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STUDIES ON 2-OXOACID DEHYDROGENASE MULTIENZYME COMPLEXES OF *AZOTOBACTER VINELANDII*

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STUDIES ON 2-OXOACID DEHYDROGENASE MULTIENZYME COMPLEXES OF *AZOTOBACTER VINELANDII*

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 21 september 1984
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

ISN: 211441-03

STELLINGEN

1. De conclusie van Akiyama en Hammes dat de door hen geobserveerde langzame fase van de incorporatie van acetylgroepen in het pyruvaatdehydrogenase complex van *Escherichia coli* afhankelijk is van de pyruvaatconcentratie is onjuist en wordt niet gestaafd door hun waarnemingen.
   - Akiyama, S.K., and Hammes, G.G. (1980) Biochemistry 19, 4208.
   - Dit proefschrift (hoofdstuk 6).

2. De sedimentatie-evenwicht techniek is niet geschikt voor de bepaling van de molecuulmassa van deeltjes met een moleculaire organisatie als die van de 2-ketozuurdehydrogenase multi-enzym complexen.
   - Munk, P., and Cox, D.J. (1972) Biochemistry 11, 687.
   - Gilbert, G.A., and Gilbert, L.M. (1980) Journal of Molecular Biology 144, 405.

3. Jaenicke en Perham hebben onvoldoende aangetoond dat de vorming van de dihydrolipoyltransacetylase "kern" niet de snelheids-beperkende stap is bij de reconstitutie van het pyruvaatdehydrogenase complex van *Bacillus stearothermophilus*.
   - Jaenicke, R., and Perham, R.N. (1982) Biochemistry 21, 3378.

4. Bij de zuivering van multi-enzymcomplexen wordt onvoldoende rekening gehouden met de mogelijke effecten van hydrostatische druk bij ultracentrifugatie.
   - Dit proefschrift (hoofdstuk 4).

5. Pefferkorn et al. hadden bij hun onderzoek aan helix-coil overgangen van aan cellulose-acetaat gebonden poly-L-glutaminezuur als referentie-polyelectrolyt beter poly-DL-glutaminezuur kunnen gebruiken dan het copolymeer van ethylvinylether en maleinezuur.
   - Pefferkorn, E., Schmitt, A., and Varoqui, R. (1982) Biopolymers 21, 1451.

6. Het is onwaarschijnlijk dat 4a-hydroxyflavine de "primaire emitter" is in de bateriële bioluminescentie-reactie.

   - Kurfürst, M., Ghisla, S., and Hastings, J.W. (1984) Proceedings of the National Academy of Sciences USA 81, 2990.

7. Het onderscheid dat in de warenwet wordt gemaakt tussen natuurlijke en natuur-identieke aroma's en aromastoffen is zuiver kunstmatig. De aanduiding "natuurlijke aromastoffen" wordt door de consument dan ook ten onrechte beschouwd als een kwaliteitswaarborg.

   - Nederlandse Warenwet.

8. De beslissing of iemand de militaire dienstplicht zal moeten vervullen wordt met een onaanvaardbare willekeur genomen. Wederinvoering van de term "loteling" zou daarom moeten worden overwogen.

9. Soms kan men SDS beter gebruiken voor het poetsen van tanden dan voor de bepaling van de subeenheid-molecuulmassa van een eiwit.

Hans Bosma
Studies on 2-oxoacid dehydrogenase multienzyme complexes of *Azotobacter vinelandii.*
21 september 1984

VOORWOORD

Een groot aantal mensen is betrokken geweest bij het totstandkomen van dit proefschrift. Ik wil van de gelegenheid gebruik maken hen hierbij te bedanken.
Mijn promotor, Cees Veeger, wil ik bedanken voor zijn belangstelling voor het onderzoek.
Mijn copromotor, Aart de Kok, wil ik bedanken voor de dagelijkse begeleiding. Aart, je hebt mij een grote mate van vrijheid gegeven bij de uitvoering van het onderzoek, terwijl je toch altijd direct betrokken bent geweest bij de essentiële 'wendingen'.
Addy de Graaf wil ik bedanken voor de inwijding in het 'complexgebeuren' en voor de uitermate gezellige sfeer die zij op lab IV wist te creëren.
Gerrit Voordouw wil ik bedanken voor zijn hulp bij de start van mijn onderzoek. Eén van de resultaten van onze gezamelijke experimenten prijkt nu op de omslag van dit proefschrift.
De hulp van Adrie Westphal is essentieel geweest bij het afronden van het experimentele werk voor enkele hoofdstukken.
Ben van Ommen, Erik van Heck, Fred van Wijk en Peter de Vries hebben in het kader van hun studie in het onderzoek geparticipeerd. Hoewel jullie ploeteren niet altijd beloond is met duidelijke resultaten, zal ieder wel iets van zijn werk in dit proefschrift kunnen terugvinden.
Adrie en Ben wil ik (alvast) bedanken voor hun assistentie bij de promotieplechtigheid.
I am very grateful to Chris Stanley and Richard Perham for their help and hospitality in Cambridge. Dear Chris, it is clear (at least to my opinion) that the model deduced by us in Cambridge has survived several experimental 'checks'.
Saskia van der Vies, Hans Grande, Johan van Leeuwen, Ton Visser en alle andere leden van de LH-vakgroep Biochemie wil ik bedanken voor discussies, experimentele hulp en prettige werksfeer.
De leden van de vakgroep Moleculaire Biologie ben ik dankbaar voor hun gastvrijheid in hun radiochemisch laboratorium.

Dr T.A.J. Payens, Dr H.J. Vreeman en Bas van Markwijk van het NIZO te Ede, en Yves Engelborghs en Karel Heremans van de Katholieke Universiteit te Leuven ben ik zeer erkentelijk voor hun hulp, discussies en adviezen bij de lichtverstrooiingsmetingen.
Jan van Breemen van de Rijksuniversiteit te Groningen wil ik bedanken voor de electronenmicroscopische opnames.
Lyda Verstege en Jenny Toppenberg-Fang hebben het manuscript uitgetypt, Bery Sachteleben en Martin Bouwmans hebben het tekenwerk voor hun rekening genomen. Roland van Helden heeft het omslagontwerp gemaakt en de lay-out van het proefschrift verzorgd. Ik wil hen allen daarvoor hierbij bedanken.

CONTENTS

LIST OF ABBREVIATIONS

ATCC	American type culture collection
CoA	Coenzyme A
DCPIP	2,6-dichlorophenol indophenol, oxidized form
E_1	pyruvate dehydrogenase
E_2	dihydrolipoyl transacetylase
E_3	lipoamide dehydrogenase
EDTA	ethylenediamine tetraacetate
FAD	flavine adenine dinucleotide, oxidized form
FMN	flavine adenine mononucleotide, oxidized form
HETPP	hydroxyethylthiamine pyrophosphate
K_i	inhibition constant
K_m	Michaelis constant
M_{app}	apparent molecular mass
M_r	relative molecular mass
n	servicing number
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
OGDC	2-oxoglutarate dehydrogenase complex
PDC	pyruvate dehydrogenase complex
PEG	polyethylene glycol
PMSF	phenyl methane sulfonyl fluoride
R_{45}	intensity of scattered light at 45°
$S_{0.5}$	substrate concentration giving half-maximal velocity
$s_{20,w}$	sedimentation coefficient in water at 293 K
SDS	sodium dodecylsulfate
TNBS	trinitrobenzene sulfonic acid
TPP	thiamine pyrophosphate
Tris	tris(hydroxymethyl)aminomethane
TTTPP	thiamine thiothiazolone pyrophosphate
U	unit of enzyme activity (1 U = 16.6 nkatal)
UV-vis	ultraviolet and visible light
V	initial enzymatic velocity at saturating substrate concentration
v_0	initial enzymatic velocity at a given substrate concentration

LIST OF ENZYMES

EC number	Systematic name	Trivial name
1.2.4.1	pyruvate:lipoate oxidoreductase	pyruvate dehydrogenase
2.3.1.12	acetyl-CoA:dihydrolipoate S-acetyltransferase	dihydrolipoyl transacetylase
1.8.1.4	NADH: lipoamide oxidoreductase	lipoamide dehydrogenase
1.2.4.2	2-oxoglutarate:lipoate oxidoreductase	2-oxoglutarate dehydrogenase
2.3.1.61	succinyl-CoA:dihydrolipoate S-succinyltransferase	dihydrolipoyl succinyltransferase.

