 and developed with a gradient of NaCl (----) at 4.0 ml/min. Absorbance was recorded at 280 nm (——). CO dehydrogenase activity was determined in the standard assay (●——●)
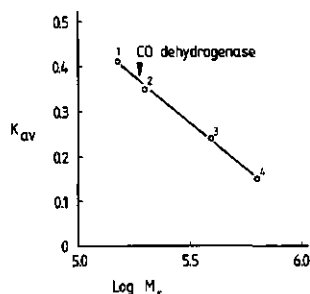

Fig. 2. *Molecular mass determination of the native CO dehydrogenase on Suparose 6 HR 10/30.* Standards were aldolase (158 kDa, 1); catalase (232 kDa, 2); ferritin (446 kDa, 3) and thyroglobulin (669 kDa, 4). The position of CO dehydrogenase is indicated by the arrow

Fig. 3. *Analysis of purity by SDS/PAGE.* Lane 1, pooled fractions from Superose 6 HR 10/30 (50 µg); lane 2, molecular mass markers: phosphorylase b (94 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa); lane 3, crude extract (80 µg); lane 4, pooled fractions from Q-Sepharose (80 µg); lane 5, pooled fractions from Mono-Q (80 µg)

The spectral characteristics of the CO dehydrogenase are illustrated in Fig. 4. The enzyme displayed a peak at 280 nm and a very broad shoulder over 380−480 nm, a region which is characteristic for Fe-S proteins. The absorbance of this broad shoulder decreased upon incubation with CO, indicating that iron-sulfur clusters play a role in the catalytic center. Atomic absorption analysis revealed 1.9 ± 0.2 ($n = 3$) mol Ni/mol and 19 ± 3 ($n = 3$) mol Fe/mol.

The activity of the enzyme did not decrease significantly in the presence of air. After a 72 h exposure to air at 4°C, the activity decreased in crude extracts from 4.5 to 4.1 U/mg and of the purified enzyme from 115 to 104 U/mg. The enzyme was highly thermostable. Heating for 10 min at 60°C resulted only in a loss of activity of about 10%. Complete inactivation occurred after heating at 72°C (Fig. 5).

Kinetics

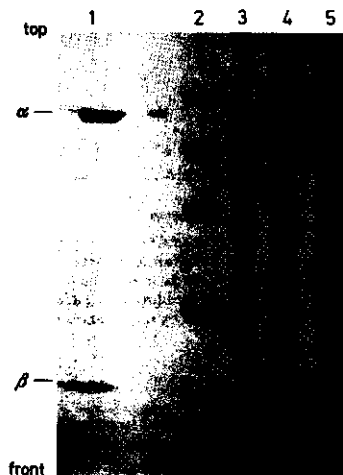The purified enzyme exhibited a high K_m for CO. It was not possible to demonstrate saturation kinetics for CO at 35°C with 1 mM methyl viologen and 2 atm CO. Extrapolation in the Lineweaver-Burke plot gave an estimation of an the K_m for dissolved CO of 0.7 mM and a V_{max} of 140 U/mg (Fig. 6). The K_m for methyl viologen was 65 µM. In 50 mM Tris/HCl the enzyme showed an optimum at pH 9. The CO dehydrogenase reduced coenzyme F_{420} at 1.5%, FMN at 0.6% and FAD at 0.3% of the specific activity in the standard assay with methylviologen. NAD was not reduced.
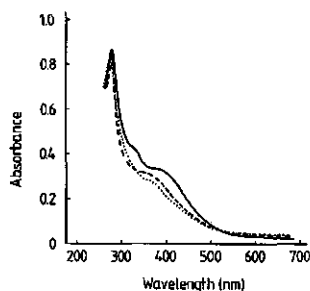
Fig. 4. *Spectral analysis of the purified CO dehydrogenase.* Spectra were recorded under air (– – – –), under N_2 (———) and after injection of CO (·······)
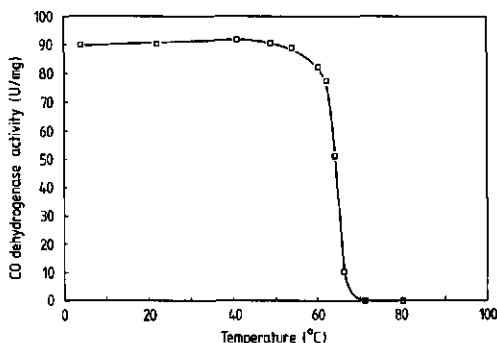

Fig. 6. *Relationship between CO concentration and specific activity of the purified CO dehydrogenase.* Conditions and calculations are described in Material and Methods. Inset is a Lineweaver-Burke plot of the same data

Fig. 5. *Heat denaturation of purified CO dehydrogenase.* Samples of 100 µl CO dehydrogenase (0.1 mg/ml) in 50 mM Tris/HCl pH 8.0 were heated in Eppendorf cups for 10 min at the temperatures indicated. After cooling to room temperature (19 °C) aliquots were withdrawn and the activity was assayed by the standard method

Fig. 7. *Time-dependent inhibition of CO dehydrogenase by potassium cyanide.* Enzyme samples (100 µl; 0.1 mg/ml) were incubated in 2.2-ml stoppered serum vials with an air, N_2 or CO atmosphere. KCN was added to a final concentration of 200 µM. Samples of 10 µl were withdrawn and the activity was measured immediately according to the standard assay, except that 200 µM of KCN was present in the assay mixture. (●) Incubation without KCN (mean of air, N_2 or CO); incubation with 200 µM KCN, in (▽) air, in (△) CO and in (□) N_2

Inhibition

When the enzyme was incubated in either an atmosphere of air, CO or N_2 and in the presence of 0.2 mM KCN, the CO dehydrogenase activity was initially reduced by 17% in the presence of air, 32% in the presence of CO and 69% in the presence of N_2. Under an air or CO atmosphere this inhibition disappeared with time (Fig. 7). The time-dependent inhibition could only be observed when 0.2 mM KCN was directly added to the assay mixture in which the enzyme activity was determined. If the enzyme was exposed to 0.2 mM KCN, but assayed in the standard mixture without KCN an average activity of 81.4 ± 3.2 ($n = 12$) µmol CO oxidized mg^{-1} min^{-1} was found for all different incubations. These experiments show that the inhibition by KCN can be removed by both oxygen and CO gas.

DISCUSSION

Carbon monoxide dehydrogenase is an abundant protein of *Methanothrix*. From the increase of the specific activity upon purification and from the 60% recovery, it can be calcu-
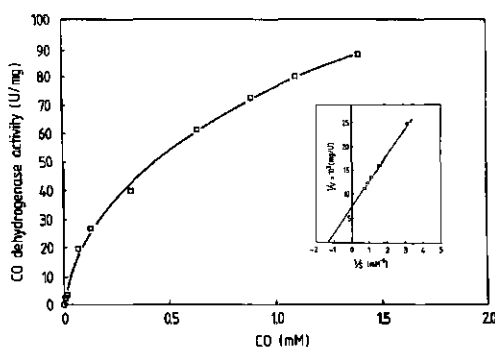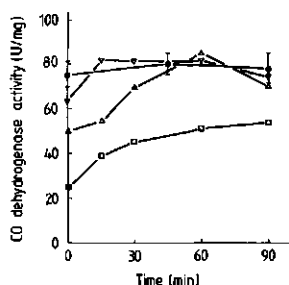
lated that up to 4% of the soluble cell protein of *Methanothrix* is CO dehydrogenase. This lies in the same range as that described for acetate grown *Methanosarcina* [15, 18]. CO dehydrogenase of *Methanothrix* also has the same subunit composition, molecular mass and metal composition as the *Methanosarcina* enzyme [15, 18]. The catalytic properties of the CO dehydrogenase of *Methanothrix* resemble those of *Methanosarcina*, since both enzymes have the same high K_m for CO. This suggests that a similar CO dehydrogenase functions in the acetate metabolism of the different types of acetoclastic methanogens. The enzyme is involved in the cleavage of an activated form of acetate. The methyl group is transferred to a C_1 carrier and the carbonyl to the nickel site, forming a nickel-iron-carbon center. A corrinoid which was found in the CO dehydrogenase complex of *Methanosarcina thermophila* could actually be the proposed C_1 carrier [17]. EPR studies of Therlesky et al. [24] showed that acetyl coenzyme A binds to the enzyme of *Methanosarcina thermophila* and induces a change in the nickel-iron-carbon

center. The activated form of acetate might be acetyl coenzyme A because high activities of acetate kinase and phosphotransacetylase are present in *Methanosarcina* and high activities of an acetate thiokinase are present in *Methanothrix* [15, 17, 20, 21, 24].

With respect to the oxygen sensitivity, the CO dehydrogenase of *Methanothrix* differs markedly from CO dehydrogenases of most other anaerobic bacteria, including *Methanosarcina*. The purified enzyme of *Methanothrix* appears to be completely insensitive to molecular oxygen and it is only slightly inhibited by cyanide. This is in contrast with CO dehydrogenases of other anaerobic bacteria, which are irreversibly inactivated by traces of oxygen (< 5 ppm) and are competitively inhibited by small amounts of cyanide ($< 10 \mu M$) [15, 17, 25]. So far only the enzymes of *Desulfovibrio desulfuricans* and of *Desulfobacterium autotrophicum* were reported to be insensitive to oxygen [26, 27]. At present no data on the kinetic properties are available to make a comparison between these CO dehydrogenases and the CO dehydrogenase of *Methanothrix*. The catalytic center has to be studied in more detail on a biochemical and a genetic level to obtain information concerning the different properties of the CO dehydrogenases towards oxygen.

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Chapter 7

Paramagnetic centers and acetyl-coenzyme A / CO
Exchange Activity of
Carbon Monoxide Dehydrogenase from *Methanothrix soehngenii*

Mike Jetten, Wilfred Hagen, Antonio Pierik, Alfons Stams and Alexander Zehnder

Paramagnetic centers and acetyl-coenzyme A/CO exchange activity of carbon monoxide dehydrogenase from *Methanothrix soehngenii*

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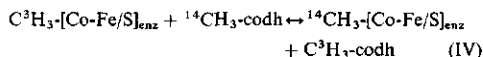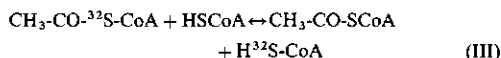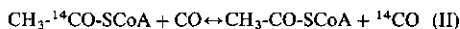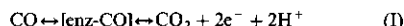
Carbon monoxide (CO) dehydrogenase was purified, both aerobically and anaerobically, to apparent homogeneity from *Methanothrix soehngenii*. The enzyme contained 18 ± 2 ($n = 6$) mol Fe/mol and 2.0 ± 0.1 ($n = 6$) mol Ni/mol. Electron paramagnetic resonance (EPR) spectra of the aerobically purified CO dehydrogenase showed one sharp EPR signal at $g = 2.014$ with several characteristics of a $[3Fe-4S]^{1+}$ cluster. The integrated intensity of this signal was low, 0.03 $S = 1/2$ spin/$\alpha\beta$ dimer. The 3Fe spectrum was not affected by incubation with CO or acetyl-coenzyme A, but could be reduced by dithionite. The spectrum of the reduced, aerobically purified enzyme showed complex EPR spectra, which had several properties typical of two $[4Fe-4S]^{1+}$ clusters, whose $S = 1/2$ spins weakly interacted by dipolar coupling. The integrated intensity was $0.1 - 0.2$ spin/$\alpha\beta$ dimer. The anaerobically isolated enzyme showed EPR spectra different from the reduced aerobically purified enzyme. Two major signals were apparent. One with g values of 2.05, 1.93 and 1.865, and an $E_{m7.5}$ of -410 mV, which quantified to 0.9 $S = 1/2$ spin/$\alpha\beta$ dimer. The other signal with g values of 1.997, 1.886 and 1.725, and an $E_{m7.5}$ of -230 mV gave 0.1 spin/$\alpha\beta$ dimer. When the enzyme was incubated with its physiological substrate acetyl-coenzyme A, these two major signals disappeared. Incubation of the enzyme under CO atmosphere resulted in a partial disappearance of the spectral component with $g = 1.997$, 1.886, 1.725. Acetyl-coenzyme A/CO exchange activity, 35 nmol · min^{-1} · mg^{-1} protein, which corresponded to 7 mol CO exchanged min^{-1} mol^{-1} enzyme, could be detected in anaerobic enzyme preparations, but was absent in aerobic preparations. Carbon dioxide also exchanged with C-1 of acetyl-coenzyme A, but at a much lower rate than CO and to a much lower extent.

Autotrophic methanogenic, acetogenic and sulfate-reducing bacteria, which use the Wood pathway for CO_2 fixation, contain carbon monoxide (CO) dehydrogenase, which catalyzes the formation of acetyl coenzyme A (acetyl-CoA) from a methyl donor and an enzyme-bound carbonyl moiety [1–8]. A reversed Wood pathway is operative in those methane bacteria, *Methanosarcina* and *Methanothrix*, which are known to degrade acetate to CH_4 and CO_2 [3, 8]. In this reversed acetyl-CoA pathway, CO dehydrogenase has been suggested to play a pivotal role in the cleavage of the C-C bond of acetate [9]. Prior to the cleavage, acetate is activated to acetyl-CoA by an acetyl-CoA synthetase in *Methanothrix* or by an acetate kinase and phosphotransacetylase in *Methanosarcina* [10, 11].

CO dehydrogenase is present in both *Methanothrix* and *Methanosarcina* and constitutes up to 5% of the soluble cell protein of these bacteria. The enzyme has been isolated from *Methanothrix* and *Methanosarcina* [9, 12–14]. The enzyme has an $\alpha_2\beta_2$ subunit structure, with subunits of approximately 90 and 20 kDa and contains iron and nickel.

Most of the knowledge about the catalytic properties of the CO dehydrogenase has been obtained from the studies of Ragsdale and Wood, who investigated the CO dehydrogenase of *Clostridium thermoaceticum* in detail [4, 5, 15, 16]. This enzyme has been shown to catalyze five reactions: the oxi-

dation of CO to CO_2 or the reduction of CO_2 to CO (I) [15], the exchange between CO and the carbonyl of acetyl-CoA (II) [16, 17], the exchange between CoA and the CoA moiety of acetyl-CoA (III) [18], the methyl exchange between methylated CO dehydrogenase and methylated corrinoid-Fe/S protein (IV) [19], and the condensation of a methyl group bound to CO dehydrogenase with CO and CoA to acetyl-CoA (V) [19, 20].

$$CO \leftrightarrow [enz\text{-}CO] \leftrightarrow CO_2 + 2e^- + 2H^+ \qquad (I)$$

$$CH_3\text{-}^{14}CO\text{-}SCoA + CO \leftrightarrow CH_3\text{-}CO\text{-}SCoA + {}^{14}CO \quad (II)$$

$$CH_3\text{-}CO\text{-}^{32}S\text{-}CoA + HSCoA \leftrightarrow CH_3\text{-}CO\text{-}SCoA$$
$$+ H^{32}S\text{-}CoA \qquad (III)$$

$$C^3H_3\text{-}[Co\text{-}Fe/S]_{enz} + {}^{14}CH_3\text{-}codh \leftrightarrow {}^{14}CH_3\text{-}[Co\text{-}Fe/S]_{enz}$$
$$+ C^3H_3\text{-}codh \qquad (IV)$$

$${}^{14}CH_3\text{-}codh + CO + HSCoA \rightarrow {}^{14}CH_3\text{-}CO\text{-}SCoA + codh \quad (V)$$
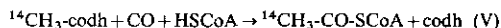
where [enz-CO] = enzyme-bound carbonyl group, codh = carbon monoxide dehydrogenase, C^3H_3-codh = methylated carbon monoxide dehydrogenase, $[Co\text{-}Fe/S]_{enz}$ = methyl-acceptor protein, containing a corrinoid and an iron-sulfur cluster.

So far only the CO:methylviologen oxidoreductase activity (I) could be demonstrated for the methanogenic CO dehydrogenases [9, 12–14]. It was found that the oxidoreductase activity of the *Methanothrix* enzyme is stable towards oxygen, whereas the CO dehydrogenase of *Methanosarcina* and of most other anaerobic bacteria is inactivated by

traces of oxygen [9, 14]. Fisher and Thauer recently reported the presence of CO_2/acetyl-CoA exchange activity in crude extracts of *Methanosarcina barkeri* [21].