1. INTRODUCTION

The pyruvate dehydrogenase complex plays an essential role in the metabolism of aerobic organisms. It catalyzes the oxidative decarboxylation of pyruvate, yielding acetyl-coenzyme A, CO_2 and NADH. This conversion requires the activity of three enzymes, *i.e.* pyruvate dehydrogenase (E_1), dihydrolipoyl acetyltransferase (E_2) and lipoamide dehydrogenase (E_3). The reaction sequence has been extensively studied and proceeds through the following steps (Reed, 1966):

$$\text{pyruvate} + \text{TPP-}E_1 \longrightarrow [\text{hydroxyethyl-TPP}]\text{-}E_1 + CO_2, \quad (1)$$

$$[\text{hydroxyethyl-TPP}]\text{-}E_1 + [\text{lip}S_2]\text{-}E_2 \rightleftarrows [\text{acetyl-S-lipSH}]\text{-}E_2 + \text{TPP-}E_1 \quad (2)$$

$$[\text{acetyl-S-LipSH}]\text{-}E_2 + \text{HSCoA} \rightleftarrows [\text{HS-Lip-SH}]\text{-}E_2 + \text{acetylCoA} \quad (3)$$

$$[\text{HS-Lip-SH}]\text{-}E_2 + \text{FAD-}E_3 \rightleftarrows [\text{Lip}S_2]\text{-}E_2 + \text{reduced FAD-}E_3 \text{ and} \quad (4)$$

$$\text{reduced FAD-}E_3 + NAD^+ \rightleftarrows \text{FAD-}E_3 + \text{NADH} + H^+. \quad (5)$$

The E_1 component contains non-covalently bound TPP, and it catalyzes the decarboxylation of pyruvate and the subsequent reductive acetylation of the lipoic acid residue of E_2. This lipoyl group is covalently bound to the E_2 chain through an amide linkage with a lysine ε-amino group (Nawa, 1960). E_2 catalyzes the transfer of the acetyl group from the dihydrolipoyl group to CoA. The E_3 component, containing FAD, reoxidizes the dihydrolipoyl group, using NAD^+ as electron acceptor.

The pyruvate dehydrogenase complex (PDC) has been isolated from a number of sources like mammals (Hayakawa, 1964 and 1966; Ishikawa, 1966; Linn, 1972), birds (Jagannathan, 1952; Furuta, 1977), plants (Reid, 1977; Rubin, 1977), *Ascaris lumbricoides* (Komuniecki, 1979), yeasts (Hirabayashi, 1972; Kresze, 1981a), *Neurospora crassa* (Harding, 1970), gram-positive bacteria (Hen-

derson, 1979 and 1980; Visser, 1980), and from the gram-negative bacteria *Escherichia coli* (Koike, 1960), *Salmonella typhimurium* (Seckler, 1982), *Pseudomonas aeruginosa* (Jeyaseelan, 1980) and from *Azotobacter vinelandii* (Bresters, 1975a).

Although the complexes of all organisms catalyze the oxidation of pyruvate by the same sequence of reactions, considerable differences do exist with respect to enzyme regulation and molecular organization.

In all organisms, the E_2 component forms a "core" to which the E_1 and E_3 components are non-covalently bound. Therefore, the structure of the E_2 component will determine the molecular organization of the assembled complex. The PDC's can be roughly divided into three classes: those of gram-negative bacteria, those of gram-positive bacteria and the eukaryotic PDC's.

The PDC of *E.coli* probably is the best studied complex. Its E_2 core is composed of 24 chains, arranged in a cubic structure with 432 symmetry (Reed, 1968b; Eley, 1972). The E_1 and E_3 components are dimers of identical chains.

The quaternary structure of the PDC of *B.stearothermophilus* is markedly different; its E_2 core is composed of 60 chains, arranged in an eicosahedral structure (Henderson, 1979 and 1980). Its E_1 component is a dimer of non-identical chains ($\alpha_2\beta_2$).

The mammalian PDC has a similar organization. However, in addition to the E_1 and E_3 components, a kinase is tightly, and a phosphatase is loosely bound to the E_2 core (Linn, 1972; Barrera 1972). The kinase inactivates the E_1 component by phosphorylation of the E_1 chain (Roche, 1972), and the activity is restored by the action of the phosphatase. The relative activities of these regulatory enzymes are controlled by concentrations of metabolites, cations and hormones, resulting in a precise control of the complex activity (Denton, 1972; Randle, 1978).

The bacterial PDC's are not controlled by such sophisticated mechanisms. The *E.coli* PDC activity is regulated by feed-back inhibition (acetyl-CoA, NADH) and by the cooperative binding characteristics of pyruvate (Schwartz, 1970; Bisswanger, 1971 and 1974). The *A.vinelandii* PDC shows similar kinetic behaviour, although a higher Hill-coefficient was found for the binding of pyruvate to the E_1 component (Bresters, 1975b). For the PDC of *P.aeruginosa*, it was shown that all substrates (*i.e.* pyruvate,

CoA, and NAD^+) show cooperativity towards the enzyme complex (Ghosh, 1981).

Although it is generally accepted that the E_2 core of *E.coli* PDC is composed of 24 chains, a long-lasting discussion exists on the amount of the periferal components that can be bound to this core. 'Optimal' chain-stoichiometries of 24:24:12 (Eley, 1972) and 48:24:24 (Bates, 1975b and 1977) have been proposed.

The 24:24:12 model proposed by the group of Reed is based on the determination of the molecular mass of the complex and its components (Eley, 1972), and on the lipoyl and flavin contents of the isolated *E.coli* PDC (Eley, 1972; Collins, 1977; White, 1980). Supporting evidence comes from reconstitution experiments (Reed 1975), and from the relative staining intensity of the components on SDS-gels (Angelides, 1979b).

The 48:24:24 model from Perham's group is essentially based on chain-ratio determinations by chemical modification of the lysine residues in the three components (Bates, 1975b; Danson, 1976 and 1979). The 2:1 optimal chain-ratio for E_1:E_2 was obtained from reconstitution experiments (Bates, 1977; Perham, 1977). The PDC of *E.coli* has a sedimentation coefficient of 53-63 S, and its molecular mass has been estimated at 3.0-6.1 MDa (Koike, 1963; Dennert, 1970a; Vogel, 1972a; Eley, 1972; Danson, 1979).

It is generally accepted that the E_2 chains of the *E.coli* PDC each carry two lipoic acid residues (Collins, 1977; Danson, 1976 and 1981a). These lipoyl groups play an essential role in the mechanism of the complex, by shuttling the acetyl groups and reducing equivalents between the active sites of the component enzymes. In the 'swinging arm' model of Koike (1963) it is suggested that the lipoyl-lysyl 'arm' rotates between the active sites of the component enzymes. By specific modification with spin-labels, it was indeed shown that the lipoyl groups are very mobile (Grande, 1975; Ambrose, 1976).

Fluorescence energy transfer measurements however indicated that the active sites of the component enzymes are at least 4 nm apart in the PDC (Moe, 1974; Shepherd, 1976 and 1977; Scouten, 1978; Angelides, 1979c). Clearly, this distance cannot be spanned by a single lipoyl-lysyl residue, and two possibilities can be considered.

In the first place, the large distance could be overcome by transacetylation reactions between neighbouring lipoyl groups. These transacetylation reactions have been observed for the *E.coli* PDC in so-called servicing experiments (Collins, 1977; Bates, 1977); the E_2 core can still be fully acetylated when only a few active E_1 chains are bound (Stanley, 1981; Packman, 1983). Until now it is however still unclear whether these transacetylation processes are fast enough to be of physiological importance (Danson, 1978; Akiyama, 1980 and 1981).

A single lipoyllysyl residue would be able to interact with the active centers of the complex, if it would be bound to a highly flexible, extended region of the E_2 chain. The existence of these flexible regions is indicated by ^{1}H-NMR spectra of the complex: some very sharp peaks are observed, indicative for a high-mobility structure (Perham, 1981b, 1983; Roberts, 1983).
The existence of such a region also follows from the work of Bleile (1979): when *E.coli* PDC is partially proteolyzed by trypsin, the lipoyl groups are excised from the E_2 core, in a protein fragment of about 30,000 Da. The other half of the E_2 chain remains associated in the E_2 core and still fulfills its function with regard to the binding of the periferal components (structural domain).
This organization of the E_2 chain is also evident from its primary structure, as derived from the DNA-sequence by Stephens (1983b); the amino acid sequence predicts three lipoyl binding sites, arranged in a 30 kDa lipoyl-binding domain. The amino acid composition of the lipoyl binding domain is compatible with an extended structure.
It has been shown (Angelides, 1978; Bleile, 1979; Berman, 1981; Stepp, 1981) that a large fraction of the lipoyl domains can be removed from the E_2 core without a strong decrease in enzyme activity, and a take-over mechanism has been proposed. From the experimental data it was calculated that each E_1 chain must be able to interact with three to four lipoyl groups (Hackert, 1983b).

The 2-oxoglutarate dehydrogenase multi-enzyme complex (OGDC) strongly resembles the PDC with respect to its working mechanism and molecular organization (Pettit, 1973; Koike, 1982). The complex is based on a (succinyl transferase) E_2 core composed of 24 subunits of 45 kDa. One lipoyl group is bound per E_2

chain (Collins, 1977; Angelides, 1979a; White, 1980). The OGDC shows the same behaviour with respect to trypsin treatment; its structural domain is also about 30 kDa, and its lipoyl binding domain has a molecular mass of 11 kDa (Stepp, 1981; Hackert, 1983a). The existence of highly mobile regions within the E_2 chains have also been indicated by NMR measurements (Perham, 1981a; Wawrzynczak, 1981). The OGDC is much more conserved in evolution than the PDC: no essential difference is observed with respect to molecular organization between the complex from *E.coli* and pig-heart. The eukaryotic complex is not regulated by phosphorylation/dephosphorylation (like the eukaryotic PDC), but merely by allosteric effectors (McCormack, 1979; Craig, 1980; Lawlis, 1981).
A third type of 2-oxoacid dehydrogenase complex is known: the branched chain oxoacid dehydrogenase complex (BCOAD). It has been purified from several sources (Namba, 1969; Pettit, 1978; Danner, 1979; Odessey, 1980; Lawson, 1983); the mammalian complex is subject to regulation by a phosphorylation/dephosphorylation mechanism.

The PDC of *A.vinelandii* is much smaller than the *E.coli* complex. Its sedimentation coefficient is 19 S and from light-scattering measurements a molecular mass of about 1.0-1.2 MDa was calculated (Bresters, 1975a). An enzymatically active 17-20 S form has also been observed for the *E.coli* complex, albeit in minor quantities (Schmitt, 1975; Danson, 1979).
The *A.vinelandii* PDC has been isolated as a four-component complex. The subunit molecular masses of the E_1, E_2, and E_3 components were reported as 89 kDa, 82 kDa and 56 kDa respectively (De Abreu, 1979), in good agreement with the values reported for the components of *E.coli* PDC (Perham, 1971; Vogel, 1977). The function of the fourth component (63 kDa) is obscure. De Abreu *et al.* have shown that this component could act as an additional transacetylase (De Abreu, 1977a). The fourth component can however be resolved from the complex, without a significant loss in overall enzyme activity (De Abreu, 1977b).
The flavin content of the *A.vinelandii* PDC preparations is somewhat lower (1.6-1.8 nmol/mg; Bresters, 1975a) than has been reported for the *E.coli* complex (1.8-2.4 nmol/mg; Eley, 1972; Speckhard, 1975). This difference is probably caused by the pre-

sence of the fourth component, since no significant difference was observed in lipoyl:FAD ratio for the complexes of *E.coli* and *A.vinelandii* (De Abreu, 1977a)

In this thesis further studies on the *A.vinelandii* PDC and OGDC are described. An optimized purification procedure for both complexes is described in chapter 2. Some properties of the OGDC are described in chapter 3. The association behaviour and the molecular organization of the PDC are described in chapters 4 and 5. Chapter 6 deals with the acetylation reactions of the PDC, and the results of all of these studies are summarized in chapter 7. A model for the quaternary structure of the PDC is discussed in relation to the model of *E.coli* PDC.

2. ISOLATION OF THE ENZYME COMPLEXES

INTRODUCTION

The pyruvate dehydrogenase complex (PDC) has been isolated from a number of sources, for instance from mammals (Hayakawa, 1966; Linn, 1972; Ishikawa, 1966; Stanley, 1980), yeast (Kresze, 1981a), *Neurospora crassa* (Harding, 1970), gram-positive bacteria (Henderson, 1979; Visser, 1980), *Escherichia coli* (Koike, 1960) and other gram-negative bacteria (Bresters, 1975a; Jeyaseelan, 1980; Seckler, 1982). The most frequently used procedure for the isolation of bacterial PDC's is the method of Reed and Willms (1965), and the original purification procedure for *Azotobacter vinelandii* PDC forms no exception in this respect. The essential steps in the procedure are: protamine sulfate precipitation, ultracentrifugation, isoelectric precipitation, ammonium sulfate precipitation and gel permeation chromatography. For the *A.vinelandii* PDC this method resulted in a complex preparation that showed four bands on SDS gels, *i.e.* it contained one component more than the *E.coli* PDC. It was suggested that this fourth component (Mr 63,000 Da) was an additional transacetylase (De Abreu, 1977a). The same author however also showed that this component could be removed from the complex without an apparent decrease in enzyme activity (De Abreu, 1977b), indicating that the fourth component was a contaminant.

The isolation procedure as developed by Bresters (1975a) resulted in complete inactivation of the *A.vinelandii* 2-oxoglutarate dehydrogenase complex (OGDC). During the protamine sulfate step the lipoamide dehydrogenase component was dissociated from the E_2 core. The resulting E_1E_2 subcomplex proved a persistent contaminant in the purification of the *A.vinelandii* PDC.

In addition to the difficulties normally encountered in the isolation of enzymes, in the isolation of multi-enzyme complexes one should be alert for the dissociation or specific inactivation of component enzymes. Even more subtle changes in the interaction between the components could be induced during purification, causing artefacts in further studies.

For these reasons we have revised the isolation procedure for *A.vinelandii* PDC, optimizing each step with respect to reproducibility and yield.