All the reported CO dehydrogenases from anaerobic bacteria contain iron and nickel. Core extrusion experiments with the CO dehydrogenase of *Methanosarcina barkeri* were interpreted to indicate the presence of six [4Fe-4S] clusters [22]. One of these clusters was detected by EPR spectroscopy and gave g values of 2.05, 1.94 and 1.90. The $E_{m9.2}$ was -390 mV. The CO dehydrogenase of *Methanosarcina barkeri* showed a second EPR signal with g values 1.91 and 1.76 and a g value around the g_e of 2.002, which was estimated to be 2.005. This cluster had an $E_{m9.2}$ of -35 mV, which according to the authors possibly played a non-redox catalytic role. The spin yield was about 1.1 $S = 1/2$ spin/$\alpha\beta$ dimer [22]. The CO dehydrogenase complex of *Methanosarcina thermophila* gave complex EPR spectra comprised of two paramagnetic species, only when the enzyme was reduced with CO [23]. Isotope substitution with [61]Ni, [57]Fe and [13]CO resulted in broadening of the EPR spectra of both the *Clostridium* and *Methanosarcina* enzyme, indicating an Ni-Fe-C spin-coupled complex [23, 24]. The EPR spectrum of both enzymes is also markedly changed by binding of acetyl-CoA [23, 24].

EPR spectroscopy of the enzyme from *Clostridium thermoaceticum* has detected a signal typical of a [4Fe-4S] cluster together with a novel signal with apparent g values of 1.86, 1.75 and a g value around the g_e of 2.002, which was estimated to be 2.01 [25]. Very recently electrochemical studies were performed with the clostridial enzyme [26]. Controlled potential coulometric reductive titrations under argon revealed four distinct EPR signals: one with $g_{av} = 1.82$ (2.01, 1.81 and 1.62) and an $E_m \approx -220$ mV (I); two signals with $g_{av} = 1.94$ (2.04, 1.94 and 1.90) and an $E_m \approx -440$ mV (IIa/b); and one signal with $g_{av} = 1.86$ (1.97, 1.87 and 1.75) and an $E_m \approx -530$ mV (III). All these $S = 1/2$ signals had low spin concentration, $0.2-0.3$ spin/$\alpha\beta$ dimer [26]. Features between $g = 6-4$ were also observed.

Mössbauer spectroscopy of the metal clusters of the CO dehydrogenase of *Clostridium thermoaceticum* demonstrated a variety of components at low potential (< -400 mV) [27]; 40% of the Fe belonged to a $[Fe_4S_4]^{1+}$ cluster, most probably EPR signals (IIa/b). EPR signal (I) yielded 18% of the Fe, while 9% of the Fe was present in a doublet with $\Delta E_Q = 2.90$ mm/s and $\delta = 0.7$ mm/s, typical of a ferrous FeS_4 complex. The Ni-Fe-C complex contributed up to 20% of the Mössbauer absorption. The complexity of the EPR and Mössbauer data forced the authors to conclude that the enzyme preparations were spectroscopically heterogeneous and therefore made assignment of the cluster types and composition premature.

Here we report on the paramagnetic centers of the aerobically and anaerobically purified CO dehydrogenase of *Methanothrix*. The EPR data of the *Methanothrix* enzyme are compared to those of the *Methanosarcina* and *Clostridium* CO dehydrogenase. Further, the acetyl-CoA exchange activity of the anaerobically purified CO dehydrogenase of *Methanothrix soehngenii* is demonstrated.

MATERIALS AND METHODS

Organism and cultivation

Methanothrix soehngenii (DSM 2139) was cultured on 80 mM sodium acetate as described previously [28]. Cells were harvested at the late-log phase by continuous centrifugation,

washed in 50 mM anaerobic Tris/HCl pH 7.6, 0.5 mM titanous [Ti(III)] citrate and frozen in liquid N_2. They were stored under N_2/H_2 (96:4) at $-20°C$.

Purification procedure

The aerobic purification procedure was carried out as described previously [14]. The anaerobic purifications were carried out under strict anaerobic conditions in an anaerobic chamber with N_2/H_2 (96:4) as gas phase, traces of oxygen were removed by a platinum catalyst. All buffers contained 0.5 mM titanous citrate or 0.5 mM sodium dithionite as reducing agent to prevent inactivation by oxygen. Cells were suspended (25%, mass/vol.) in 50 mM Tris/HCl pH 7.6. The cell suspension was disrupted by passing through a French pressure cell at 135 MPa. Cell debris was removed by centrifugation for 30 min at $35000 \times g$ and the supernatant was used as crude extract. The crude extract ($20-40$ ml) was applied to a Q-Sepharose (fast flow) column (2.2×8 cm) equilibrated with 50 mM Tris/HCl pH 7.6 (buffer A). After the column was washed with 60 ml buffer A, the CO dehydrogenase was eluted at 230 mM NaCl in a discontinuous gradient of 250 ml 1 M NaCl at 4 ml/min as described in [14]. The fractions with CO dehydrogenase activity were combined and concentrated to 5 ml in an Amicon Diaflo ultrafiltration cell equipped with a PM 30 filter. The concentrated enzyme preparation was diluted 1:1 with 2 M sodium chloride in buffer A. The protein solution was then applied to a Phenyl-Sepharose CL-4B column (2.2×6 cm) equilibrated with 2 M NaCl in buffer A. Impurities of methyl-CoM reductase did not bind to the column and were collected in the void volume. After the methyl-CoM reductase was washed off the column with 20 ml 2 M NaCl in buffer A, a 60-ml linear gradient of $2-0$ M NaCl in buffer A was applied at a flow rate of 1 ml/min. The CO dehydrogenase was washed off the column in a 60-ml linear gradient of 50% ethyleneglycol in buffer A at 10% ethyleneglycol. Fractions with CO dehydrogenase were combined and concentrated to 2 ml in a PM 30 centricon unit. The concentrated protein solution was applied to Superose 6 HR 10/30 column equilibrated with 10% ethyleneglycol in buffer A. The CO dehydrogenase was eluted after 14.5 ml at 0.5 ml/min. The active fractions of several runs were concentrated to 1 ml with a PM 30 centricon unit. The enzyme was either used immediately for activity determinations or frozen in liquid N_2 and stored at $-80°C$ under N_2 until use.

Activity determination

The CO:methylviologen oxidoreductase activity was determined by following the reduction of methylviologen as described previously [14]. The acetyl-coenzyme A/CO exchange activity was determined according to Raybuck et al. [17]. The assay mixture contained 0.78 ml 100 mM Tris/HCl + 1 mM dithiothreitol pH 7.5, 0.2 mM methylviologen, 5 nmol [1-^{14}C]acetyl-coenzyme A (specific activity 59 Ci/mol), 195 nmol acetyl-coenzyme A and 0.15 mg CO dehydrogenase. The assay was performed at $35°C$ in 8-ml serum vials and an appropriate gas phase. At regular intervals, 100-µl aliquots were taken, mixed with 50 µl 0.1 M H_2SO_4 and flushed to remove labeled gas. The extent of exchange was calculated from the difference in the amount of radioactivity remaining in the aqueous phase. Label in CO was determined after removal of CO_2 as described by Ragsdale and Wood [16].

Analytical determinations

The nickel content of the enzyme was analyzed with an atomic absorption spectrometer (ICP 5500, Perkin-Elmer) equipped with an HG-400A graphite furnace atomizer (Perkin-Elmer). The iron content was determined by atomic absorption spectrometry for the aerobically purified protein preparations and photometrically with bathophenanthroline disulfonate [29] for the anaerobically purified samples. Protein was routinely estimated according to Bradford [30]. For more accurate determinations, a modified procedure of the microbiuret method was used [31].

The molecular mass of the protein was calculated from the amino acid composition determined by sequence analysis of the CO dehydrogenase gene (unpublished results). The molecular mass of the α subunit is 89461 Da, of the β subunit 21008 Da.

Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance (EPR) spectra were obtained with a Bruker ER-200 D spectrometer. Sample cooling was with a home-built helium-flow cryostat. The temperature was calibrated with a dummy sample containing two 5-kΩ Allen-Bradley carbon resistors just below and above the 1.5-cm measuring area of the standard rectangular cavity. The spectrometer was interfaced to an Olivetti M24 PC with software written in Asyst for data acquisition, correction of background signals, double integration procedures and g value determinations. Spin quantification used 10 mM CuSO$_4$ in 10 mM HCl/2 M NaClO$_4$ as a standard. Anaerobic reduction and oxidation of the protein samples was carried out in EPR tubes connected to a scrubbed argon/vacuum manifold as previously described [32]. Redox potentiometry followed the method of Dutton and used 40 μM end concentration of each of the following redox mediators: methyl and benzyl viologen, neutral red, safranine-T, phenosafranine, 2-hydroxy-1,4-naphthoquinone and anthraquinone 2-sulfonate, indigo disulfononic acid, resorufin, methylene blue, phenazine ethosulfate and 2,6-dichloroindophenol [33]. Prior to titration titanous citrate was removed by ultrafiltration with 50 mM Tris/HCl pH 7.5, 20% ethyleneglycol in a PM 30 Centricon unit. The enzyme was diluted to 6.8 mg protein ml^{-1} and mixed in an 1:1 ratio with redox mediators. Reducing titrations used 5 mM dithionite in 0.5 M Tris/HCl pH 7.5 as titrant. Oxidizing titrations used 50 mM potassium ferricyanide in 0.5 M Tris/HCl pH 7.5 as titrant.

Chemicals

All chemicals were at least of analytical grade. Dithiothreitol and methylviologen were obtained from Janssen Chimica (Beerse, Belgium). Acetyl-coenzyme A was purchased from Sigma Chemical Co. (Amsterdam). [1-^{14}C]Acetyl-coenzyme A (59 Ci/mol) was obtained from Amersham (Houten, NL). Gases were purchased from Hoekloos (Schiedam, NL). Platinum catalyst was a gift of BASF (Arnhem, NL). Q-Sepharose (fast flow), Phenyl-Sepharose CL-4B and Superose 6 HR 10/30 were obtained from Pharmacia Fine Chemicals (Woerden, NL). Titanium(III) (titanous) citrate was prepared from TiCl$_3$ and sodium citrate as described in [34].

Table 1. *Anaerobic purification of CO dehydrogenase of* Methanothrix soehngenii

One unit (U) is the amount of enzyme catalyzing the oxidation of 1 μmol CO/min

Step	Volume	Protein	Activity	Specific activity	Purification	Yield
	ml	mg	U	U/mg	-fold	%
Crude extract	38	1024	5230	5.1	1	100
Q Sepharose	40	128	4736	37	7	90
Phenyl Sepharose	10	45	4184	92	18	80
Superose	1	28	3670	131	26	70

RESULTS AND DISCUSSION

Enzyme purification

The results of a typical anaerobic purification are summarized in Table 1. In three steps a 26-fold purified protein was obtained with 70% recovery. The final CO:methylviologen oxidoreductase activity was 130 μmol CO oxidized · mg protein^{-1} · min^{-1}. The purification, recovery and final activity were in the same range as in the aerobic procedure. The only modification in the anaerobic procedure was the introduction of a Phenyl-Sepharose step, which was very effective in the removal of contaminating methyl-CoM reductase.

Metal analysis

The metal content of the CO dehydrogenase was determined either by graphite furnace atomic absorption spectrometry for nickel and also for iron in the aerobic samples or photometrically with bathophenanthroline disulfonate for iron in the anaerobic samples. The aerobically purified protein contained 1.9 ± 0.1 ($n = 3$) mol Ni/mol $\alpha_2\beta_2$ and 19 ± 3 ($n = 3$) mol Fe/mol $\alpha_2\beta_2$. The metal content of the anaerobic enzyme preparations was 2.0 ± 0.1 ($n = 3$) mol Ni/mol $\alpha_2\beta_2$ and 18 ± 2 ($n = 3$) mol Fe/mol $\alpha_2\beta_2$, which is in good agreement with metal content of the aerobically purified samples.

EPR spectroscopy

EPR spectra of the aerobically purified CO dehydrogenase are presented in Fig. 1. The protein as isolated exhibited a sharp signal (cf. Fig. 1 A) with several characteristics that are typical for spectra from the [3Fe-4S]$^{1+}$ cluster. There was a small g anisotropy around the free electron value. The signal peaked at $g = 2.014$ and had a zero crossing at $g \approx 1.99$. Spin-lattice relaxation was relatively fast: the onset of saturation was above a microwave power of 8 mW at 12 K. The signal rapidly broadened above 12 K and became undetectable at 40 K. Double integration at low temperature yielded $0.03 - 0.04$ $S = 1/2$ spin/half-protein molecule (i.e. per $\alpha\beta$ unit). Thus the signal represented a minor component, possibly created by oxidative damage of another Fe/S structure. In the thionin-oxidized CO dehydrogenase of *Methanosarcina barkeri* a similar signal with $g = 2.016$ was observed, which was also not detectable above 20 K. Because the integration of the entire spectrum indicated a total of 1.1 spin/enzyme molecule, the signal represented a major oxidized component of that enzyme. The 3Fe spectrum of the *Methanothrix* enzyme was unaffected by anaerobic incubation of the enzyme with either CO (101 kPa) or acetyl-CoA (5 mM). Reduction with
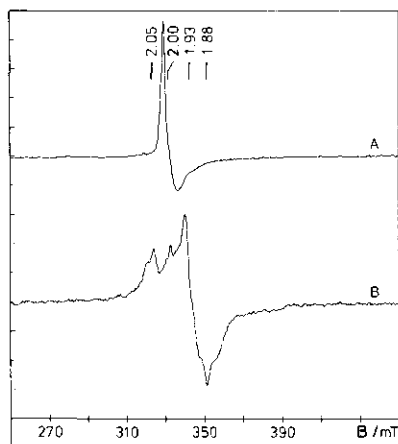
Fig. 1. *EPR spectrum of aerobically purified CO dehydrogenase*. EPR conditions: microwave frequency, 9.33 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; microwave power, 8 mW; temperature, 11.5 K. Trace A, enzyme as isolated (23.4 mg/ml, 0.22 mM $\alpha\beta$ dimer). Trace B, enzyme reduced by dithionite
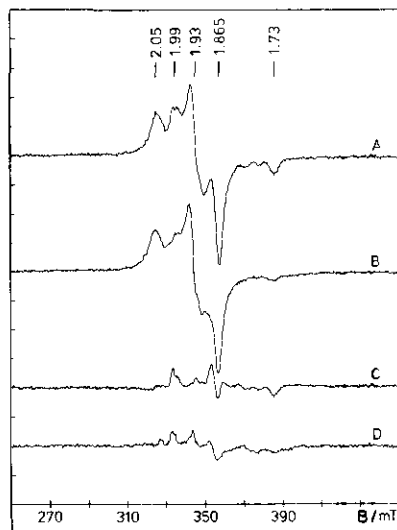

Fig. 2. *EPR spectrum of anaerobically purified CO dehydrogenase*. EPR conditions: microwave frequency, 9.33 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; microwave power, 8 mW; temperature, 13 K. Trace A, enzyme (9 mg/ml, 0.085 mM $\alpha\beta$ dimer) as prepared [after removal of 0.5 mM Ti(III) citrate and addition of 0.5 mM dithionite]. Trace B, enzyme under CO atmosphere. Trace C, difference spectrum of A minus B. Trace D, enzyme incubated with 5 mM acetyl-CoA

dithionite resulted in the spectrum given in Fig. 1 B. The following characteristics were observed: the spectrum exhibited multiple features, notably more than the three expected for a single, rhombic g tensor; there were broad low-intensity wings; the zero-crossing was at $g \approx 1.925$; broadening of the individual features set in at $T \approx 20$ K, the spectrum collapsed into a single, broad line at $T \approx 40$ K and broadened beyond detectability above $T \approx 50$ K. These properties taken together are reminiscent of the EPR from reduced bacterial eight-iron ferredoxins, i.e. two [4Fe-4S]$^{1+}$ cubanes whose individual $S = 1/2$ spins weakly interact by dipolar coupling, as they are separated by a distance through space of typically ≈ 1 nm. Quantification of two fully developed 4Fe/S spectra is expected to give a stoichiometry of two $S = 1/2$ systems/protein unit. The actually observed spin intensities were considerably lower than the expected value of 2.0, namely, $0.11-0.16$ spin/$\alpha\beta$ dimer, even though these aerobically isolated preparations contained more than enough iron to accommodate two cubanes/dimer.

When the isolation and purification were carried out under strict anaerobic conditions, the CO dehydrogenase as isolated was in the reduced state. The EPR spectrum (Fig. 2, trace A) was very different from that of the dithionite-reduced, aerobically isolated protein. Two major signals were apparent: one with g values of 2.05, 1.93, 1.865 and the other which is recognizable from the feature with apparent g value of 1.73. The spectrum (i.e. shape and intensity) was consistently that of Fig. 2, trace A, irrespective of whether the reductant was titanous citrate or sodium dithionite. The spectrum in Fig. 2 was taken after the removal of the Ti$^{3+/4+}$ solution because the titanous [Ti(III)] citrate is paramagnetic (d^1; $S = 1/2$) and its EPR spectrum ($g = 1.967$, 1.941, 1.874; not shown) seriously interferes with that of the enzyme.