MATERIALS AND METHODS

Materials

1,4-Butanediol diglycidyl ether, DL-lipoamide and protamine sulfate (Salmon, grade X) were obtained from Sigma (St. Louis, USA). All other biochemicals were from Boehringer (Mannheim, BRD). Polyethylene glycol 6000 (PEG 6000) was obtained from Merck (Schuchardt, BRD). All other chemicals were analytical grade. Sepharose 2B, 4B and 6B were obtained from Pharmacia (Uppsala, Sweden), Biogel A-5.0m and A-15m were from Bio-Rad laboratories (Richmond, USA). *A.vinelandii* (ATCC 478) cells were grown on a nitrogen-free medium on a large scale, as previously described by Bresters *et al.* (1975a). The cell paste was frozen and stored at 253 K.

Methods

Ethanol-Sepharose was prepared from Sepharose 2B in 50 gram batches as described by Sundberg and Porath (1974) and Visser *al.* (1978). Ethanolamine (0.1 M) was coupled to the diglycidyl-activated gel in 0.01 N NaOH, 2 mg/ml $NaBH_4$ was also added. Protein concentrations were measured according to Lowry *et al.* (1951). PDC overall activity was measured at 298 K essentially as described by Bresters *et al.* (1975a) and by Schwartz and Reed (1970). The assay cuvette (final volume 1 ml) contained: 50 mM potassium phosphate buffer pH 7.0, 5 mM pyruvate, 1.25 mM $MgCl_2$, 0.13 mM CoA, 1.5 mM DTT, and 1 mM NAD^+. The reaction was started by the addition of 0.2-20 µg PDC (approx. 0.004-0.4 U). The reaction was followed by the change in absorbancy at 340 nm, due to the formation of NADH. One unit of activity was defined as the amount of enzyme required for the production of 1 µmol of NADH per minute (1 U = 16.6 nkatal). The assay for OGDC activity was based on the same mixture, only the pyruvate was replaced by 5 mM 2-oxoglutarate.

To prevent interference from NADH-oxidase activity that was present in impure samples, the cuvette was made semi-anaerobic

in these cases by the addition of 10 µl 1 M glucose and 10 µl of a 20 mg/ml solution of glucose oxidase. The cuvette was sealed with several layers of Parafilm (American Can co. Greenwich, USA), and left for at least 10 minutes at 298 K prior to the assay.
The assay for lipoamide dehydrogenase activity was performed by a variation of the method of Van den Broek (1971). The measurements were performed in 1 ml 1 M potassium phosphate buffer pH 7.0, containing 0.8 mM DL-lipoamide, 0.1 mM NAD^+ and 0.1 mM NADH. The reaction was started by addition of the enzyme, and it was followed by the absorbancy change at 340 nm due to the oxidation of NADH. One unit of enzyme activity was defined as the amount of lipoamide dehydrogenase required for the oxidation of 1 µmol NADH per minute (1 U = 16.6 nkatal).

RESULTS AND DISCUSSION

The revised isolation procedure for *A.vinelandii* PDC presented here does not contain any 'new' purification principles, it is composed of purification steps that have already been described by others (Reed, 1965; Eley, 1972; Bresters, 1975a; Visser, 1978). Its uniqueness lies in the omission of several steps and the combination of others.
Isoelectric precipitation and ammonium sulfate precipitation steps are omitted, since these treatments result in irreproducible, irreversible inactivation of *A.vinelandii* PDC. In the new procedure, *A.vinelandii* PDC is not precipitated with protamine sulfate, since such a precipitation is accompanied by the inactivation of the 2-OGDC. The addition of protamine sulfate is therefore stopped just before the precipitation of PDC would occur. In comparison to the 'old' isolation procedure for *A.vinelandii* PDC, polyethylene glycol precipitation and ethanol-Sepharose chromatography are additional steps.

Isolation of the pyruvate and 2-oxoglutarate dehydrogenase complexes

All steps are performed at 277 K. Standard buffer is 50 mM potassium phosphate pH 7.0, containing 1 mM EDTA and 50 µM PMSF.

Step 1. Preparation of the cell-free extract

250 Grams (wet weight) of frozen *A.vinelandii* cells (strain ATCC 478) are thawed in 1 litre 50 mM potassium phosphate buffer pH 7.0. After one washing, the cells are resuspended in standard buffer, containing 2 mM EDTA, and the total volume is brought to 800 ml. The cells are broken by passing the suspension once through a precooled Manton-Gaulin laboratory homogenizer at a pressure of 50 MPa (500 bar, 7000 psi.). The homogenizer is rinsed out with some buffer to remove the bacterial mass quantitatively. The resulting homogenate is centrifuged at 20,000 x g for 30 minutes to remove unlysed cells and celldebris. The supernatant (approximately 1 litre) contains most of the 2-OGDC and PDC enzyme activity.

Step 2. Protamine sulfate fractionation

With gentle stirring, 150 ml of a 2% (w/vol.) protamine sulfate solution is added to the cell-free extract. After 15 minutes the suspension is centrifuged at 20,000 x g for 15 minutes. More protamine sulfate is added to the supernatant in 20 ml aliquots until an additional 20 ml addition would result in a detectable precipitation of PDC (as monitored by small-scale pilot experiments). 15 Minutes after the last protamine sulfate addition, the solution is centrifuged and the pellet is discarded. 50 ml of a 2% (w/vol.) yeast RNA solution is then added to the supernatant to precipitate excess protamine sulfate, which is then removed by centrifugation.

Step 3. Polyethylene glycol fractionation

The supernatant of step 2 is made 10% (w/vol.) in polyethylene glycol 6000 (PEG 6000) by dropwise addition of a 50% (w/vol.) stock solution in standard buffer. 90% Of the OGDC-activity is then precipitated by the addition of $MgCl_2$, usually a concentration of 1.5 mM is sufficient. The precipitation must be carefully followed by enzyme assays, since precipitation of more than 90% of the OGDC activity results in coprecipitation of PDC. The precipitate is collected by centrifugation (15 min., 20,000 x g). About 90% of the PDC activity is precipitated in an analogous way between 4 and 15 mM $MgCl_2$, almost free from OGDC activity. The pellet resulting from the 1.5-4 mM $MgCl_2$ step con-

tains several hundred units of OGDC and PDC activity in almost equal amounts, and is therefore discarded.

Step 4A. Ethanol-Sepharose chromatography of PDC

The PDC pellet from step 3 is resuspended by gentle stirring overnight in 200 ml standard buffer containing 3 mM EDTA. Undissolved material and a brown contaminant are removed by centrifugation (60,000 x g, two times 20 minutes).
The supernatant is applied on a 150 ml ethanol-Sepharose column as described by Visser *et al.* (1978). The column is equilibrated with 10 mM potassium phosphate buffer pH 7.0, containing 1 mM EDTA and 50 µM PMSF. The column is eluted with a 500 ml 0-100 mM KCl gradient in this buffer. All PDC-containing fractions are pooled and concentrated by ultracentrifugation (40,000 x g, 16 hr.).

Step 5A. Gelpermeation chromatography of PDC

The PDC-pellet from the ultracentrifugation step (step 4A) is resuspended by gentle rolling in a small volume of supernatant. The protein solution (7-15 ml, protein concentration approx. 30 mg/ml) is applied on a 2.6 x 95 cm Biogel A5.0m column, eluted with standard buffer at 20 ml/hr, fractions of 5 ml are collected. The fractions that appear to be pure as judged from SDS-gels are pooled. After concentration by centrifugation, the preparation is stored in 0.5 ml portions in liquid nitrogen at a protein concentration of 20-40 mg/ml.

Step 4B. Gelpermeation chromatography of OGDC

The OGDC pellet from step 3 is resuspended by gentle stirring overnight in 80 ml standard buffer. Undissolved material is removed by centrifugation (60,000 x g, two times 20 minutes).
The OGDC is concentrated by centrifugation overnight at 30,000 x g. The resulting pellets are resuspended in a small volume of supernatant by gentle rolling for several hours. The volume at this stage is about 10 ml. The protein solution is applied on a 2.6 x 100 cm Biogel A15m column, eluted with standard buffer at 20 ml/hr, fractions of 5 ml are collected. After control by SDS-gelelectrophoresis, the pure fractions are stored in liquid nitrogen after concentration by centrifugation, like the PDC from *A.vinelandii*.

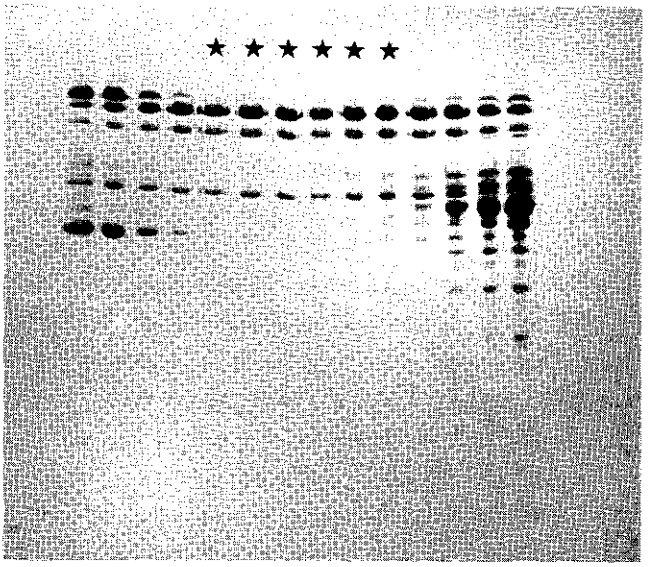


Fig. 2.1. SDS-gelelectrophoresis patterns of the *A.vinelandii* PDC-containing fractions collected after gelpermeation chromatography on Biogel A-5.0m. Each sample contained 0.2 U of PDC activity. The fractions that were pooled are marked.

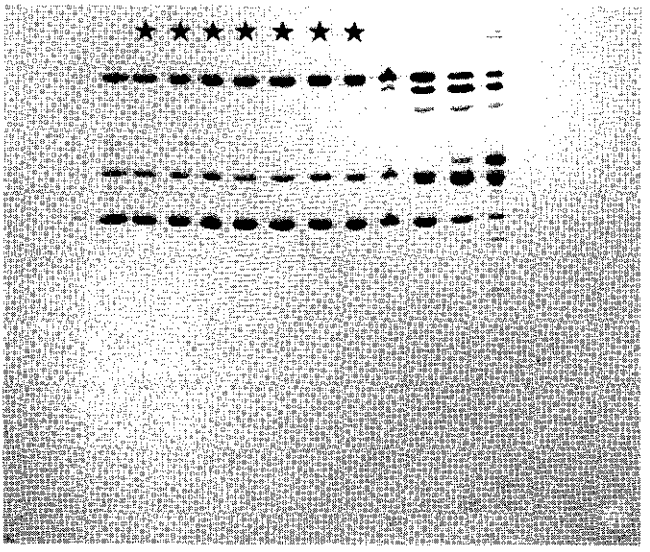


Fig. 2.2. SDS-gelelectrophoresis patterns of *A.vinelandii* OGDC-containing fractions collected after gelpermeation chromatography on Biogel A-15m. Each sample contained 0.15 U of OGDC activity. The fractions that were pooled are marked.

Table 2.1

Purification of *A.vinelandii* PDC

Step	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Ratio PDC/OGDC (U/U)
1. Cell-free extract	1065	8900	18100	0.5	-	1.4
2. Protamine sulfate	1030	11100	12400	0.9	100	1.7
3. PEG 6000/$MgCl_2$	170	8700	2500	3.4	78	13
4A. Ethanol-Sepharose	240	8200	830	9.8	74	54
5A. Biogel A-5,0m	10	4900	280	17.5	44	65

Table 2.2

Purification of *A.vinelandii* OGDC

Step	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Ratio PDC/OGDC (U/U)
1. Cell-free extract	1065	6400	18100	0.4	-	0.7
2. Protamine-sulfate	1030	6700	12400	0.5	100	0.6
3. PEG 6000/$MgCl_2$	94	5200	750	7.0	78	21
4A. Biogel A-15m	6	3400	290	11.9	51	49

This method results in an essentially pure *A.vinelandii* PDC preparation, with an overall yield of 40-50% (Table 2.1). The specific activity of the final preparation is about twice as high as can be obtained with the old procedure (Bresters, 1975a). Moreover, about 80% of the losses can be accounted for in the form of discarded side-fractions, indicating that the complex is hardly inactivated during its isolation. Furthermore, the *A.vinelandii* OGDC also is obtained in a high yield, essentially free from contaminants.

The described conditions for the preparation of cell-free extract proved to be optimal: at lower pressures the lysis of cells decreased significantly, whereas at higher pressures the yield in PDC activity also decreased, probably due to high-shear or heat-inactivation. At these high pressures (up to 80 MPa), some protein denaturation could be observed.

The ratio buffer/cellmass also was optimal: when more cells were suspended in the same volume, an identical yield in PDC activity was obtained, *i.e.* PDC activity per unit weight of bacteria decreased.

The protamine sulfate fractionation (Step 2) is performed at pH 7.0 instead of pH 6.1 as prescribed in the previous procedure, since we have observed a decrease in PDC activity upon acidification of the solution. Also in contrast to the old procedure, PDC is not precipitated with protamine sulfate. The first reason for this decision is the non-quantitative, irreproducible recovery of PDC activity from the protamine sulfate pellet (50-80%). Secondly, and even more important, it proved that the *A.vinelandii* OGDC is inactivated by protamine sulfate: the lipoamide dehydrogenase component is dissociated from the complex and the resulting E_1E_2 subcomplex is coprecipitated with the PDC (see also chapter 3). This subcomplex proved to be a very persistent contaminant of *A.vinelandii* PDC. It could only be removed by isoelectric precipitation, a step that should be avoided, as discussed above. The addition of protamine sulfate is therefore stopped before precipitation of PDC (and the simultaneous inactivation of OGDC) takes place. The addition of protamine sulfate must be carefully monitored by small-scale pilot experiments, since small changes in the composition of the cell-free extract and the quality of the protamine sulfate will lead to variations in the amount of protamine sulfate solution that has to be added.

The separation of PDC and OGDC is performed by PEG/$MgCl_2$ precipitation, as has already been described for the isolation of the complexes from *E.coli* (Eley, 1972). This step leads to an almost complete separation of PDC and OGDC activities (Tables 2.1 and 2.2, Fig. 2.3). The protamine sulfate step is an essential pretreatment, since a PEG/$MgCl_2$ fractionation on cell-free extract leads to coprecipitation of PDC and OGDC.