Part of the spectrum was responsive to incubation with CO. Under 101 kPa of CO, a spectral component with $g \approx 1.99$, 1.89, 1.73 partly disappeared (cf. Fig. 2 B and C). The K_m for CO of the enzyme was previously established to

be 0.7 mM and an aqueous solution equilibrated with 101 kPa of CO contained about 0.8 mM CO [14]. This may explain why the CO-sensitive spectral component did not disappear quantitatively, i.e. its partial disappearance reflects the extent of binding of CO.

Krzycki et al. reported that the EPR spectrum of the *Methanosarcina* CO dehydrogenase broadened by incubation of the enzyme with CO. Another effect of the addition of CO to the reduced enzyme was the substantial shifts of the $g = 1.76$ to $g = 1.73$ and of the $g = 1.90$ feature to $g = 1.89$. However, the $g = 1.76$ feature did not disappear [22]. When the enzyme of *Clostridium thermoaceticum* was incubated under CO, a new intense signal appeared with g values of 2.07 and 2.02, which was assigned as an Fe-Ni-C center. However, the feature at $g = 1.75$ disappeared partially under a CO atmosphere [25].

In trace D of Fig. 2 it is shown that reacting the *Methanothrix* enzyme with acetyl-CoA (5 mM) diminished the CO-dependent spectral component to the same extent as with CO itself. In addition, however, the other spectral component, with main features at $g \approx 2.05$, 1.93, 1.865 disappeared completely. The g values of this component are usual for the [2Fe-2S]$^{1+}$ or [4Fe-4S]$^{1+}$ structure. As no signal was observed at liquid nitrogen temperatures, it is likely that we are dealing with the relatively faster relaxing [4Fe-4S]$^{1+}$ cubane. However, the spectrum differed considerably from that of the aerobically isolated enzyme in that no apparent broadenings and splittings from dipolar interaction were detected. In other words, the signal appears to come from magnetically isolated cubane(s).
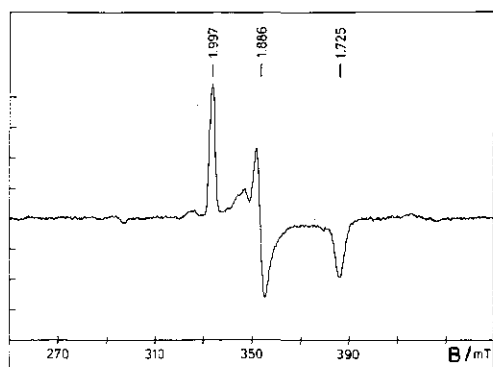
Fig. 3. *EPR spectrum of partially reduced CO dehydrogenase.* EPR conditions: microwave frequency, 9.33 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; microwave power, 8 mW; temperature, 13 K. Enzyme concentration 34 mg/ml (0.3 mM $\alpha\beta$ dimer)

Fig. 4. *Redox titration of carbon monoxide dehydrogenase.* The lines drawn through the points are Nernst $n = 1$ curves. Signal I (Fig. 2A) was monitored at $g = 2.05$ ($\times$) and signal II (Fig. 3) at $g = 1.73$ ($\bullet$). The intensities are given as percentage of the maximal intensity. The potential axis is defined versus the normal hydrogen electrode (NHE)

Double integration of the spectrum from Ti(III)-reduced enzyme (Fig. 2, trace A) gave 0.96 $S = 1/2$ spin/$\alpha\beta$ dimer. About 10% of this intensity was from the CO-sensitive component. Thus, we observe EPR from ≈ 0.9 cubane/$\alpha\beta$ dimer (2.0 expected) and $\approx 0.1/\alpha\beta$ dimer of a second as yet unassigned component.

The same major paramagnetic species were also observed in the reduced CO dehydrogenase from *Methanosarcina barkeri* and *Clostridium thermoaceticum* [22, 25]. Incubation of the clostridial enzyme with acetyl-CoA or HSCoA under N_2 made the enzyme EPR-silent. An effect of acetyl-CoA on the EPR spectrum of the CO dehydrogenase was only observed under a CO atmosphere. Under these circumstances, two signals were found: (S1) with $g = 2.074, 2.028$ and (S2) with $g = 2.062, 2.047, 2.028$, which only could be observed by EPR simulation. The EPR spectrum of the CO-reduced CO dehydrogenase of *Methanosarcina thermophila* was only influenced by acetyl-CoA under a N_2 atmosphere. In this case one signal was found in the spectrum with $g = 2.057, 2.049, 2.027$.

A particularly intriguing facet of the signal from the CO-sensitive component of the *Methanothrix* enzyme was that all three g values appeared to be less than the free electron value. This fact was more firmly established from the isolated spectrum of this component as given in Fig. 3. The spectrum is from enzyme 'as isolated' in the presence of 0.5 mM dithionite. Apparently, the nominally present amount of dithionite resulted in a redox potential not low enough to reduce the cubane center also. Incubation with 5 mM dithionite at ambient temperature for $2-3$ min resulted in a reduced spectrum as in Fig. 2A. Double integration of the spectrum in Fig. 3 again gave 0.1 spin/$\alpha\beta$ dimer.

The shape of the spectrum in Fig. 3 is very similar to that reported for CO dehydrogenase from *Methanosarcina barkeri* obtained after 'exchanged into' dithionite-free buffer and incubated for 48 h [22]. In the EPR spectrum of CO dehydrogenase of *Clostridium thermoaceticum* a similar feature appeared under an CO atmosphere with $g = 2.01, 1.86$ and 1.75 [25]. An essential difference, however, was observed in the g values: where as Krzycki et al. reported $g = 2.01, 1.91, 1.76$ (i.e. one g valu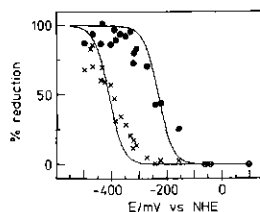e $> g_e$), we find $g = 1.997, 1.886, 1.725$ (i.e. all g-values $< g_e$). A feature was very recently described for the clostridial enzyme also with all g values $< g_e$ [26]. Similar signals were also reported for *Rhodospirillum rubrum* CO dehydrogenase after depletion of nickel or after cyanide treatment [35]. Isolated low-spin nickel (d^7 or d^9) has all g values $> g_e$ because the d shell is more than half filled. The EPR spectrum observed for an NiFe-containing cluster of unknown structure in CO dehydrogenase of *Clostridium thermoaceticum* and *Methanosarcina thermophila* is also characterized by all g values $> g_e$ [23, 24]. On the other hand, spectra with all g values $< g_e$ are very unusual for iron-sulfur clusters. We have recently reported the first example of such a spectrum from a 6Fe-containing protein and we tentatively assigned the signal to a $[6Fe\text{-}6S]^{5+}$ prismane cluster [32]. In this latter protein the intensity of the signal with all g values $< g_e$ was also 0.1 $S = 1/2$/molecule. Very recently we have found the balance of the spin intensity in the 6Fe protein in the form of an $S = 9/2$ system with characteristic lines at low magnetic field (unpublished results). We have therefore scrutinized the EPR of *Methanothrix* CO dehydrogenase taken under the conditions appropriate for the detection of high-spin systems (i.e. low magnetic field, high microwave power, $T = 4-60$ K). We have not detected any high-spin signals in CO dehydrogenase.

An EPR-redox titration was carried out on titanous citrate-free enzyme in the presence of a mixture of mediators. No signals other than the ones present in Fig. 2A were observed in the potential range $+400$ mV to -500 mV. The cubane was monitored at $g = 2.05$ and the second center at $g = 1.73$. These intensities are given in Fig. 4 as percentage of maximal intensity. Thus, the 100% of the $g = 2.05$ signal corresponds to 0.9 spin and 100% of the $g = 1.73$ signal is 0.1 spin. The cubane signal titrated with a midpoint potential $E_{m7.5} = -410$ mV. This number is similar to the $E_{m9.2} = -390$ mV reported for the corresponding signal in the *Methanosarcina* CO dehydrogenase. Since the E_0 of the CO/CO_2 couple is -520 mV, it is likely that this cluster accepts the electrons from the oxidation of CO. The corresponding signal of the clostridial enzyme S IIa/b $g_{av} = 1.94$ (2.04, 1.94, 1.90) had an E_m of ≈ -440 mV, which is also in the same range.

For the second species of the *Methanosarcina* enzyme, a midpoint potential $E_{m9.2} = -35$ mV is reported. For the *Methanothrix* enzyme, we find a significantly different value of $E_{m7.5} = -230$ mV. The EPR signal associated with the reduced site of this transition proved to be sensitive to incubation with CO (i.e. it partially disappeared). The redox center is subject to two redox transitions. The first reducing equivalent can be provided by Ti(III) or by dithionite and this

Table 2. *Acetyl-CoA/CO exchange activity of carbon monoxide dehydrogenase of* Methanothrix soehngenii
The assay mixture consisted of 0.78 ml 100 mM Tris/HCl + 1 mM dithiothreitol pH 7.5, 0.01 ml purified CO dehydrogenase (0.15 mg) and 0.02 ml acetyl-CoA (200 nmol, 10912 Bq) in 8-ml serum vials. MV = methyl viologen

Gas phase	Addition	Specific activity	$[1-^{14}C]$Acetyl-CoA : CO isotope exchanged	CO formed	CO_2 formed
		$nmol \cdot min^{-1} \cdot mg^{-1}$	nmol		
CO 100%	0.2 mM MV no CO-dehydrogenase	0	0.2	< 0.1	< 0.1
N_2 100%	none	1.3	4.3	2.8	< 0.1
CO_2 5%	none	3.7	10.1	8.3	< 0.1
CO_2 5%	0.2 mM MV	6.9	28.9	18.7	4.2
CO 5%	none	10.6	38.7	30.3	< 0.1
CO 5%	0.2 mM MV	23.7	56.4	44.2	0.4
CO 100%	0.2 mM MV	35.4	74.8	58.3	1.4

produces the intermediate, paramagnetic state, which is 10% $S = 1/2$ (with all g values $< g_e$). Additional low-potential reducing equivalent(s) can be conveyed by CO and this results in a state for which we have not yet found an EPR signal. Note that also in the putative biological [6Fe-6S] cluster, the $S = 1/2$ signal (with 10% spin intensity and with all g values $< g_e$) does exhibit two redox transitions with the paramagnetic $S = 1/2$ state being the intermediate state [32]. Krzycki et al. suggest that the cluster with $E_{m9.2} = -35$ mV plays a nonredox catalytic role, as has been reported for citrate binding to aconitase [22]. In the clostridial enzyme an even more negative potential is reported for the $g_{av} = 1.86$ (1.97, 1.87, 1.75) signal $E_m \approx -530$ mV; the redox properties of this complex can not be explained easily.

The midpoint potential of the Ni-Fe-C cluster of the clostridial enzyme was difficult to obtain, since the titration data reflect the redox state of the cluster during catalytic turn over. The E_m was estimated between -350 mV and -520 mV.

Acetyl-coenzyme A/CO exchange activity

The CO:methylviologen oxidoreductase activity of CO dehydrogenase from *Methanothrix* does not require strict anaerobic conditions for purification [14]. However, the acetyl-CoA/CO exchange activity, as assayed by the decarbonylation/recarbonylation of $[1-^{14}C]$acetyl-CoA in the presence of CO, was extremely sensitive to oxygen and was strongly inhibited by dithionite. An enzyme preparation anaerobically purified in the presence of 0.5 mM titanous citrate as reducing agent showed an acetyl-CoA/CO exchange activity of 35 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} protein, equivalent to 7 mol CO exchanged $\cdot$ min^{-1} $\cdot$ mol^{-1} enzyme), which was approximately 4000-fold lower than the conversion of CO to CO_2 with reduction of the artificial electron acceptor methylviologen. When 5% CO was replaced with 5% CO_2 as the gas phase in the exchange reaction, 28.9 nmol acetyl-CoA was exchanged as compared to 56.4 nmol with 5% CO as gas phase. When 100% N_2 was the gas phase, 2% of the radioactivity was lost from the C-1 during the reaction (Table 2). Methylviologen stimulated the exchange reaction between $[1-^{14}C]$acetyl-CoA and CO and between $[1-^{14}C]$acetyl-CoA and CO_2. These results indicate that CO and CO_2 undergo exchange with the C-1 of acetyl-CoA and that the role of the low-potential electron carriers was to stimulate an internal electron transfer that occurs during the cleavage and resynthesis of acetyl-CoA.

CONCLUDING REMARKS

The CO dehydrogenase of *Methanothrix soehngenii* is an example of a CO dehydrogenase that exhibits significant CO:methylviologen oxidoreductase activity **after** aerobic purification [14]. However, we have shown here that the acetyl-CoA/CO exchange activity for this enzyme is very sensitive to oxygen. We have also found considerable differences in EPR properties between aerobically and anaerobically isolated and purified enzyme. One important conclusion from these observations is that detection of a high CO:methylviologen oxidoreductase activity bears no direct relevance to the identification of this enzyme's full integrity.

Our reading of the combined EPR measurements on the aerobic and anaerobic preparations leads us to propose the following working hypothesis for the prosthetic groups in this CO dehydrogenase: the $\alpha\beta$ dimer unit contains one or two ferredoxin-type cubanes, which are expected to function in single-electron transfer. In addition, there is a third Fe/S cluster, whose O_2-sensitive structure is not yet established but which has some electronic and magnetic properties similar to those of the putative [6Fe-6S] prismane cluster [32]. Nonbiological [6Fe-6S] model clusters can be combined with an ion of a different metal without affecting the magnetic properties of the cluster [36]. Analogously, one could envisage the single nickel ion/$\alpha\beta$ dimer to be associated with a similar Fe/S, forming the CO-activating site.

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Chapter 8

EPR Characterization of a high spin system in
carbon monoxide dehydrogenase from *Methanothrix soehngenii*

Mike Jetten, Antonio Pierik and Wilfred Hagen (1991)

EPR Characterization of a high spin system in carbon monoxide dehydrogenase from *Methanothrix soehngenii*

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Enzymes: Carbon monoxide dehydrogenase (E.C. 1.2.99.2) ; Methyl-coenzyme M reductase (E.C. 1.8.99.-)

Carbon monoxide dehydrogenase and methyl-coenzyme M reductase were purified from ^{61}Ni-enriched and natural abundance Ni grown cells of the methanogenic archae *Methanothrix soehngenii*. The nickel EPR signal from cofactor F-430 in methyl-CoM reductase was of substoichiometric intensity and exhibited near-axial symmetry with g = 2.153, 2.221 and resolved porphinoid nitrogen superhyperfine splittings of $\approx$ 1 mT. In the spectrum from ^{61}Ni-enriched enzyme a well-resolved parallel I = 3/2 nickel hyperfine splitting was observed, $A_{||}$ = 4.4 mT. From a computer simulation of this spectrum the final enrichment in ^{61}Ni was estimated to be 69%, while the original enrichment of the nickel metal was 87%. CO dehydrogenase isolated from the same batch exhibited four different EPR spectra, however, in none of these signals any splitting or broadening from ^{61}Ni could be detected. Also, the characteristic "g = 2.08" EPR signal found in some other CO dehydrogenases, and ascribed to a Ni-Fe-C complex, was never observed by us under any condition of detection (4 to 100 K) and incubation in the presence of ferricyanide, dithionite, CO, coenzyme A, or acetyl-coenzyme A. Novel, high-spin EPR was found in the oxidized enzyme with effective g-values at g = 14.5, 9.6, 5.5, 4.6, 4.2, 3.8. The lines at g = 14.5 and 5.5 were tentatively ascribed to an S = 9/2 system ($\approx$ 0.3 spins per $\alpha\beta$) with rhombicity E/D = 0.047 and D < 0. The other signals were assigned to an S = 5/2 system (0.1 spins per $\alpha\beta$) with E/D = 0.27. Both sets of signals disappear upon reduction with $E_{m,7.5}$ = -280 mV. With a very similar reduction potential, $E_{m,7.5}$ = -261 mV, an S = 1/2 signal (0.1 spins per $\alpha\beta$) appears with the unusual g-tensor 2.005, 1.894, 1.733. Upon further lowering of the potential also the putative double cubane signal appears. At a potential E $\approx$ -320 mV the double cubane is only a few percent reduced and this allows for the detection of individual cubane EPR not subjected to dipolar interaction: a single spectral component is observed with g-tensor 2.048, 1.943, 1.894.