The PDC preparation from step 3 is further purified on an ethanol-Sepharose column, as described by Visser *et al.* (1978). The type of interaction between solutes and the column material is yet unclear, but it is probably based on a combination of hydrophobic and electrostatic forces. This type of chromatography uses very mild elution conditions, resulting in high yields in

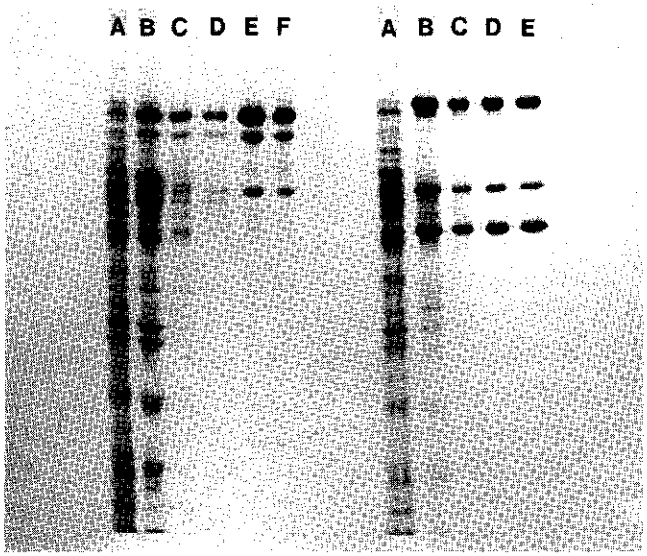


Fig. 2.3. SDS-gelelectrophoresis patterns of samples taken at different stages in the isolation procedure of *A.vinelandii* PDC and OGDC. On the left side of the gel PDC-containing samples were applied: (A), cell-free extract; (B), after PEG/$MgCl_2$ fractionation; (C), after ethanol-Sepharose chromatography; (D), after ultracentrifugation; (E), after gelpermeation chromatography; (F), final preparation.
On the right half some OGDC-samples were applied: (A), cell-free extract; (B), after PEG/$MgCl_2$ fractionation; (C), after ultracentrifugation; (D), after gelpermeation chromatography; (E), final preparation.

combination with a reasonable increase in specific activity. The complex is then concentrated by ultracentrifugation. Previously, this was performed at 144,000 x g for 4.5 hours. We have obtained more quantitative sedimentation of the complex at 40,000 x g during 16 hours. An additional advantage of the latter conditions is the relative ease with which the pellets can be resuspended in a small volume of supernatant: this is performed by gentle rolling or swirling for several hours. This method for the concentration of PDC does not lead to a detectable amount of inactivation, but up to 2 units per milliliter will remain in the supernatant.

The gel permeation chromatography step gives rise to some inactivation (5-20%). This is probably caused by dissociation

of E_1 and/or E_3 from the E_2 core. We have obtained best results with a 2-3% loading (vol./vol.) of a highly concentrated (up to 50 mg/ml) protein solution on the column. The column material should be chosen so that the peak of interest will be eluted at about $K_{av} = 0.8$. In this way, the complex is kept as concentrated as possible, minimizing dissociation effects.

The OGDC preparation from step 3 is already pure enough for gelpermeation chromatography. It is also concentrated by ultracentrifugation (30,000 x g, 16 hours). Care should be taken in sedimenting this complex at higher angular velocities, since it is inactivated by high hydrostatic pressure (*cf.* chapter 4). Fig. 2.3 shows the effect of the several steps in the purification procedure of *A.vinelandii* PDC and OGDC, as visualized on SDS-gels. The isolation procedure yields three-component OGDC and PDC complexes.

Since we have used a relatively mild isolation procedure, the conclusion seems justified that the formerly observed fourth component in the *A.vinelandii* PDC merely was a contaminant. This has been confirmed by sedimentation experiments: no physical interaction between the fourth component and *A.vinelandii* PDC has been detected. The fourth component is a pentameric structure composed of 63,000 Da chains. Its sedimentation coefficient is 19 S, which could explain its copurification (Bosma, 1982; G. Voordouw, personal communication).

3. SOME OBSERVATIONS ON THE 2-OXOGLUTARATE DEHYDROGENASE COMPLEX OF *AZOTOBACTER VINELANDII*

INTRODUCTION

The 2-oxoglutarate dehydrogenase complex (OGDC) has a molecular organization similar to that of the pyruvate dehydrogenase complex (PDC). It is composed of multiple copies of three component enzymes: 2-oxoglutarate dehydrogenase (E_1, EC 1.2.4.1), lipoate succinyltransferase (E_2, EC 2.3.1.61) and dihydrolipoamide dehydrogenase (E_3, EC 1.8.1.4). The three enzymes cooperate in this sequence in the oxidative decarboxylation of 2-oxoglutarate, yielding the succinyl-coenzyme A, CO_2 and NADH.
The complex has been isolated from a number of pro- and eukaryotic sources (Koike, 1960; Hirashima, 1967; Poulsen, 1970; Hirabayashi, 1971; Tanaka, 1972; Parker, 1973; Kornfeld, 1977), the OGDC of *Escherichia coli* being the best-studied complex.
Its E_2 core is composed of 24 chains with a subunit molecular mass of about 45 kDa. The subunits are arranged in a cubic structure with 432 symmetry (DeRosier, 1971; Pettit, 1973). Each E_2 chain carries one lipoic acid residue (Collins, 1977; Angelides, 1979a; White, 1980), and the optimal chain-stoichiometry has been estimated at 12:24:12 (Pettit, 1973).
The OGDC seems much more conserved in evolution than the PDC; the molecular organization of the *E.coli* and pig-heart complexes shows no significant differences (Linn, 1969; Tanaka, 1972 and 1974), and unlike eukaryotic PDC, the OGDC is not regulated by phosphorylation and dephosphorylation in eukaryotes (Koike, 1982).

It proved impossible to isolate the *Azotobacter vinelandii* OGDC with the procedure of Reed and Mukherjee (1968a). The protamine sulfate precipitation step caused dissociation of the E_3 component from the E_2 core, and the resulting E_1E_2 subcomplex was irreversibly inactivated. With the revised isolation procedure described in chapter 2, we were able to obtain a highly purified active complex; some of its characteristics are described in this chapter.

MATERIALS AND METHODS

Materials

The oxoglutarate dehydrogenase complex was isolated from *Azotobacter vinelandii* (ATCC 478) as described in chapter 2. N-ethyl-(2,3-^{14}C)maleimide and 2-oxo(5-^{14}C)glutarate were obtained from the Radiochemical Centre Amersham (UK). Sepharose 6B and Sephacryl S300 were obtained from Pharmacia (Uppsala, Sweden). All biochemicals were from Boehringer (FRG), all other chemicals were analytical grade.

Methods

Enzyme activities were assayed essentially according to Bresters *et al.* (1975a), except that pyruvate was substituted by 2-ketoglutarate (see also chapter 2). Protein concentrations were measured according to the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. The flavin content of the complex preparations was determined by the method of Wassink and Mayhew (1975), using FMN standards.

The lipoyl content of the OGDC was determined by incorporation of ^{14}C-labeled succinyl groups in the E_2 component, after incubation with 2-oxo(5-^{14}C)glutarate in the absence of coenzyme A, analogous to the lipoyl determination of *A.vinelandii* PDC (chapter 6, De Abreu, 1977a).

SDS-gelelectrophoresis was performed according to a modification of the method of Leammli (1970), as described by Dorssers *et al.* (1982). Antisera against *A.vinelandii* lipoamide dehydrogenase (E_3) were obtained as described by De Abreu (1978). Sedimentation analysis was performed with a MSE centriscan 75 centrifuge, equipped with an UV-vis monochromator. Corrections for solvent density and viscosity were made according to standard procedures (Schachman, 1959), assuming a partial specific volume of 0.74 ml/g. The light-scattering measurements were performed as described for *A.vinelandii* PDC in chapter 4; a value of 0.191 ml/g was assumed in dn/dC for the calculation of the molecular mass.

Isolation of the lipoamide dehydrogenase component

The E_3 component of *A.vinelandii* OGDC can be isolated from either purified OGDC or from cell-free extract by the same procedure. All steps are performed at 277 K. To the OGDC-containing solution (2-100 U/ml E_3 activity) in 50 mM potassium phosphate buffer pH 7.0, containing 0.1 mM EDTA, a 2% (w/vol.) solution of protamine sulfate is added until overall OGDC activity is no longer detected.

After centrifugation (15 min, 15,000 x g), the lipoamide dehydrogenase-containing supernatant is brought to 40% saturation in ammonium sulfate. This solution is applied on a sepharose 6B column that is equilibrated with the 40% ammonium sulfate buffer. Under these conditions, lipoamide dehydrogenase strongly binds to the column material. The column is further eluted with 0.5 bed volume of the 40% ammonium sulfate buffer, followed by 0.2 bed volume of 20% ammonium sulfate, and finally it is eluted with 10 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA. Lipoamide dehydrogenase is concentrated on the boundary of the latter two buffers. After elution, the lipoamide dehydrogenase preparation can be concentrated by ultrafiltration, using an Amicon YM 10 filter. If desired, additional purification can be performed by gelfiltration on Sephacryl S300 in 100 mM potassium phosphate buffer pH 7.0, containing 0.5 mM EDTA. The final preparation is pure as judged from SDS-gels, specific activity, the UV-vis absorption spectrum and sedimentation analysis.

RESULTS AND DISCUSSION

Composition of the isolated complex

The subunit molecular masses of the three component enzymes were estimated by SDS-gelelectrophoresis. No abnormal behaviour was observed, as judged from the relative mobilities of the components in gels of varying acrylamide content (7.5-17.5% (w/vol.)). The subunit molecular masses thus obtained are: 103±2 kDa for E_1, 45.2±0.4 kDa for E_2 and 57±2 kDa for E_3. These values are in good agreement with those reported for the components of *E.coli* and pig-heart OGDC (Perham, 1971; Pettit, 1973; Tanaka, 1972 and 1974; Koike, 1974).

The flavin content of the *A.vinelandii* OGDC preparations varied

between 2.8 and 3.5 nmol/mg protein. This value is somewhat low in comparison to that of the *E.coli* OGDC (4.0 nmol/mg, Pettit, 1973); this is probably caused by dissociation of E_3 from the complex during the isolation procedure. For instance, when a complex preparation with a flavin content of 3.2 nmol/mg was subjected to size-exclusion chromatography on Biogel A-15m, its flavin content was reduced to 2.1 nmol/mg. Such a relatively high degree of dissociation of E_3 from the complex during the isolation has also been observed for the OGDC of cauliflower (Poulsen, 1970).

The relative ease with which the E_3 component can be dissociated from the complex can also be used to obtain an E_1E_2 subcomplex. When the complex is bound to hydroxyl appetite in 10 mM potassium phosphate buffer (through the E_3 component), an E_1E_2 subcomplex can be eluted with 100 mM potassium phosphate (pH 7.0), in a 30% yield. This subcomplex can be reconstituted with isolated E_3, yielding an enzymatically active complex. An optimal chain-stoichiometry can however not be obtained from these experiments, since there is no linear correlation between the amount of E_3 added and the overall activity; *i.e.* the binding of the first E_3 to the E_2 core has a more pronounced effect on the overall activity than the binding of following E_3 dimers (Fig. 3.1).

A large excess of E_3 can be bound to the complex; in reconstitution experiments we have obtained complex preparations with a flavin content of about 10 nmol/mg protein. Similar observations have been made for the complex of *E.coli* (Pettit, 1973).

We have determined the amount of lipoyl groups by the incorporation of radioactively labeled succinyl groups. ^{14}C-labeled 2-oxoglutarate was added to the complex in the absence of coenzyme A. Under these conditions, desuccinylation cannot take place, and the (covalently bound) radioactive succinyl groups will be coprecipitated with the protein upon addition of trichloroacetic acid, analogous to the acetyl incorporation in PDC, as described in chapter 6.

These experiments yielded lipoyl contents of 3-5 nmol/mg OGDC, which is a very low value in comparison to the lipoyl contents reported for the *E.coli* OGDC. For the OGDC of that organism, a value of about 9 nmol/mg was reported, which corresponds to one

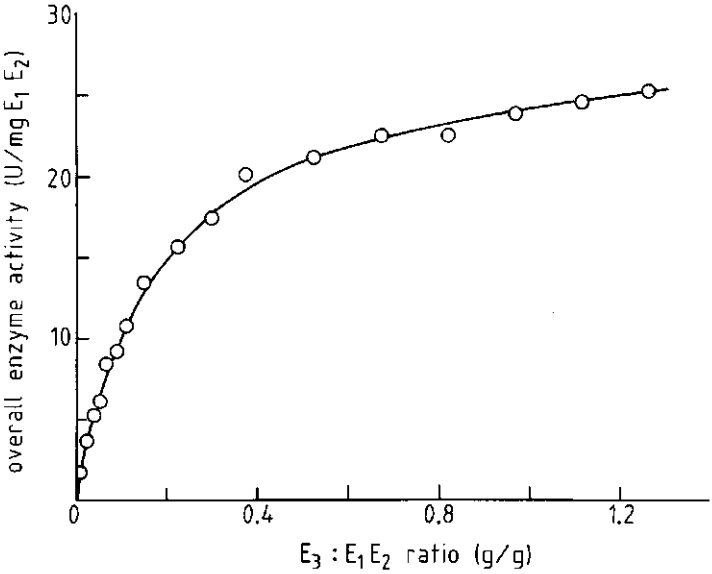


Fig. 3.1. Reconstitution of *A.vinelandii* OGDC from its isolated E_3 component and an E_1E_2 subcomplex (obtained by hydroxyl appetite chromatography). Varying amounts of E_3 (0-9 µg) were added to 1 ml overall enzyme assay mixture in a cuvette. The assay was started by the addition of a fixed amount (6.2 µg) of the E_1E_2 subcomplex.

lipoyl group per E_2 chain (Collins, 1977; Angelides, 1979a, White, 1980). Before the paper of Collins and Reed, reports on the lipoyl content of *E.coli* OGDC mentioned a value of 5 nmol/mg (Koike, 1960; Pettit, 1973), as we have also found for the *A.vinelandii* OGDC. Clearly, the determination of lipoyl contents by this method can give false results. In one experiment, we directly compared the incorporation of ^{14}C-succinyl groups in the OGDC's from *E.coli* and *A.vinelandii*; we obtained a value of 4.9 nmol/mg for both complexes. This indicates that the observed discrepancy between our results and those reported by others probably resides in the method of determination, and does not reflect a difference between the complexes of the two sources.

The *A.vinelandii* OGDC has a sedimentation coefficient of 35±1 S (9 experiments with 3 preparations). From laser light-scattering experiments we calculated a molecular mass of 2.5 ± 0.2 MDa for a complex preparation with a flavin content of 2.1

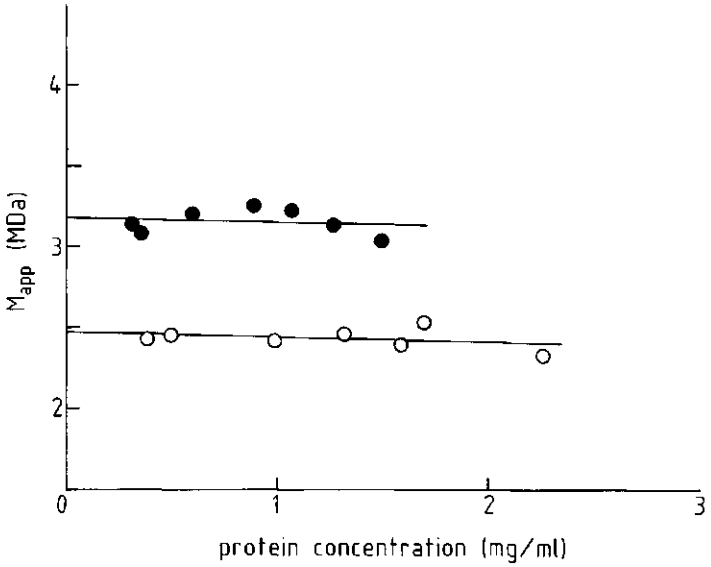


Fig. 3.2. The apparent molecular mass of *A.vinelandii* OGDC, as determined by laser light-scattering at various protein concentrations. A value of 0.191 ml/g was used in the calculation of the molecular weight from the experimental results: (o), an OGDC preparation with a flavin content of 2.1 nmol/mg (•), the same preparation with E_3 added to a flavin content of 4.0 nmol/mg.

nmol/mg. When E_3 was added to this preparation, resulting in a flavin content of 4.0 nmol/mg, a molecular mass of 3.2 ± 0.3 MDa was found (Fig. 3.2). These values are comparable to those reported for the complexes from *E.coli* and pig-heart (Hirashima, 1967; Pettit, 1973).