Carbon monoxide dehydrogenase (CDH) is the central enzyme in the Wood pathway for autotrophic acetyl-coenzyme A (Ac-SCoA) synthesis by homoacetogenic bacteria [1-3]. In these bacteria the enzyme catalyses several reactions, the most important being the reduction of CO_2 to CO and the condensation of a methyl group bound to CDH with CO and CoASH to Ac-SCoA [4-6]. CDH also plays an important role in the acetate degradation by methanogenic archae, where the enzyme catalyses the oxidation of CO to CO_2 and the cleavage of Ac-SCoA to a methyl-, carbonyl- and CoASH moiety [7-10]. Although the CDH activity has opposite functions in the metabolisms of these two microbial groups, on the molecular level the enzymes have several properties in common. CDH constitutes up to 2-5 % of the soluble protein, and this reflects its metabolic importance. The enzyme has a tetrameric structure $(\alpha\beta)_2$, but it can be part of a complex [4,7-10]. The size of the subunits is about 90 + 20 kDa in the methanogens and 78 + 72 kDa in the homo-acetogenic bacteria [4,7-10]. The genes for the subunits are clustered in an operon-like structure and contain several conserved cysteine residues [11,12]. In addition all the CDH's contain several Fe and acid-labile sulfur and 2-4 Ni per enzyme. These atoms are arranged into several complexes, which can be studied by electron paramagnetic resonance (EPR) spectroscopy.

In all these CDH's at least one, sometimes more $[4Fe-4S]^{2+/1+}$ cubane structures, with typical g= 2.05, 1.93 and 1.87 values, have
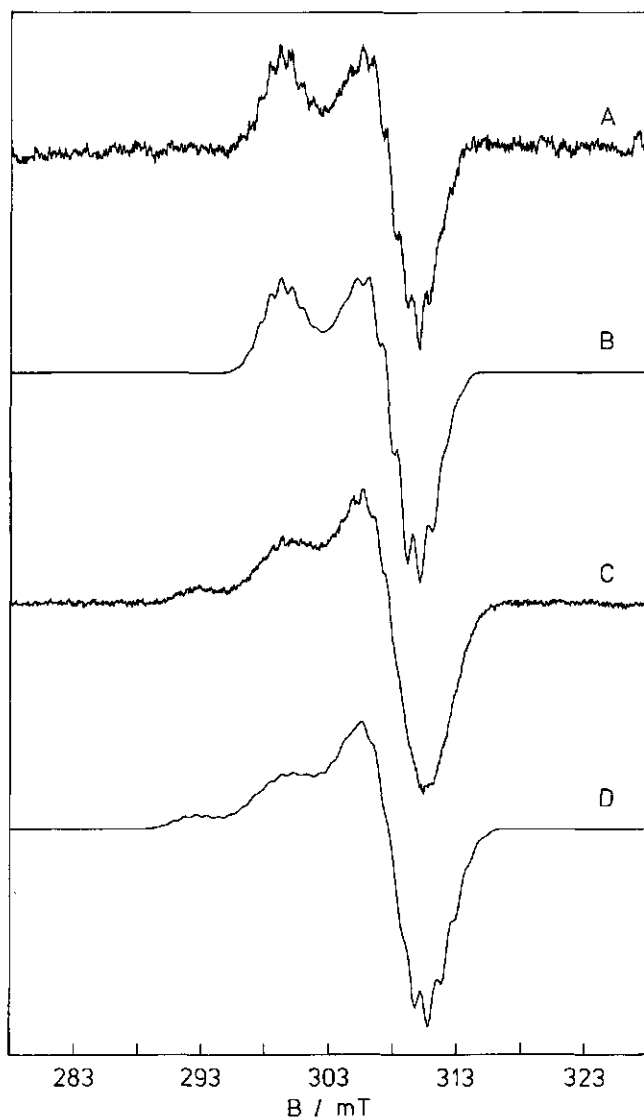
Fig.1. Nickel EPR spectra of factor F-430 in anaerobically purified *M. soehngenii* methyl-coenzyme M reductase.

Trace A, enzyme (90 mg/ml) as isolated, with natural abundance nickel; trace B, computer simulation on the basis of 160 orientations in axial symmetry with parameters: $g_{||} = 2.2214$, $g_{\perp} = 2.1525$, linewidth $W_{||} = 5.5$ mT, $W_{\perp} = 8$ mT, nitrogen hyperfine splitting $A_{||} = 0.87$ mT, $A_{\perp} = 1.05$ mT.; Trace C; ^{61}Ni-enriched enzyme (70 mg/ml); Trace D, simulation with the same parameters as in trace B, but now including an enrichment of 69% in ^{61}Ni with $I = 3/2$ and $A_{||} = 4.4$ mT, $A_{\perp} = 1.2$ mT. Experimental EPR conditions; microwave frequency, 9.30 GHz; modulation frequency, 100 KHz; modulation amplitude, 0.4 mT; microwave power, 2 mW; temperature, 68 K.

been detected [13-16]. The E_m of this cluster varied between -440 and -390 mV over the different species. It is likely that the cluster functions as an e-acceptor in the oxidation of CO or as an e-donor in the reduction of CO_2 [13-16]. In the CDH of *Clostridium thermoaceticum* (*C. thermoaceticum*) and *Methanosarcina thermophila* (*M. thermophila*) a very characteristic EPR spectrum is observed with g= 2.08, 2.05 and 2.03 values, when the enzyme is incubated with CO [14,17]. Isotope substitution with ⁶¹Ni, ⁵⁷Fe or ¹³CO each resulted in broadening of the signal from CDH of both microorganisms, indicating an Ni-Fe-C spin coupled complex [17,18]. A $Ni_1Fe_{3-4}S_{\geq 4}C_1$ stoichiometry has been proposed for the complex in the enzyme of *C. thermoaceticum* on the basis of Q-band Electron Nuclear Double Resonance (ENDOR) spectroscopy [19].

This characteristic spin coupled EPR signal has not been observed in spectra of the CO reduced enzyme from *Methanosarcina barkeri* (*M. barkeri*) and *Methanothrix soehngenii* (*M. soehngenii*) [13,16]. The EPR signal, which most notably changed (mainly reduced in intensity) upon incubation with CO of the enzyme of these two archae was a feature with $g_{ave} \approx 1.87$ (2.00, 1.89 and 1.73) [13,16]. Interestingly, a similar signal with $g_{ave} \approx 1.86$ (1.97, 1.87 and 1.75) was also observed in the reduced enzyme of *C. thermoaceticum*, in addition to the Ni-Fe-C spin-coupled complex [15]. However, the E_m and the spin intensity of these signals varied considerably in the 3 species: -35 mV (pH 9.2), 1.1 spins / $(\alpha\beta)_2$ for *M. barkeri*, -230 mV (pH 7.5) 0.1 spins/$(\alpha\beta)$ for *M.soehngenii* and 530 mV (pH 7.2), 0.3 spins /$(\alpha\beta)$ for *C. thermoaceticum* [13,15,16]. The biological function of the cluster that gives rise to this signal is not clear. It has been suggested that it could play a non-redox, catalytic role in substrate binding [13].

Spectra with all *g*-values < g_e are very unusual for iron-sulfur clusters. Recently, an example of such a spectrum was reported and tentatively assigned to a [6Fe-6S]⁵⁺ prismane cluster [20]. Also in this case the spin intensity was substoichiometric. Very recently the balance of the spin intensity was found in the form of an S = 9/2 system with characteristic lines at

low magnetic field [21].

In order to determine whether this "low-spin/very high-spin" description would also apply to the unusual CDH cluster, concentrated samples of CDH from *M. soehngenii* were prepared and analyzed by EPR spectroscopy under the appropriate conditions for the detection of very high spin systems [21]. In this paper we report on the detection of such a high spin system in the CDH of *M. soehngenii* and we describe the redox behaviour of this spin system together with the $g_{ave} \approx 1.87$ signal in oxidizing and reducing titrations. The characteristics of this high spin system are compared to other recently described systems. In addition, we report on the complete absence of characteristic EPR signals from Ni-Fe-C spin complex in ⁶¹Ni enriched, CO reduced CDH preparations.

MATERIALS AND METHODS

Organism and cultivation

Methanothrix soehngenii was the Opfikon strain isolated by Huser *et al.* [22]. It was cultured on 80 mM sodium acetate as described previously [22]. For growth on acetate in the presence of ⁶¹Ni, trace element solutions were prepared without $NiCl_2.6H_2O$. Nickel oxide (87% enriched in ⁶¹Ni) was dissolved in concentrated HCl and added to a final concentration of 2 μM Ni. Cells were harvested anaerobically under a stream of N_2 at the late log phase by continuous centrifugation (Heraeus Sepatech, Osterode, FRG), washed in 50 mM anaerobic Tris-HCl pH 7.6, 0.5 mM Ti(III)citrate and frozen in liquid N_2. The cells were stored under N_2/H_2 (96:4) at -20 °C.

Purification procedure

The purifications were carried out under strict anaerobic conditions in a glove box with N_2/H_2 (96:4) as the gas phase. Traces of oxygen were removed by a palladium catalyst. All buffers contained 0.5 mM Ti(III)citrate as the reducing agent to prevent any damage by oxygen. Cells were suspended (25% w/v) in 50 mM Tris-HCl, pH 7.6. The cell suspension was disrupted by passing it twice through a
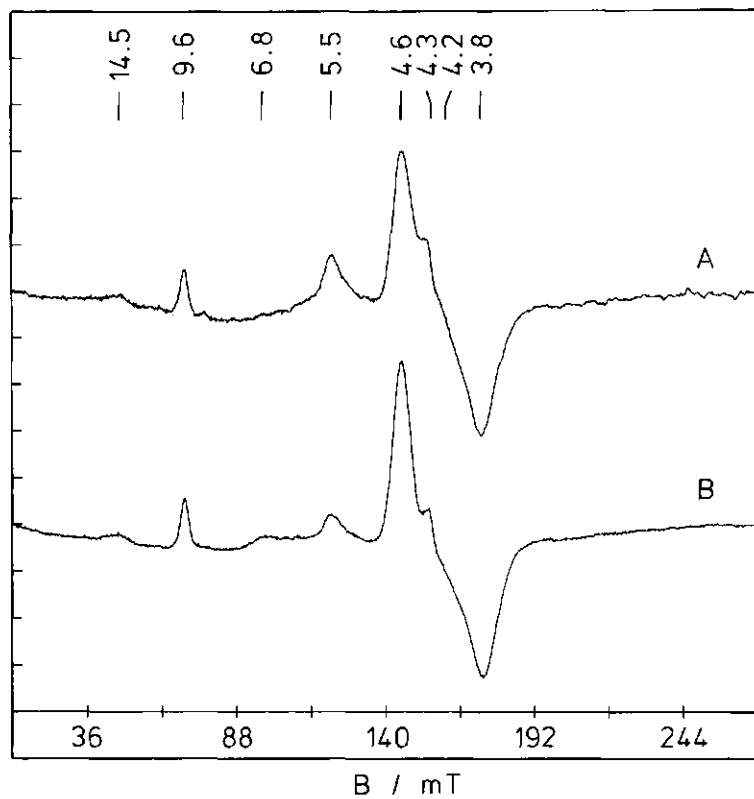
Fig. 2. High-spin EPR signals in ^{61}Ni-enriched and natural CO dehydrogenase.

Trace A, ^{61}Ni-enriched enzyme (10 mg/ml); trace B, natural abundance enzyme (31 mg/ml). The two traces have been normalized with respect to protein concentration. EPR conditions; microwave frequency, 9.30 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.6 mT; microwave power, 200 mW; temperature, 4.2 K.

French pressure cell at 135 MPa. Cell debris was removed by centrifugation for 90 min at 120,000 x g and the supernatant was used as crude extract. The CDH was purified 25-fold to apparent homogeneity using anion exchange chromatography, hydrophobic interaction and gel filtration as described previously [16]. Before the enzyme was frozen in liquid nitrogen, the Ti(III)citrate was removed by dialysis in an PM30 centricon unit (Grace, Rotterdam, NL) with 50 mM Tris-HCl pH 7.5 in 5% ethylene glycol. The CO:methylviologen oxidoreductase activity was determined from the rate of methylviologen reduction as described previously [10].

From the crude extracts also the methyl-coenzyme M reductase was purified as previously described [23], and these preparations were used to determine the ^{61}Ni enrichment in the CDH enzyme preparations by a novel method described below in the RESULTS section.

Analytical determinations

The nickel content of the enzyme was analyzed with an atomic absorption spectrometer (ICP 5500, Perkin-Elmer) equipped with a HG-400A graphite furnace atomizer (Perkin-Elmer). The iron content was determined photometrically with ferene [24]. Acid-labile sulfur was determined as described in [25]. Protein was routinely estimated according to Bradford [26]. For more accurate determinations a modified procedure of the microbiuret method was used [27].

The molecular mass of the protein was calculated from the amino acid composition determined by sequence analysis of the CO dehydrogenase gene [11]. The molecular mass of the α subunit is 89,461 Da, of the ß subunit 21,008 Da.

Electron paramagnetic resonance spectroscopy

X-band EPR data were taken on a Bruker ER-200 D spectrometer with peripheral equipment and data acquisition as in [21]. Redox potentiometry was done according to [28] at 22 °C in an anaerobic cell under purified argon with the bulk potential of the stirred solution measured at a Radiometer P-

1312 micro-platinum electrode with respect to the potential of a Radiometer K-401 saturated calomel electrode. Reported potentials were recalculated with respect to the normal hydrogen electrode (NHE). The following redox mediators were used at 40 μM end concentration : methyl and benzylviologen, neutral red, phenosafranin, safranin O, anthraquinone disulfonate, 2-hydroxy-1,4-naphtoquinone, indigo disulfonic acid, resorufin, methylene blue, phenazine ethosulfate and 2,6-dichlorophenol indo-*o*-chlorophenol and N,N,N',N'tetramethyl-p-phenylenediamine. Samples were drawn and transferred to EPR tubes under a slight overpressure of nitrogen, and directly frozen in liquid nitrogen. Oxidizing titrations were done with 5 mM potassium ferricyanide as the titrant in 0.5 M Tris-HCl, pH 7.5. Reducing titrations used 5 mM sodium dithionite in 0.5 M Tris-HCl, pH 7.5.

Chemicals

All chemicals were at least of analytical grade and purchased from Janssen Chimica (Beerse, Belgium) or Merck (Darmstadt, FRG). Acetyl-Coenzyme A and coenzyme A were from Boehringer Mannheim (Almere, the Netherlands). ^{61}NiO was obtained from the USSR via Intersales (Hengelo, the Netherlands). Gases were purchased from Hoekloos (Schiedam, the Netherlands). Palladium catalyst was a gift of BASF (Arnhem, the Netherlands). Titanium(III)-citrate was prepared from $TiCl_3$ and sodium citrate as described in [29].

RESULTS

Elemental analyses and isotope enrichment determination

The Ni and Fe content of the CDH were previously established to be 2.0 $\pm$ 0.1 mol Ni/$(\alpha\beta)_2$ and 9 $\pm$ 2 mol Fe/$(\alpha\beta)$ [16]. The samples used in this study also contained 2.0 $\pm$ 0.1 (n=4) mol Ni/$(\alpha\beta)_2$; the iron content determined with the more sensitive ferene method was 12.5 $\pm$ 2.2 (n=8) mol Fe/$(\alpha\beta)$. In addition, also the acid-labile sulfur content was determined colorimetrically to be 11.8 $\pm$ 1.7 (n=4) mol $S^{2-}/(\alpha\beta)$.

Table 1. EPR detectable components in M. soehngenii CO dehydrogenase.

The effective g-values in parentheses are calculated values assuming a real g-value of 2.00 and a rhombicity of E/D = 0.27 (for S = 5/2) or E/D = 0.047 for S = 9/2). The spin intensity is expressed as stoichiometry with respect to the $\alpha\beta$-dimer. Abbreviations: ox, oxidized; red, reduced; nd, not detected.

system spin	doublet $\|\pm m_s>$	g_z	g_y	g_x	spin intensity	$E_{m7.5}$ (mV)	EPR-form
S = 5/2	$\|\pm 3/2>$	4.60 (4.64)	3.85 (3.90)	4.20 (4.17)	≈0.1	-280	ox
	$\|\pm 1/2>$	nd (0.84)	9.6 (9.49)	nd (1.28)			
S = 9/2	$\|\pm 3/2>$	5.5 (5.49)	nd (4.77)	nd (4.83)	≈0.3	-280	ox
	$\|\pm 1/2>$	nd (1.54)	14.5 (14.5)	nd (4.86)			
S = 1/2		2.005	1.894	1.733	0.1	-261	red
S = 1/2		2.048	1.943	1.894	0.9	-410	red

The study of hyperfine interactions in EPR spectra from isotopically enriched preparations can be a useful approach to cluster classification. A meaningful analysis, however, requires some way of determining the final enrichment at the time of cell harvesting. This is especially important for trace elements that are commonly present as contaminants of mineral salts in the growth medium and/or in parts of the reactor, e.g., nickel.

The percentage of ^{61}Ni enrichment in the enzyme preparations was determined by computer simulation of EPR spectra from methyl-coenzyme M reductase, purified from the same extracts as CDH. The methyl-CoM reductase contains per $(\alpha\beta\gamma)_2$ two tightly bound cofactors F-430, which carry one Ni each [30]. The spectrum plus simulation (assuming axial symmetry) of the Ni-related signal form *M. soehngenii* Methyl-CoM reductase, purified from ^{nat}Ni-grown cells, are presented in Fig 1, traces A and B. The spectrum is virtually identical to that published by Albracht *et al.* for the enzyme from *Methanobacterium thermoautothrophicum* [31,32]. As with the previous work on *M. thermoautothrophicum*, also here the activity of the purified enzyme is low, and the integrated intensity of this S = 1/2 signal is substoichiometric. Therefore, the relevance of this EPR signal to the study of the enzyme Methyl-CoM reductase is questionable. The signal is, however, a good means to determine ^{61}Ni enrichment in preparations from methanogens.

Trace C of Fig. 1 is the spectrum form *M. soehngenii* Methyl-CoM reductase purified from ^{61}Ni cells. The sum-simulation, trace D, was obtained on the basis of identical parameters as used for trace B, except for the inclusion of an axial ^{61}Ni hyperfine tensor ($A_{||}$ = 4.4 mT; $A_\perp \approx$ 1.2 mT) and a ^{61}Ni/^{nat}Ni ratio. From this latter fitting parameter the percentage enrichment in ^{61}Ni was found to be 69%. This final benchmark is a very reasonable one in view of the original 87% enrichment in ^{61}Ni of the nickel metal that was dissolved and added to the growth medium.

High spin EPR signals in oxidized CDH

We have previously reported on two S = 1/2 signals in (partially) reduced CDH [16]. We have now found a set of high-spin signals at low temperature in concentrated samples of anaerobically purified CDH, which were rigorously freed from Ti(III)citrate. Overview spectra at T = 4.2 K are presented in Fig 2 for both ^{61}Ni-enriched and natural abundance enzyme. Several high-spin components are observed.

The most prominent feature is a set of lines at g = 3.8 to 4.6. A very similar spectrum has thus far once been reported - and not further commented on - in the single subunit CDH from the photosynthetic *Rhodospirillum rubrum* [33]. The spectrum is typical that from the middle doublet, $|\pm 3/2>$, of an S = 5/2 multiplet of intermediate to high rhombicity. The low-field peak from the $|\pm 1/2>$ doublet spectrum is also observed at g $\approx$ 9.6. The $|\pm 3/2>$ spectrum is slightly disturbed by the weak, sharp g = 4.3 line from a trace of adventitious iron. The effective g-values of the S = 5/2 spectrum are readily fit, under the "weak-field assumption" [21], to the usual spin Hamiltonian (1) with g = 2.00 and E/D = 0.27 (see Table 1).

$$H = g \beta B \cdot S + D[S_z^2 - S(S+1)/3] + E(S_x^2 - S_y^2) \quad [1]$$

A peak at g-effective $\approx$ 6.8 is visible in one of the traces of Fig. 2 but not in the other. We found this line to vary from preparation to preparation. All the other lines visible in Fig. 2 were, within experimental error, found proportional to CDH concentration. We have not assigned the g $\approx$ 6.8 line.

There is a weak, but distinct peak at the effective g = 14.5. Under the assumption that the spin system is half-integer, an effective g-value > 14 indicates S = 9/2 [21]. The above spin Hamiltonian would fit with an effective g = 14.5 and S = 9/2 for a rhombicity of E/D = 0.047. Under this model the g = 14.5 line stems from the $|\pm 1/2>$ doublet. The rhombicity fit properly predicts a line at g $\approx$ 5.5 from the $|\pm 3/2>$ doublet as the most prominent feature (see Table 1), other potentially observable lines being overshadowed by the S = 5/2 spectrum.
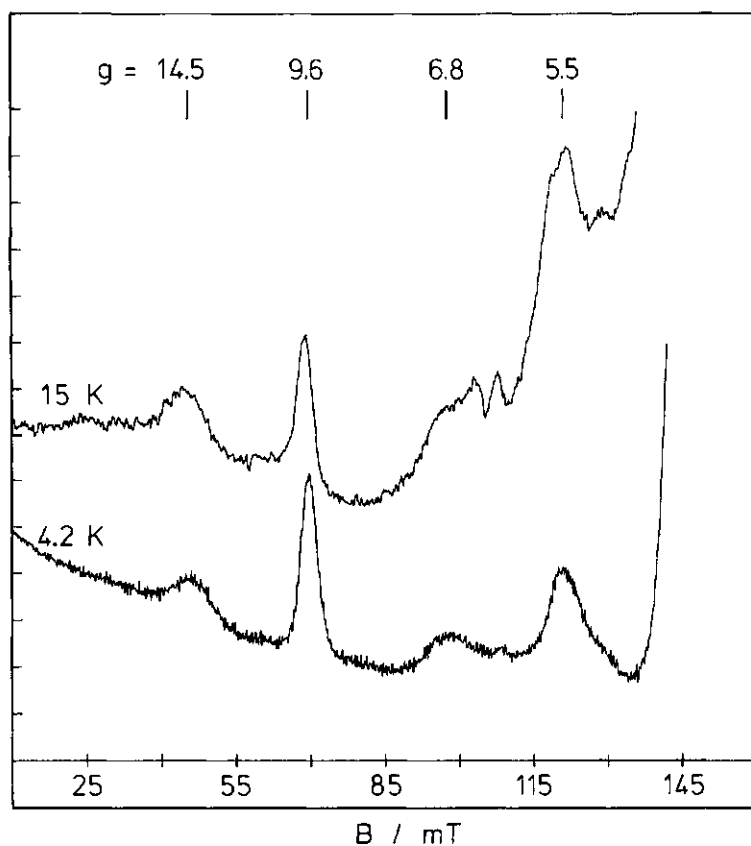
Fig. 3. **Temperature dependence of S = 5/2 and S = 9/2 signals in** *M. soehngenii* **CO dehydrogenase.**

Spectra of CO dehydrogenase (31 mg/ml) were taken at temperatures of 4.2 and 15 K, conditions otherwise being as in Fig. 2. The intensity of the T = 15 K spectrum has been multiplied by a factor of (15/4.2) with respect to the T = 4.2 K spectrum.

The putative $S = 5/2$ and $S = 9/2$ lines in the spectra of concentrated CDH can be seen in Fig. 2 to have line widths independent of whether or not the protein is 69% enriched in [61]Ni. This means that either nickel is not part of the structure(s) giving rise to these signals, or the 61-nickel hyperfine splitting is smaller than the line width.

Fig. 3 illustrates the Boltzmann redistribution effect of raising the temperature to 15 K on the low-field lines. The amplitude of the two traces has been corrected for the $1/T$ Curie-law dependence. Under this correction the spectrum from an isolated ground state should exhibit constant intensity. Increasing intensity with temperature is observed for low-lying excited states, decreasing intensity is observed for ground states within a closely spaced spin multiplet.

The $g = 9.6$ line is seen to slightly decrease in intensity, which identifies it (consistent with the g-value analysis of Table 1) as a ground state resonance in a multiplet with zero-field splittings of a few wavenumbers. The decrease is consistent with $D \approx + 2$ cm^{-1}.

The intensity of the lines at $g = 14.5$ and at $g = 5.5$ slightly increase with increasing temperature. Under the model of $S = 9/2$ this points to a small, negative D-value, with the $|\pm 1/2>$ doublet being the highest in energy. From the increase with temperature of the $g = 14.5$ line from this doublet we can estimate $D \approx -0.4$ cm^{-1}.

We can now determine the stoichiometry of the two high-spin systems by integration of the isolated peaks at $g = 14.5$ and at $g = 9.6$, using the estimated zero-field parameters to correct for the fractional population of the relevant doublets. The $S = 5/2$ system represents ≈ 0.1 spin per $\alpha\beta$ dimer of CDH as it is isolated. The $S = 9/2$ spectrum corresponds to ≈ 0.3 spins per $\alpha\beta$ dimer.

Redox titrations

An EPR redox titration was carried out on Ti(III)citrate free enzyme in the presence of a mixture of mediators. The $S \approx 5/2$ system was monitored at $g = 4.6$ and $g = 9.6$, the $S = 9/2$ was detected at $g = 5.5$. In the same series of experiments we also monitored the S $= 1/2$ system with $g_{ave} \approx 1.87$ by the

amplitude of its low-field peak at $g = 1.73$. For this latter component we have previously determined a reduction potential $E_{m,7.5} = -230$ mV [16].

The intensities in Fig. 4 are presented as fractions of the maximal signal amplitude. The disappearance of the $S = 5/2$ signals upon reduction indicated an $n = 1$ acceptor with $E_{m,7.5} = -280$ mV. The $S = 9/2$ signal at $g = 5.6$ also titrated with an $E_{m,7.5}$ of -280 mV, however, the apparent n value was ≈ 0.6. The $S = 1/2$ signal at $g = 1.73$ appeared upon reduction with an $n \approx 0.5$ and with a midpoint potential $E_{m,7.5}$ of -261 mV. This latter value is some 30 mV more negative than our previously reported number [16].

S = 1/2 EPR signals from [61]Ni-enriched CDH

In the course of the redox titration spectrally rather well isolated signals from the $S = 1/2$ component with $g_{ave} \approx 1.87$ were observed at low potentials. An example is the spectrum in Fig. 5, trace B from a [61]Ni-enriched sample poised at $E = -280$ mV. By comparison of this spectrum with that of [nat]Ni samples (cf. Fig. 3 in ref. [16]) no line-broadening contribution whatsoever from nickel to this signal was indicated.

Due to a slight error of calibration in our previous work [16] we must upwardly correct the three g-values of this signal with an amount of ≈ 0.008 to 2.005, 1.894, 1.733. It is possible that this small correction is of theoretical importance, because it makes one of the g-values just greater than the free electron value.

The spectrum in trace A of Fig. 5 was obtained from a sample poised at a potential some 40 mV more negative than that of trace B. Extra peaks are observed signalling the onset of reduction of the "regular" (i.e. according to the sequence data [11]) cubanes. We have previously assigned the bulk of the EPR intensity in the dithionite and/or CO reduced enzyme to two cubanes separated by the typical distance of 10-15 Å as to make them subject to weak dipolar electron spin interaction [16]. When the two individual clusters in such a double cubane have approximately the same reduction potential, and when they do not experience significant

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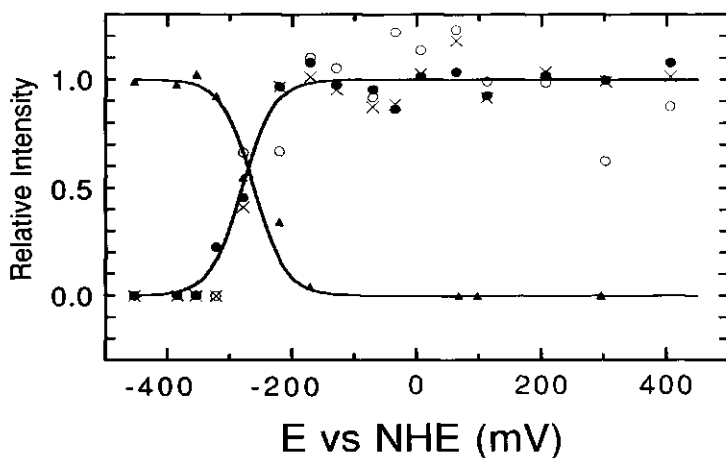
Fig. 4. Redox titration of M. soehngenii CO dehydrogenase.

The S = 5/2 system was monitored at g = 9.6 (●) g = 4.6 (o), the S = 9/2 system at g = 5.5 (x) and the S = 1/2 system (with $g_{ave} \approx 1.87$) at g = 1.733 (▲). The intensities are given as fraction of the maximal intensity. The potential axis is defined versus the Normal Hydrogen Electrode. The solid lines corresponds to the Nernstian n = 1 curves.

mutual redox interaction than in the early stages of the reduction, the situation with a single cluster reduced per protein unit ($\alpha\beta$ dimer) is statistically favoured over the situation with two reduced and dipolarly interacting, clusters. This situation is illustrated in Fig. 5 in which the spectrum of the individual cubane in trace C as the difference between traces A and B is given. Indeed, the spectrum is that of a regular ferredoxin-like [4Fe-4S]$^{1+}$ cluster with g-values 2.048, 1.943, 1.894.

In a series of attempts to detect a Ni-Fe-C center in the CDH from *M. soehngenii* both natural and ^{61}Ni-enriched preparations were incubated with CO in the presence or absence of CoASH or Ac-ScoA. Under no circumstances a Ni-Fe-C like signal was observed as reported for the CDH of *C. thermoaceticum* or *M. thermophila* [15,17]. CoASH additions had no significant effect on any spectrum, while incubation with Ac-SCoA perturbed the spectrum of the reduced enzyme completely as previously reported [16]. In none of these spectra any broadening from ^{61}Ni was detectable.

DISCUSSION

In the CO dehydrogenase from anaerobic microorganisms at least one putative [4Fe-4S] cubane structure has been detected by EPR spectroscopy [13,14-17,33]. It is likely that this cluster with an E_m around -400 mV functions as an e-acceptor in the oxidation of CO [7-10]. In addition to this cubane signal, a very unusual EPR signal has been observed in the spectra of CDH from several anaerobic microorganisms [13,15,16,32]. This latter signal represents substoichiometric amounts; it has all g-values $\leq$ the free electron value [15,16]. Recently, another example of an Fe/S spectrum with all g-values $< g_e$ was reported and tentatively assigned to an [6Fe-6S] prismane cluster [20]. The balance of the spin intensity was found in the form of a S=9/2 system [21]. Because of this striking similarity, new studies have been initiated to determine whether this "low spin / very-high spin" description would also apply to the unusual cluster of the CDH from *M. soehngenii*. Under

the appropriate conditions for the detection of high spin signals with low intensity (i.e. high microwave power and low temperatures), several high spin components were found in the enzyme from *M. soehngenii*.

The most prominent high spin feature was a spectrum typically from the middle doublet of an S = 5/2 multiplet. This signal with lines at g = 3.8 to 4.6 has also been reported for the CDH of *R. rubrum* [33]. In that work the signal was only observed in methylviologen oxidized holo-CDH, but not in the oxidized apo-enzyme, which lacks Ni. Although the authors carried out ^{61}Ni isotope substitution experiments, they did not report that the g = 4 signal was broadened by hyperfine splitting, nor did they mention the spin intensity of this feature [33]. In our 69% ^{61}Ni- enriched CDH preparations of *M. soehngenii* no broadening of this S = 5/2 feature was observed, which argues against assignment of this signal to a Ni containing cluster. Another, less pronounced, high spin feature was observed at g = 14.5 and g = 5.5. We assign this set of signals to a putative S = 9/2 system on the basis of the following arguments: i) one of the effective g-values is > 14, which excludes half-integer spins up to S = 7/2; ii) the second observed g-value is consistent with the rhombicity, E/D, determined from the first one; iii) all other lines of the subspectra within the five Kramers doublets of the S = 9/2 system are likely to be more difficult to detect because of their low transition probability (cf. [21]) and/or their being overshadowed by the S = 5/2 spectrum; iv) the spectrum titrating in concert with the S = 5/2 spectrum indicates the spin to be half integer; v) we fail to envisage any other reasonable explanation. If the above assignment is correct, then we have found the third (cf. [21]) S = 9/2 system in a metalloprotein. All these "superspin" proteins share other properties as well: a) they are catalysts of reactions involving multiple electron transfer; b) they are Fe/S proteins with unusually high Fe and acid-labile S content. These ions are possibly arranged in "super-clusters", i.e. clusters larger than the common [4Fe-4S] cubane [20, 21].

We set out to determine whether the unusual S = 1/2 signal is part of a low spin/high spin
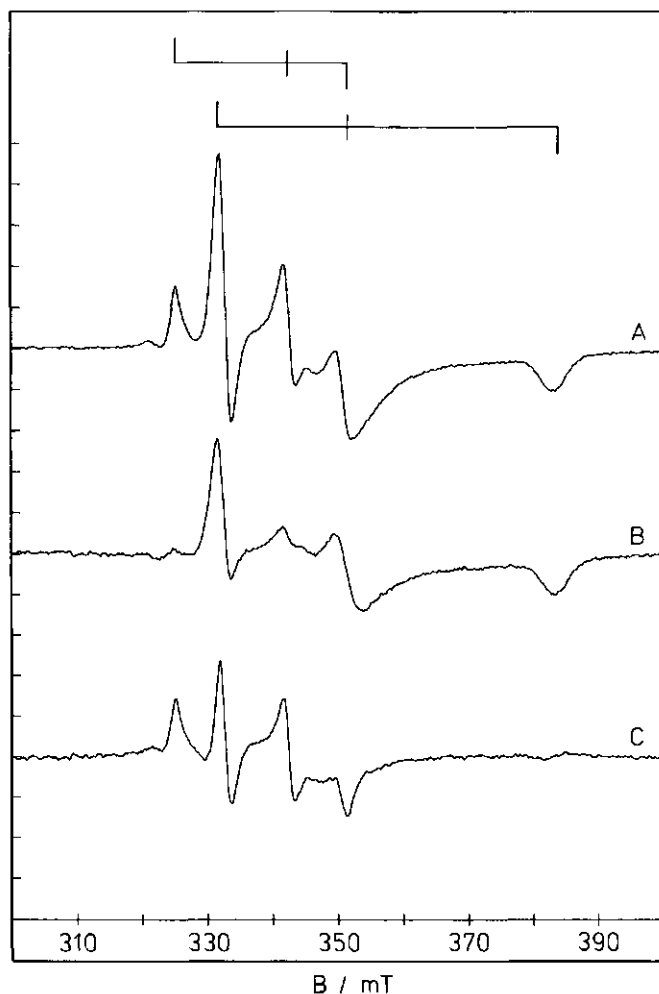
Fig. 5. S = 1/2 EPR signals in *M. soehngenii* CO dehydrogenase poised at intermediate redox potentials.

The enzyme was 14 mg/ml (0.15 Mm αß-dimer) in 50 mM Tris-HCl buffer, pH 7.5 in the presence of 40 μM each of the redox mediators specified in the experimental section. Trace A, potential poised at E = -322 mV ; Trace B, E = -280 mV; Trace C, difference spectrum of A-B. The bar symbols on top of the figure indicate the g-values of magnetically-isolated [4Fe-4S]$^{1+}$ cubane (2.048, 1.943, 1.894), and of the $g_{ave} \approx 1.87$, S = 1/2 system (2.005, 1.894, 1.733), respectively. EPR conditions; microwave frequency, 9.32 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; microwave power, 20 mW; temperature, 17 K.

system. To this goal the two high spin systems were monitored in EPR redox titrations together with the $S=1/2$ system. The high spin signals disappeared upon reduction with an $E_{m,7.5}$ of -280 mV. The low spin system appeared upon reduction with an $E_{m,7.5}$ of -261 mV. This near crossover of the low and high spin system(s) could reflect a redox transition within a single paramagnetic center. However, as all spin systems involved are half-integer, the redox transition would have to involve two electrons. The observed apparent values of $n \leq 1$ argue against this interpretation. Alternatively, the $S = 1/2$, $g_{ave} \approx 1.87$ system and the high-spin system ($S = 5/2$ and $9/2$) may belong to two different redox centers, which happen to have similar reduction potentials, and possibly even exhibit mutual redox interaction. A third explanation would be that the low-spin / high-spin crossover does not represents a redox transition *per se*, but rather a conformational change induced by a redox event somewhere else in the protein. One of the more pressing problems hampering a decision on these models, is the substoichiometric spin intensity of the different EPR signals, which we have found here and in our previous work on *M. soehngenii* CO dehydrogenase [16] and others have found for other CO dehydrogenases [5,13,15].

An characteristic EPR signal at *g* values of 2.08, 2.05 and 2.03, is also observed in substoichiometric amounts, when the CDH from *C. thermoaceticum* and *M. thermophila* is incubated with CO [15,17]. This signal can be observed at liquid nitrogen temperatures and is broadened by unresolved [61]Ni hyperfine splitting. In order to determine the Ni site in the *M. soehngenii* CDH, [61]Ni enriched preparations reduced with CO were subjected to EPR spectroscopy. However, no broadening or hyperfine splitting could be observed in any of the low or high spin EPR signals. Thus, there is no evidence for a Ni-Fe-C center in the CDH of *M. soehngenii*, although the enzyme unambiguously contains Ni. Also, no isolated Ni(III) or Ni(I) signals are observed. This indicates that either the Ni is not related to the observed signals or that the clusters have structures in which the [61]Ni

hyperfine splitting is significantly smaller than the inhomogeneous line width. The magnetic properties of non-biological [6Fe-6S] model clusters are not drastically affected, when they are combined with an ion of a different metal [34]. Analogously, one could envisage the nickel ion in CO dehydrogenase to be associated in a similar manner with an Fe/S supercluster. In addition to CO reduction the CDH of acetoclastic methanogens is involved in the catalysis of the acetyl-CoA cleavage [7-10]. Therefore, also the influence of Ac-ScoA and CoASH binding on the paramagnetic centers was studied. The EPR spectrum of the reduced enzyme was not significantly altered by incubation with CoASH, which was also observed for the enzyme from *M. thermophila* [17]. This is in sharp contrast to observations made with the clostridial enzyme, where CoASH induced a change in the Ni-Fe-C spin coupled complex [4]. This difference may reflect the ability of the enzymes to catalyze similar reactions, albeit in opposite directions. However, when the CDH of *M. soehngenii* was incubated with Ac-ScoA the EPR spectrum was completely perturbed. This is in sharp contrast to enzymes of *C. thermoaceticum* and *M. thermophila*, which form an $g=2.08$, 2.05 and 2.03 signal upon incubation with Ac-ScoA [4,17]. The perturbation of the EPR signal by Ac-ScoA indicates that the binding of the biological substrate causes either an oxidation of the reduced clusters or induces a conformational change, which renders the enzyme completely EPR silent.

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Chapter 9

Purification and some properties of
the Methyl-Coenzyme M Reductase of *Methanothrix soehngenii*

Purification and some properties of the methyl-CoM reductase of *Methanothrix soehngenii*

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1. SUMMARY

The methyl-CoM reductase from *Methanothrix soehngenii* was purified 18-fold to apparent homogeneity with 50% recovery in three steps. The native molecular mass of the enzyme estimated by gel-filtration was 280 kDa. SDS-polyacrylamide gel electrophoresis revealed three protein bands corresponding to M_r 63 900, 41 700 and 30 400 Da. The methyl-coenzyme M reductase constitutes up to 10% of the soluble cell protein. The enzyme has K_m apparent values of 23 μM and 2 mM for N-7-mercaptoheptanoylthreonine phosphate (HS-HTP = component B) and methyl-coenzyme M (CH_3-CoM) respectively. At the optimum pH of 7.0 60 nmol of methane were formed per min per mg protein.

2. INTRODUCTION

Acetate has been shown to be the precursor of 70% of the methane in anaerobic digestors [1,2].

Two genera of methanogenic bacteria, *Methanosarcina* and *Methanothrix*, are known to degrade acetate to CH_4 and CO_2 [3]. In these bacteria acetate is metabolized via acetyl-coenzyme A, which is supposed to be cleaved by the carbon monoxide dehydrogenase [4–6]. The carbonyl-moiety is further oxidized to CO_2 to generate reducing equivalents and the methylgroup is transferred to coenzyme M [7–11]. The reductive demethylation of methyl-coenzyme M to methane is a common step in all methanogenic bacteria [12–15]. This reaction, which has been studied in detail in *Methanobacterium thermoautotrophicum*, is catalyzed by a complex enzymatic system [16–18]. Component C of this system has earlier been suggested to be the methyl-CoM reductase per se [19]. The enzyme has been found to contain coenzyme M, component B and two tightly bound molecules of coenzyme F430 [20–22]. Evidence has been presented that N-7-mercaptoheptanoyl-threonine phosphate (HS-HTP = component B) functions as an electron-carrier in the methyl-CoM reduction to methane [23–26]. This report summarizes the purification and some properties of the methyl-CoM reductase from *Methanothrix soehngenii*.

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3. MATERIAL AND METHODS

3.1. Organism and cultivation

Methanothrix soehngenii (DSM 2139) was cultured on 80 mM sodium acetate as described previously [27]. Cells were harvested at the late logphase by continuous centrifugation, washed in 50 mM anaerobic Tris-HCl pH 7.6, 0.15 M NaCl and 1 mM Ti(III) citrate and stored under N_2 at $-20°C$.

The following procedures were carried out under strict anaerobic conditions in anaerobic chamber with 95% N_2/5% H_2 as gas phase, traces of oxygen were removed by a platina catalyst.

3.2. Preparation of cell-free extract

Cells were suspended 25% (w/v) in 50 mM Tris-HCl pH 7.6 containing 0.15 M NaCl and 1 mM Ti(III)citrate. The cell suspension was disrupted by sonication for 10×30 s alternating with 30 s cooling periods. Cell debris was removed by centrifugation at $8\,000 \times g$ and the supernatant was used as crude extract.

3.3. Enzyme assay

All enzyme activities were measured at $37°C$ by following the methane production from methyl-coenzyme M gas-chromatographically [26]. The assays were performed in 8-ml serum vials containing 0.4 ml assay mixture (1–5 mg protein, 50 mM KH_2PO_4, pH 7.0, 6.25 mM CH_3-CoM, 0.2 mM H-S-HTP, 10 mM DTT, 0.1 mM aquo-B_{12} and 1.2 mM Ti(III)citrate). Samples of 0.2 ml of the headspace were removed and analyzed for methane on a Becker gaschromatograph model 417 equiped with FID detector. Methane was quantified by relating peak height to a standard curve. One unit of methyl-CoM reductase activity is the amount catalyzing the production of 1 μmol of methane per min at $37°C$.

3.4. Protein determination

Protein was measured with Coomassie brillant blue G250 as described by Bradford [28]. Bovine serum albumin was used as standard.

3.5. Enzyme purification

The crude extract (20 ml) was applied to a Q-Sepharose (fast flow) column (2.2×8 cm) equilibrated with 50 mM Tris-HCl pH 7.6, 0.15 M NaCl. The Methyl-CoM reductase was eluted at 300 mM NaCl in a linear gradient of 250 ml (0.15-0.5 M NaCl) at 4 ml/min. The fractions with methyl-CoM reductase activity were combined and concentrated to 5 ml in an Amicon Diaflo ultrafiltration cell equipped with a PM 30 filter. The concentrated enzyme preparation was diluted 1:1 with 2 M potassium acetate in 50 mM KH_2PO_4 pH 6.9. The protein solution was then applied to a Phenyl-Superose HR 5/5 column equilibrated with 25 mM KH_2PO_4 pH 6.9 and 1 M potassium acetate at 0.5 ml/min. The methyl-CoM reductase was not retarded and collected in the void volume. The void volumes of several runs were combined and concentrated to 1 ml in a PM 30 centricon unit. The concentrated protein solution was applied to Superose 6 HR 10/30 column equilibrated with 50 mM Tris-HCl pH 7.6 and 150 mM NaCl. The methyl-CoM reductase was eluted after 13.5 ml at 0.5 ml/min. The active fractions were combined and concentrated to 200 μl with a PM 30 centricon unit, and stored at $4°C$ until use.

3.6. Electrophoresis

Polyacrylamide gel electrophoresis was carried out on 15% polyacrylamide gel at pH 8.3 according to the method of Laemmli [29].

3.7. Chemicals

All chemicals were at least of analytical grade. Sodium dodecyl-sulfate and acrylamide were from Biorad (Utrecht, The Netherlands). Aquo-vitamin B_{12} was purchased from Hicol (Oud Beijerland, The Netherlands). Gases were purchased from Hoekloos (Schiedam, The Netherlands). Platina catalyst was a gift of BASF (Arnhem, The Netherlands). Q-Sepharose (fast flow), Phenyl-Superose HR 5/5, Superose 6 HR 10/30 and molecular mass standards for gel-filtration and PAGE were obtained from Pharmacia Fine Chemicals (Woerden, The Netherlands). Titanium(III)citrate was prepared from $TiCl_3$ and sodium citrate as described by Zehnder [30]. *N*-7-mercaptoheptanoylthreonine phosphate (HS-HTP) and methyl-coenzyme M (CH_3-CoM) were generous gifts from Dr. J.T. Keltjens (Department of Microbiology, University Nijmegen, The Netherlands).

4. RESULTS

4.1. Enzyme purification

The results of a typical enzyme purification are summarized in Table 1. A 18-fold purification was achieved in three steps with a recovery of 50%. The final specific activity was 50 nmol of $CH_4 \cdot min^{-1} \cdot mg^{-1}$. Chromatography and fraction collection were performed in an anaerobic chamber with 95% N_2/5% H_2 as gas phase. All the buffers used were supplied with 1 mM Ti(III)citrate to prevent enzyme inactivation by oxygen.

4.2. Molecular mass estimates

The native enzyme M_r was determined by gel-filtration on Superose 6 HR 10/30 and appeared to be 280 kDa, when compared with standards of known molecular mass. SDS-polyacrylamide gel electrophoresis of the purified enzyme revealed the presence of three major protein bands (Fig. 1). These protein bands represent the α, β and γ subunits of the methyl-CoM reductase with molecular masses of 63.7, 41.7 and 30.4 kDa, respectively.

4.3. Kinetic properties

Recently it was shown that HS-HTP acted as an electron donor in the reduction of methyl-CoM to CH_4 by the methyl-CoM reductase [24–26]. Ellerman et al. showed that methane and the heterodisulfide (CoM-S-S-HTP) of HS-CoM and HS-HTP were the products of the reaction [26]. The reaction rate at different HS-HTP and CH_3-CoM concentrations followed Michaelis-Menten
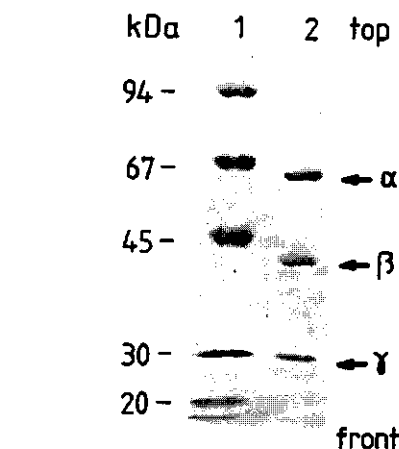

Fig. 1. SDS-polyacrylamide gel electrophoresis of methyl-CoM reductase. 40 μg of methyl-CoM reductase were separated on 15% acrylamide gels by SDS-PAGE and stained with Coomassie blue (lane 2). The M_r of the subunits were calculated using trypsin inhibitor (M_r 20100), carbonic anhydrase (M_r 30000), ovalbumin (M_r 45000), bovine serum albumine (M_r 67000) and phosphorylase b (M_r 94000) as calibration proteins (lane 1).

kinetics. Half maximal rates were obtained at 2 mM CH_3-CoM and at 23 μM HS-HTP. At pH 7.0 the maximal rate of methane formation from methyl-coenzyme M was 60 nmol $\cdot$ $min^{-1} \cdot mg^{-1}$.

5. DISCUSSION

Methyl-CoM reductase is an abundant protein of *Methanothrix*. From the increase of the specific activity upon purification and from the 50% recovery it can be calculated that up to 10% of the soluble cell protein of *Methanothrix* is methyl-CoM reductase. This is within the same range as described for other methanogens [12–15]. Methyl-CoM reductase of *Methanothrix* has also the same subunit composition and molecular mass as the enzymes of other methanogenic bacteria [31]. The methyl-CoM reductase of *Methanothrix* has about the same apparent K_m for methyl-CoM and HS-HTP as the enzyme of *Methanobacterium thermoautotrophicum*. The methane formation rate observed in cell suspensions of *Methanothrix* is 70

Table 1

Purification of Methyl-CoM reductase of *Methanothrix soehngenii*

	protein (mg)	units (mU)	sp. act. (mU/mg)	purification fold	yield (%)
crude extract	280	750	3 [a]	1	100
Q-Sepharose	58	752	13	4	100
Phenyl-superose	24	610	29	10	77
Superose	11	408	51	18	54

[a] = nmol CH_4 formed per min per mg protein.

$nmol \cdot min^{-1} \cdot mg^{-1}$ [27]. Since 10% of the soluble cell protein consists of methyl-CoM reductase, a maximum specific activity of 700 $nmol \cdot min^{-} \cdot mg^{-1}$ would be expected for the purified enzyme. The maximal observed rate is only 6–8% of this value. The same decrease in activity has been observed for the enzyme of *Methanobacterium* [26]. This indicates that either the structural arrangement of the enzyme is destroyed upon breakage of the cells or that the proper assay conditions have not yet been found.

The methyl-CoM reductase catalyses the formation of CH_4 and CoM-S-S-HTP from methyl-CoM and HS-HTP. The heterodisulfide is most probably reduced by the reducing equivalents generated in the oxidation of the carbonyl-moiety to CO_2. Future research is in progress to study the electron transport processes in *Methanothrix*.

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Chapter 10

Discussion and Summary

Discussie en Samenvatting

DISCUSSION and SUMMARY

Acetate is quantitatively the most important intermediate in the anaerobic degradation of soluble organic matter. The conversion rate of acetate by methanogenic bacteria is proposed to be the rate limiting step in this degradation The study of acetoclastic methanogens, therefore is of relevance to our understanding of anaerobic processes and their optimal application in treatment of waste water from various sources.

Until now only two genera of methane bacteria have been described which are able to use acetate as sole energy source: *Methanosarcina* and *Methanothrix*. Because *Methanothrix* has a long generation time and low growth yield, most of the research on acetoclastic methanogenesis was done with *Methanosarcina* until now. The aim of this work was to extend the knowledge of the acetate metabolism in *Methanothrix* and to compare the results with the knowledge about *Methanosarcina*.

The study of the acetate metabolism in *Methanothrix soehngenii* was concentrated around three major subjects:

- Acetate activation to acetyl-coenzyme A by acetyl-coenzyme A synthetase and the energetic consequence of this activation mechanism (Chapters 2-5).
- Cleavage of acetyl-coenzyme A in to a methyl-, carbonyl- and coenzyme A moiety by the enzyme carbon monoxide dehydrogenase and concomitant oxidation of the CO group to CO_2 by the same enzyme (Chapters 6-8).
- Reduction of the methyl group to methane (Chapter 9).

Chapters 2 to 5 deal with several aspects of the acetate degradation and acetate activation in *Methanothrix*. In chapter 2 the threshold concentrations of acetate utilization and the enzymes responsible for acetate activation of several methanogenic bacteria, including *Methanothrix* and *Methanosarcina*, are presented and compared with literature data. The minimum acetate concentrations reached by the acetoclastic *Methanosarcina* are between 0.2 and 1.2 mM and by *Methanothrix* between 7 and 70 μM, whereas the hydrogenotrophic methane bacteria, which can use acetate as an additional carbon source, achieve acetate concentrations between 0.4 and 0.6 mM. *Methanosarcina* uses an acetate kinase / phosphotransacetylase system to activate acetate with high V_{max} but low affinity. *Methanothrix* and most hydrogenotrophic methane bacteria have an acetyl-CoA synthetase to activate acetate with relatively high affinity for acetate. The difference in affinity for acetate of *Methanosarcina* and *Methanothrix* are consistent with the general model by which *Methanosarcina* dominates in environments with high acetate concentrations while low acetate concentrations favour *Methanothrix*. Although the affinity for acetate of the hydrogenotrophic methane bacteria was high, these methanogens were not able to remove acetate to lower concentrations than the acetoclastic methane bacteria. Therefore it is not likely that these hydrogenotrophic methanogens compete strongly for acetate with the acetoclastic methanogens.

In chapter 3 the purification procedure and properties of the acetate activating enzyme of *Methanothrix*, acetyl-coenzyme A synthetase (ACS), are presented and compared to the acetate activating system of *Methanosarcina*. ACS activates acetate to acetyl-coenzyme A. ACS is a homodimeric (α_2) enzyme with molecular mass of 148 kDa and constitutes up to 4 % of the soluble cell protein. Comparison of the kinetic properties of the ACS from *Methanothrix* (V_{max} = 55 U/mg, $appK_m$ for acetate = 0.86 mM) with the properties of the acetate activating system of *Methanosarcina*

(V_{max} = 660 U/mg, $appK_m$ for acetate = 22 mM) confirmed again the hypothesis that *Methanothrix* dominates in environments with low acetate concentrations while high acetate concentrations favour *Methanosarcina*.

With varying amounts of ATP weak sigmoidal kinetics were observed for the ACS system of *Methanothrix*. The Hill-plot gave a slope of 1.58 ± 0.12, suggesting two interacting substrates sites for the ATP. The possible presence of different ATP binding sites was later confirmed by analysis of the deduced amino acid sequence of the *ACSa* gene.

The energy for the activation of acetate by ACS is provided by the hydrolysis of ATP to AMP and PP_i. In *Methanothrix* the conversion of AMP to ADP and the hydrolysis of PP_i to 2 P_i are catalyzed by an adenylate kinase and inorganic pyrophosphatase, respectively.

A sum of these reactions leads to the suggestion that for acetate activation in *Methanothrix* two ATP are needed. Since it is believed that methane formation from acetate can only yield one ATP, it is difficult to envisage how *Methanothrix* is able to grow at all. One possible site of energy conservation may be coupled to the hydrolysis of pyrophosphate. To investigate the possibility that the energy of the PP_i bound could be used to drive endergonic reactions, the pyrophosphatase was isolated from *Methanothrix*. The properties of the purified inorganic pyrophosphatase are described in Chapter 4. The enzyme is composed of subunits with molecular masses of 35 and 33 kDa in an $\alpha_2\beta_2$ oligomeric structure, giving a molecular mass of 139 ± 7 kDa for the native enzyme. The enzyme catalyzed the hydrolysis of inorganic pyrophosphate, tri- and tetrapolyphosphate, but no activity was observed with a variety of other phosphate esters. The cation Mg^{2+} was required for activity. The enzyme was heat-stable, insensitive to molecular oxygen, not inhibited by fluoride and constituted upto 0.2 % of the soluble protein. When cells were rigorously disrupted in a Frech Pressure cell,

the inorganic pyrophosphatase was found in the soluble cell fraction. However, when gentle lysis procedures were applied up to 5 % of the inorganic pyrophosphatase was associated with the membrane fraction. This membrane association could indicate that hydrolysis of of the pyrophosphate is not solely used to displace the equilibrium of acetate activation. In analogy with the proton translocating inorganic pyrophosphatase of plant vacuoles and phototrophic bacteria, one could envisage a similar proton translocation function for the enzyme in *Methanothrix*.

Since the energetic aspects formed an intriuging facet of the acetate metabolism in *Methanothrix*, the interconversion of adenine nucleotides during acetate fermentation in concentrated cell suspensions of *Methanothrix soehngenii* were investigated and described in chapter 5. Starved cells of *Methanothrix* contained high levels of AMP (2.2 nmol/mg protein), but had hardly any ADP or ATP. The Energy Charge (EC) of these cells was 0.1. Immediately after the addition of the substrate acetate, the level of ATP started to increase, reaching a maximum of 1.4 nmol/mg protein, corresponding to an EC of 0.7 when half of the acetate had been consumed. Once the acetate was depleted, the ATP concentration decreased to its original level of 0.1 nmol/mg protein, (EC = 0.1). These results showed that although the free energy gain on acetate is very low, *Methanothrix* is able to conserve some of this free energy in net ATP synthesis.

In chapters 6 to 8 several aspects of the central enzyme in the acetate metabolism of *Methanothrix soehngenii*, the carbon monoxide dehydrogenase (CDH) are presented.

Chapter 6 decribes the aerobic purification procedure together with several kinetic properties of the CDH. In contrast with the CDH's from most other anaerobic bacteria, the CO oxidizing activity of the purified enzyme of *Methanothrix soehngenii* was

remarkably stable towards oxygen and it was only slightly inhibited by cyanide. The enzyme constitutes 4 % of the soluble cell protein and showed a high degree of thermostability. Analysis of enzyme kinetic properties revealed a K_m of 0.7 mM for CO and of 65 μM for methylviologen. At the optimum pH of 9.0 the V_{max} was 140 μmol of CO oxidized per min per mg protein. Acetyl-coenzyme A / CO exchange activity, 35 nmol.min^{-1}.mg^{-1} of protein, could be detected in anaerobic enzyme preparations, but was absent in aerobic preparations. The enzyme has a tetrameric $(\alpha\beta)_2$ subunit structure. The M_r of the α subunit is about 89 kDa and of the ß subunit about 20 kDa. The enzyme contained about 12 Fe, 12 acid-labile sulfur and 1 Ni per αß-dimer, which are present in clusters. These iron-sulfur clusters can be studied by Electron Paramagnetic Resonance (EPR) spectroscopy.

In chapter 7 the paramagnetic iron-sulfur centers of purified CDH are described. In EPR spectra of the anaerobically isolated enzyme two major signals were apparent. One with g-values of 2.05, 1.93 and 1.865, and an $E_{m7.5}$ of -410 mV, which quantified to 0.9 S=1/2 spins per αß-dimer. This signal resembles EPR spectra of two dipolarly interacting, ferredoxin-like [4Fe-4S] clusters. Analysis of the deduced amino acid sequence of the CDHa gene confirmed that there is a strech of 64 amino acids, which could be identified as a ferredoxin domain of the archaebacterial type. Taken together the low redox potential and the ferredoxin like sructure of this cluster, it is likely that this center function as electron acceptor in the oxidation of the CO group. The other signal with g-values of 1.997, 1,886 and 1.725, and an $E_{m7.5}$ of -230 mV, gave 0.1 spin per αß-dimer. Until now no structure could be assigned to this unusual signal, although it resembles in some aspects the putative [6Fe-6S] prismane clusters. When the enzyme was incubated with its physiological substrate acetyl-CoA, these two major signals disappeared. Incubation of the enzyme under CO atmosphere resulted in a partial disappearance of the spectral component with g = 1.997, 1.886, 1.725.

In chapter 8 a novel high-spin EPR signal in the oxidized CDH with effective g-values at g = 14.5, 9.6, 5.5, 4.6, 4.2, 3.8 is described. The lines at g = 14.5 and 5.5 were tentatively ascribed to a S = 9/2 system with about 0.3 spins per $\alpha\beta$-dimer. The other signals were assigned to a S = 5/2 system with 0.1 spins per $\alpha\beta$-dimer. Both sets of signals disappear upon reduction with $E_{m7.5}$ = -280 mV. With a very similar reduction potential, $E_{m7.5}$ = -261 mV, an S = 1/2 signal (0.1 spins per $\alpha\beta$) appears with the unusual g-tensor 2.005, 1.894, 1.733. Whether these signals belong to the same paramagnetic center exhibiting different spin states is not yet clear. CO dehydrogenase, 69 % enriched in ^{61}Ni, showed the same EPR signals as enzyme preparations isolated from cells grown in media with native Ni. In none of these signals any splitting or broadening from ^{61}Ni could be detected. Also, the characteristic "g = 2.08" EPR signal found in some other CO dehydrogenases, and ascribed to a Ni-Fe-C complex, was never observed under any condition of detection (4 to 100 K) and incubation (ferricyanide, dithionite, CO, coenzyme A, acetyl-CoA).

Chapter 9 describes the purification and some properties of the methyl-CoM reductase from *Methanothrix soehngenii*. The enzyme catalyzes the final step in the conversion of acetate: the reduction of methyl-coenzyme M to methane. The enzyme had a native molecular mass of 280 kDa in a $(\alpha\beta\gamma)_2$ subunit structure. The methyl-coenzyme M reductase constituted upto 10 % of the soluble cell protein. The enzyme has K_m apparent values of 23 μM and 2 mM for N-7-mercaptoheptanoyl threonine phosphate (HS-HTP) and methyl-coenzyme M, respectively. At the optimum pH of 7.0, 60 nmol of methane were formed per min per mg protein. These properties are comparable to those of methyl-CoM reductase of other

methanogenic bacteria, although the specific activity is relatively low.

Concluding remarks

This thesis clearly showed that the acetate metabolism of the specialist *Methanothrix soehngenii* has peculiar features. The high energy input in the activation of acetate by an acetyl-CoA synthetase results in a high affinity and a low threshold value, which makes *Methanothrix* the dominant acetoclastic methanogen in anaerobic ecosystems with low acetate concentrations. The other consequences of the energy input are the low growth yield and long generation time, which causes an outcompetition by *Methanosarcina* in systems with high acetate concentrations. Although the energy input is quite clear, the energy conservation is not yet well understood. The reduction of the heterodisulfide between coenzyme M and mercaptoheptanoyl threonine phosphate is proposed to be the common site for energy conservation in all methanogens. The high activity of heterodisulfide reductase in cell extracts of *Methanothrix* indicates that this site is also operative in *M. soehngenii*. *Methanothrix*, however, needs additional sites of energy conservation to compensate for its high input. One possible site could be the oxidation of CO to CO_2, another site could be formed by the partially membrane associated pyrophosphatase.

The central enzyme in the acetate metabolism of *Methanothrix* is Carbon monoxide dehydrogenase. This enzyme showed several surprising and novel characteristics: The CO-oxidizing activity appeared to be insensitive towards oxygen, the anaerobically purified enzyme was able, although at low activity, to exchange the carbonyl group of acetyl-CoA with CO and EPR spectroscopy indicated the presence of an unusual iron-sulfur center.

De oxygen-insensitivity is recently observed for the CDH of some sulfate-reducing bacteria which also use a reversed acetyl-CoA pathway for the degradation of acetate. The reason for this insensitivity is not yet clear. The exchange reaction between CO and the carbonylgroup of acetyl-CoA was recently described for the enzyme isolated from *Methanosarcina thermophila*. This enzyme had also low activity, which indicates that either the right assay conditions are not yet found or that this activity is extremely instable.

Concerning the iron-sulfur centers there are little similarities between the enzymes of the different anaerobic bacteria. One reason why the research to the clusters of CDH of anaerobic bacteria is hampered at the moment is the low spin recovery of the different centers. This makes statements about structure and function difficult. There are good indications that at least one and possibly two ferredoxin-like [4Fe-4S] clusters are present, which play a role in the electron transfer of the CO oxidation. For the presence of a Ni-Fe-C complex in the CDH of *Clostridium thermoaceticum* and *Methanosarcina thermophila*, there also good spectroscopic indications. This complex has until now not been observed in the CDH of *Methanothrix*. The CDH of *Methanothrix* and also of *Clostridium thermoaceticum* show in EPR spectroscopy another unusual signal. This signal could be assigned to a putative [6Fe-6S] prismane cluster, which could function in multi-electron transfer or in substrate binding. More clear indications are necessary to identify the structure and function of this cluster.

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DISCUSSIE en SAMENVATTING

Acetaat is kwantitatief het belangrijkste inter-mediair in de anaërobe afbraak van oplosbaar organisch materiaal. De omzettingssnelheid van acetaat door methanogene bacteriën wordt verondersteld de snelheidsbepalende stap in deze afbraak te zijn. Het onderzoek naar acetoclastische methanogenen is daarom van belang voor een begrip van anaërobe processen en hun optimale toepassing in de behandeling van verschillende types afvalwater.

Tot nu toe zijn er echter slechts twee methaanbacteriën beschreven, die in staat zijn acetaat als enige energiebron te gebruiken: *Methanosarcina* and *Methanothrix*. Omdat *Methanothrix* een lange generatie tijd en lage groei opbrengst heeft, zijn de meeste onder-zoeken tot nu toe uitgevoerd met *Methanosar-cina*. Het doel van deze studie was het uit-breiden van de kennis van het acetaat metabolisme in *Methanothrix* en het vergelijken van de resultaten van die studie met de gegevens over het acetaatmetabolisme van *Methanosarcina*.

Het onderzoek naar het acetaatmetabolisme van *Methanothrix soehngenii* was gecon-centreerd rond drie thema's:

- Acetaat activatie tot acetyl-CoA door acetyl-CoA synthetase en de energe-tische consequenties van dit activatie mechanisme (Hoofdstukken 2-5).
- Splitsing van acetyl-coenzym A in een methyl-, carbonyl- en coenzym A groep door het enzym koolmonoxide dehy-drogenase en de daarop volgende oxi-datie van de CO groep tot CO_2 door het zelfde enzym (Hoofdstukken 6-8).
- Reductie van de methyl groep tot me-thaan (Hoofdstuk 9).

De hoofdstukken 2 tot 5 beschrijven verschil-lende aspecten van de acetaat afbraak en acetaat activatie in *Methanothrix*. In hoofdstuk 2 worden de "threshold" concentratie voor acetaat gebruik en de enzymen, die verant-woordelijk zijn voor acetaat activatie van verschillende methanogene bacteriën, inclusief *Methanothrix* and *Methanosarcina*, beschreven en vergeleken met literatuur gegevens. De minimum acetaat concentratie voor *Methano-sarcina* ligt tussen 0.2 en 1.2 mM, die voor *Methanothrix* tussen 7 en 70 µM, terwijl de hydrogenotrofe methaanbacteriën, die acetaat als additionele koolstofbron kunnen gebruiken, acetaat concentraties tussen 0.4 en 0.6 mM bereiken. *Methanosarcina* bezit een acetaat kinase / phosphotransacetylase sys-teem om acetaat te activeren met een hoge V_{max} maar een lage affiniteit. *Methanothrix* en de meeste hydrogenotrofe methaan bacteriën hebben een acetyl-CoA synthetase om acetaat te activeren met een relatief hoge affiniteit voor acetaat. Het verschil in affiniteit voor acetaat van *Methanosarcina* en *Methanothrix* voldoet aan het algemene model, waarin *Methanosarcina* domineert in milieus met hoge acetaat concentraties, terwijl lage acetaat concentraties *Methanothrix* begunstigen. Ofschoon de affiniteit voor acetaat van de hydrogenotrofe methaanbacteriën hoog was, waren deze methanogenen niet in staat om acetaat te verwijderen tot lagere concentraties dan de acetoclastische methaanbacteriën. Daarom is het niet waarschijnlijk dat deze hydrogenotrofe methanogenen sterk zullen competeren voor acetaat met de acetoclastische methanogenen.

In hoofdstuk 3 worden de zuiveringsprocedure en de eigenschappen van het acetaat ac-tiverende enzym van *Methanothrix*, acetyl-coenzym A synthetase (ACS), gepresenteerd en vergeleken met het acetaat-activerende systeem van *Methanosarcina*. ACS activeert acetaat tot acetyl-coenzym A. ACS is een homodimeer (α_2) enzym met een moleculaire massa van 148 kDa. Vier procent van het oplosbare cel eiwit is ACS. Vergelijking van de kinetische eigenschappen van het ACS uit

Methanothrix (V_{max} = 55 U/mg, *appK*$_m$ voor acetaat = 0.86 mM) met de eigenschappen van het acetaat activerende systeem van *Methanosarcina* (V_{max} = 660 U/mg, *appK*$_m$ voor acetaat = 22 mM) bevestigen opnieuw de hypothese dat *Methanothrix* domineert in milieus met lage acetaat concentraties terwijl hoge acetaat concentraties *Methanosarcina* bevoordelen. Het ACS vertoonde met variërende hoeveelheden ATP zwak sigmoidale kinetiek. De Hill-plot had een helling van 1.58 ± 0.12, dit suggereert dat ACS twee interactieve substraatbindingsplaatsen voor ATP bezit. De mogelijke aanwezigheid van verschillende ATP bindingsplaatsen werd later bevestigd door analyse van de uit het *ACSa* gen afgeleide aminozuurvolgorde.

De energie voor de activatie van acetaat door ACS wordt geleverd door de hydrolyse van ATP tot AMP en PP$_i$. In *Methanothrix* wordt de omzetting van AMP tot ADP en de hydrolyse van PP$_i$ tot 2 P$_i$ gekatalyseerd door respectievelijk een adenylaat kinase en een anorganisch pyrofosfatase.

De som van deze reacties leidt tot de suggestie dat voor acetaat activatie in *Methanothrix* twee ATP nodig zijn. Omdat wordt verondersteld dat methaanvorming uit acetaat slechts één ATP kan opleveren, is het moeilijk voor te stellen hoe *Methanothrix* überhaupt in staat is te groeien. Een mogelijke plaats van energieconservering zou gekoppeld kunnen zijn aan de hydrolyse van pyrofosfaat. Om de mogelijkheid te onderzoeken of de energie uit de PP$_i$ binding gebruikt zou kunnen worden om endergone reacties te drijven, werd het anorganisch pyrofosfatase geïsoleerd uit *Methanothrix*. De eigenschappen van het gezuiverde anorganisch pyrofosfatase zijn beschreven in Hoofdstuk 4. Het enzym is samengesteld uit subunits met moleculaire massa's van 35 en 33 kDa in een $\alpha_2\beta_2$ oligomere structuur. De molecuul massa van het natieve eiwit is 139 ± 7 kDa. Het enzym katalyseert de hydrolyse van anorganisch pyrofosfaat, tri- en tetrapolyfosfaat,

maar er werd geen activiteit waargenomen met andere organische fosfaatesters. Het kation Mg^{2+} was nodig voor volledige activiteit van het enzym. Het enzym was hittestabiel, ongevoelig voor zuurstof, werd niet geremd door fluoride en maakte 0.2 % van het oplosbare eiwit uit. Wanneer de cellen werden opengebroken met behulp van een "French Press", werd het anorganisch pyrofosfatase aangetroffen in de oplosbare celfractie. Echter wanneer een milde lysisprocedure werd toegepast, was 5 % van het anorganisch pyrofosfatase geassocieerd met de membraanfractie. Deze membraan associatie duidt aan dat de hydrolyse van het pyrofosfaat niet alleen gebruikt kan worden om het evenwicht van de acetaat activatie te verschuiven. In analogie met het proton-translocerend anorganisch pyrofosfatase van plantvacuoles en fototrofe bacteriën, kan men zich een proton-translocerende functie voor het enzym in *Methanothrix* voorstellen .

Omdat de energetische aspecten een intrigerend facet vormden van het acetaat metabolisme in *Methanothrix*, werd de omzetting van adenine nucleotiden gedurende acetaat fermentatie in geconcentreerde celsuspensies van *Methanothrix soehngenii* onderzocht en beschreven in hoofdstuk 5. Uitgehongerde cellen van *Methanothrix* hadden hoge niveaus aan AMP (2.2 nmol/mg eiwit), maar bevatten nauwelijks enig ADP of ATP. De Energy Charge (EC) van deze cellen bedroeg 0.1. Onmiddellijk na de toevoeging van acetaat steeg het niveau van ATP tot 1.4 nmol/mg eiwit, overeenkomend met een EC van 0.7. Wanneer de acetaat was opgebruikt, daalde de ATP concentratie tot het begin niveau van 0.1 nmol/mg eiwit (EC = 0.1). Deze resultaten toonden aan dat, ofschoon de Gibb's vrije energiewinst op acetaat erg klein is, *Methanothrix* toch in staat is een gedeelte van deze energie te gebruiken voor de vorming van ATP.

In hoofdstukken 6 tot 8 worden verschillende aspecten van het centraal enzym in het acetaatmetabolisme van *Methanothrix soehngenii*, het koolmonoxide dehydrogenase (CDH) gepresenteerd.

Hoofdstuk 6 beschrijft de aerobe zuiveringsprocedure samen met verschillende kinetische eigenschappen van het CDH. In tegenstelling met de CDH's uit de meeste andere anaërobe bacteriën, is de CO- oxiderende activiteit van de gezuiverde enzymen van *Methanothrix soehngenii* redelijk stabiel ten opzichte van zuurstof en het werd slechts licht geremd door cyanide. Het enzym maakt 4 % van de oplosbare celeiwit uit en toont een hoge mate van thermostabiliteit. Analyse van de kinetische eigenschappen leverden een K_m van 0.7 mM voor CO en van 65 µM voor methylviologen op. Bij de optimum pH van 9.0 was de V_{max} 140 µmol CO geoxideerd per min per mg eiwit. De Acetyl-CoA / CO uitwisselingsactiviteit van 35 $nmol.min^{-1}.mg^{-1}$ eiwit, werd waargenomen in anaërobe enzympreparaten, maar was afwezig in aerobe preparaten. Het enzym had een tetramere $(\alpha\beta)_2$ subunit structuur. De M_r van de α subunit was ongeveer 89 kDa, van de ß subunit ongeveer 20 kDa. Het enzym bevatte ongeveer 12 Fe, 12 zuur-labiele zwavel en 1 Ni per αß-dimeer, die gerangschikt zijn in clusters. Deze ijzer-zwavel clusters kunnen bestudeerd worden met behulp van Electron Paramagnetische Resonantie (EPR) spectroscopie.

In hoofdstuk 7 worden de paramagnetische ijzer-zwavel centra van het gezuiverde CDH beschreven. In de EPR spectra van het anaeroob geïsoleerde enzym zijn er twee hoofdsignalen zichtbaar. Eén met g-waarden van 2.05, 1.93 en 1.865, en een $E_{m7.5}$ van -410 mV, die integreerde tot 0.9 S=1/2 spins per αß-dimeer. Dit signaal leek op EPR spectra van twee middels dipool interacterende, ferredoxine-achtige [4Fe-4S] clusters. De analyse van de uit het *CDHa* gen afgeleide aminozuur volgorde bevestigde, dat er in het eiwit 64

opeenvolgende aminozuren zijn, die geïdentificeerd kunnen worden als een ferredoxine domein van het archaebacteriële type. De lage redoxpotentiaal en het ferredoxine achtige van dit cluster, maken het aannemelijk dat dit centrum functioneert als electron acceptor in de oxidatie van de CO groep. Het andere signaal met g-waarden van 1.997, 1.886 en 1.725, en een $E_{m7.5}$ van -230 mV, gaf 0.1 spins per αß-dimeer. Tot nu toe kon er geen structuur worden toegekend aan dit ongebruikelijke signaal, ofschoon het in sommige aspecten lijkt op vermeende [6Fe-6S] prismaan clusters. Wanneer het enzym wordt geincubeerd met het fysiologische substraat acetyl-CoA, verdwijnen deze twee signalen. Incubatie van het enzym onder een CO atmosfeer resulteerde in een partiële verdwijning van de spectrale component met g = 1.997, 1.886, 1.725.

In hoofdstuk 8 wordt een nieuw "high-spin" EPR signaal in het geoxideerde CDH met effectieve g-waarden van g = 14.5, 9.6, 5.5, 4.6, 4.2, 3.8 beschreven. De lijnen bij g = 14.5 en 5.5 werden voorlopig toegeschreven aan een S = 9/2 systeem met ongeveer 0.3 spins per αβ-dimeer. De andere signalen werden toegeschreven aan een S = 5/2 systeem met 0.1 spins per αβ-dimeer. Beide signalen verdwenen bij reductie met $E_{m7.5}$ = -280 mV. Met een vergelijkbare redoxpotentiaal, $E_{m7.5}$ = -261 mV, verscheen het S = 1/2 signaal (0.1 spins per αβ) met de ongebruikelijke g-waarden van 2.005, 1.894, 1.733. Of deze signalen behoren tot hetzelfde paramagnetische centrum, dat verschillende spin toestanden vertoont, is nog niet geheel duidelijk. CO dehydrogenase, 69 % verrijkt met ^{61}Ni, vertoont dezelfde EPR signalen als enzympreparaten geïsoleerd uit cellen gekweekt in medium met natief Ni. In geen van deze signalen kon enige splitsing of verbreding van ^{61}Ni worden waargenomen. Eveneens werd het karakteristieke "g = 2.08" EPR signaal, wat in sommige andere CO dehydrogenases aanwezig is, en wordt toege-

schreven aan een Ni-Fe-C complex, nooit waargenomen onder welke detectieconditie (4 to 100 K) en incubatieconditie (ferricyanide, dithioniet, CO, coenzyme A, acetyl-CoA) dan ook.

Hoofdstuk 9 beschrijft de zuivering en enkele eigenschappen van het methyl-Coenzym M reductase uit *Methanothrix soehngenii*. Dit enzym katalyseerd de laatste stap in de omzetting van acetaat: de reductie van methyl-CoM tot methaan. Het enzym heeft een natieve molecuul massa van 280 kDa in een $(\alpha\beta\gamma)_2$ subunit structuur. Het methyl-coenzym M reductase maakt 10 % uit va het oplosbare cel eiwit. Het enzym heeft een $appK_m$ waarde van 23 μM voor N-7-mercaptoheptanoyl threonine fosfaat (HS-HTP) en van 2 mM voor methyl-coenzym M. Bij de optimum pH van 7.0 wordt 60 nmol methaan gevormd per min per mg eiwit. Deze eigenschappen zijn vergelijkbaar met die van methyl-CoM reductases van andere methanogene bacteriën, ofschoon de specifieke activiteit relatief laag is.

Conclusies

Deze dissertatie heeft duidelijk aangetoond dat het acetaat metabolisme van de specialist *Methanothrix soehngenii* bijzondere karakteristieken vertoond. De hoge energie investering in de activatie van acetaat door acetyl-CoA synthetase resulteert in een hoge affiniteit en lage "threshold" waarde voor acetaat, dit maakt *Methanothrix* tot de dominante acetoclastische methanogeen in anaërobe ecosystemen met lage acetaat concentraties. De andere consequenties van de hoge energie investering zijn de lage groei opbrengst en de lange generatietijd, dit zorgt ervoor dat *Methanosarcina* in systemen met hogere acetaat concentraties de overhand krijgt. Ofschoon de energie investering van *Methanothrix* redelijk duidelijk is, wordt de

manier van energieconservering nog niet geheel begrepen. De reductie van het heterodisulfide tussen coenzym M en mercaptoheptanoyl threonine fosfaat wordt verondersteld de gemeenschappelijke plaats voor energieconservering van alle methanogenen te zijn. De hoge activiteit van het heterodisulfide reductase in celextracten van *Methanothrix* duidt erop dat deze conserveringsplaats ook werkzaam is in *M. soehngenii*. *Methanothrix* heeft echter additionele plaatsen van energie conservering nodig ter compensatie van zijn hoge energie investering. Een mogelijke plaats zou de oxidatie van CO tot CO_2 kunnen zijn, een andere plaats zou gevormd kunnen worden door het partieël membraan geassocieerde pyrofosfatase.

Het centrale enzym in het acetaat metabolisme van *Methanothrix* is koolmonoxide dehydrogenase. Dit enzym vertoonde verschillende verrassende en nieuwe eigenschappen. De CO-oxiderende activiteit bleek ongevoelig voor zuurstof, het anaëroob gezuiverde enzym was instaat, ofschoon met lage activiteit, de uitwisselingsreactie tussen de carbonylgroep van acetyl-CoA en CO te katalyseren en EPR spectroscopie gaf aanwijzingen voor de aanwezigheid van ongebruikelijke ijzer-zwavel centra.

De zuurstofongevoeligheid is recent ook waargenomen voor het CDH van enkele sulfaatreducerende bacteriën, die eveneens de ongekeerde acetyl-CoA route bezitten om acetaat af te breken. De reden van deze ongevoeligheid is echter nog niet bekend.

De uitwisselingsreactie tussen CO en de carbonylgroep van acetyl-CoA is inmiddels ook beschreven voor het enzym geïsoleerd uit *Methanosarcina thermophila*. Dit enzym heeft eveneens een lage activiteit, wat erop duidt dat óf de juiste assay condities nog niet gevonden zijn óf dat deze activiteit zeer instabiel is.

Wat betreft de ijzer-zwavel centra zijn er op dit moment weinig parallellen te trekken. Een

reden waardoor het onderzoek naar de clusters in de CDH's van alle anaërobe bacteriën wordt beperkt, is de lage spinopbrengsten van de verschillende centra. Dit maakt gegronde uitspraken over de structuur moeilijk. Er zijn voldoende aanwijzingen, die de aanwezigheid van tenminste één en mogelijk twee ferredoxine achtige [4Fe-4S] clusters, welke een rol spelen in de electron overdracht bij de CO oxidatie, aannemelijk maken. Voor de aanwezigheid van een Ni-Fe-C complex in het CDH van *Clostridium thermoaceticum* en *Methanosarcina thermophila* zijn verschillende spectroscopische indicaties. Dit complex is tot nu toe in het CDH van *Methanothrix* niet waargenomen. Het CDH van *Methanothrix* en ook van *Clostridium thermoaceticum* vertonen in EPR spectroscopie echter een ander ongewoon signaal. Dit signaal is mogelijk afkomstig van een vermeend [6Fe-6S] prismaan cluster, dat zou kunnen functioneren in multi-electron overdracht of substraat-binding. Meer eenduidige aanwijzingen zijn echter noodzakelijk om de structuur en functie van dit cluster op te helderen.

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NAWOORD

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CURRICULUM VITAE

Op 16 maart 1962 werd ik als Michaël Silvester Maria Jetten geboren te Roermond. Na mijn verhuizing in 1965 naar Horn bezocht ik daar de katholieke bewaar school St. Jozef. In 1980 behaalde ik het einddiploma Gymnasium ß aan de plaatselijke scholengemeenschap St. Ursula. In het zelfde jaar begon ik met de studie Moleculaire Wetenschappen aan de toen nog Landbouw Hogeschool te Wageningen. Het kandidaats B examen legde ik in juni 1984 met lof af. Het doktoraalprogramma bevatte de 2 bijvakken, Organische Chemie (Wijnberg/De Groot) en Filosofie (Mertens/Koningsveld) en het verzwaarde hoofdvak Chemische Microbiologie (Schraa/Zehnder). Mijn stage voerde ik uit aan de vakgroep Angewandte Mikrobiologie, Universität Ulm, BRD (Schauder/Fuchs), waar ik werd ingewijd in de anaerobe microbiële enzymologie. Het ingenieursdiploma ontving ik in november 1986, het jaar waarin de krijgsmacht mij op allergische basis ongeschikt achtte voor de "verdediging" van het "vaderland". Na enige tegenstribbelingen van de afdeling personeelszaken, begon ik op 2 februari 1987 voor vier plus een half jaar als promovendus "oude stijl" bij de vakgroep microbiologie van de LU. Het onderzoek over het acetaat metabolisme in *Methanothrix soehngenii* werd in augustus 1991 afgerond met de vervaardiging van het proefschrift dat nu voor u ligt.

On 16 March 1962 I was born as Michaël Silvester Maria Jetten in Roermond. In 1965 I moved to Horn, where I visited the primary school St. Jozef. I finished my secondary school in 1980 at the St. Ursula Gymnasium. In the same year I started my study Molecular Sciences at the Wageningen Agricultural University. I received my bachelors degree in june 1984 with honours. The master degree consisted of the minors Organic Chemistry and Philosophy and the extended major Chemical Microbiology. My practical period was carried out at the Department of applied Microbiology of the University of Ulm, where I was introduced in the anaerobic microbial enzymology. I received my master degree in November 1986, the same year the army refused my service on allergic basis. After some objections of the personnel department I started my PhD study for four and one half year on 2 february 1987 at the Department of Microbiology of the WAU. The study on the acetate metabolism in *Methanothrix soehngenii* was finished in august 1991 with this thesis.