Enzyme kinetics

The bacterial OGDC's can be divided into two classes: the *E.coli* complex mainly being regulated by the NAD/NADH and succinyl-CoA/CoA ratios, whereas the complexes of *Acinetobacter lwoffi* and *Acetobacter xylinum* are also regulated by cooperative binding of 2-oxoglutarate and nucleotides (Parker, 1973; Kornfeld, 1977 and 1978). The complex of *A.xylinum* is also different with respect to the subunit molecular mass of the E_2 component; a subunit molecular mass of 75 kDa was found and the E_2 core is composed of 12 chains (De Kok, 1980).

Table 3.1.

Kinetic parameters of *A.vinelandii* OGDC

Substrate	K_m (μM)	K_i (μM)	Type of inhibition, remarks
CoASH	14		
NAD^+	180		
2-oxoglutarate	60		no cooperativity
NADH		13	competitive with NAD^+
oxaloacetate		150	competitive with 2-keto-glutarate
2-ketoadipate	28		3% of activity of 2-ketoglutarate

The effects of some compounds on the *A.vinelandii* OGDC activity are summarized in table 3.1; the substrate specificity resembles that of the OGDC from yeast (Hirabayashi, 1971). Pyruvate, 2-oxobutyrate, glutamine and glutamic acid have no effect on the OGDC activity. The *A.vinelandii* OGDC resembles the complex of *E.coli* with respect to its relatively simple regulation mechanism. Due to the anaplerotic function of the citric acid cycle in the metabolism of *A.xylinum* and *A.lwoffi* (gluconeogenesis), the complexes of these organisms probably need to be more finely tuned (Weinhouse, 1972).

Comparison of the lipoamide dehydrogenase component in the PDC and OGDC of Azotobacter vinelandii

In the case of *E.coli*, it has been assessed that the lipoamide dehydrogenase component (E_3) of the PDC and OGDC are identical (Pettit, 1967); there is only one E_3 gene in *E.coli* (Guest, 1973).

To establish the identity of *A.vinelandii* E_3 in PDC and OGDC, we have performed a blotting experiment. We treated the PDC and OGDC of *A.vinelandii* with various amounts of chymotrypsin and papain. The proteolytic breakdown was stopped after 15 minutes by incubating the sample in SDS-containing buffer at 373 K for 5 min. After electrophoresis, the peptides were trans-

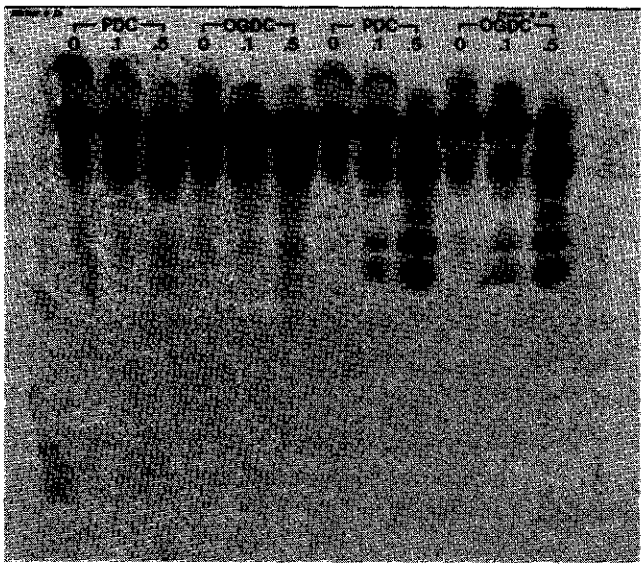


Fig. 3.3. Comparison of the lipoamide dehydrogenase component of *A.vinelandii* PDC and OGDC. 150 µg samples of *A.vinelandii* PDC and OGDC in 40 µl 0.05 M Tris-Cl pH 8.0 containing 2% (w/vol.) SDS were heated for 1 minute at 373 K. After cooling to room temperature, 0, 0.1 or 0.5 µg of chymotrypsin (left side of the blot) or papain (right side) was added. After 15 minutes, the proteolytic breakdown was stopped by heating the samples at 373 K for 5 minutes.
After electrophoresis, the peptides were transferred to nitrocellulose (Western blotting; Bowen, 1980; for experimental details: Voordouw, 1982). The nitrocellulose sheet was incubated with antilipoamide dehydrogenase serum (obtained from OGDC-E_3) and ^{125}I-protein A. The sheet was autoradiographed for 24 hours at 203 K, using an intensifying screen.

ferred to nitrocellulose (Western blotting; Bowen, 1980). The nitrocellulose blot was then treated with antiserum raised against (OGDC) E_3. To those antibodies, ^{125}I-labeled protein A was coupled, and the antigenic bands were visualized by autoradiography. Fig. 3.3 shows that identical breakdown patterns are observed for the OGDC and PDC of *A.vinelandii* which is a good indication that the E_3 components of the two complexes are identical.

Conclusion

Although we have not extensively studied the OGDC of *A.vinelandii* we conclude from our measurements that the molecular organization of this complex probably is similar to that of *E.coli* and pig-heart OGDC. In its kinetic behaviour it resembles the *E.coli* complex. This conclusion agrees with the idea that the complex is rather well conserved in evolution.

4. THE SIZE OF THE PYRUVATE DEHYDROGENASE COMPLEX OF *AZOTOBACTER VINELANDII*; ASSOCIATION PHENOMENA

INTRODUCTION

The pyruvate dehydrogenase complex (PDC) from *Azotobacter vinelandii* is smaller than PDC's from other sources. Its sedimentation coefficient has been determined at 19 S and from light-scattering measurements a molecular mass of 1.0 - 1.2 MDa was calculated (Bresters, 1975a). The well-studied PDC from *Escherichia coli* has a sedimentation coefficient of 53-60 S and estimates of its molecular mass range from 3.75 - 6.1 MDa (Vogel, 1972b; Eley, 1972; Danson, 1979). PDC's from gram-positive bacteria and eukaryotes are even larger (Reed, 1974; Henderson, 1980; Stanley, 1980; Kresze, 1981b).

A good estimation of the molecular mass of a multienzyme complex is essential for the interpretation of measurements concerning its composition. This can be demonstrated for the PDC of *E.coli*. It is generally accepted that its E_2 core is composed of 24 chains, arranged in a cube with 432 symmetry. E_1 dimers and E_3 dimers are non-covalently bound to this core and E_1:E_2:E_3 stoichiometries of 48:24:24 and 24:24:12 have been proposed (Bates, 1975b; Eley, 1972). Clearly, a good estimation of the molecular mass of the complex would distinguish between the models. The estimation of molecular masses of this size is however extremely difficult and it is even further complicated by the heterogeinity of the PDC preparations (Schmitt, 1980; Gilbert, 1980).

The *A.vinelandii* PDC preparations that were used in the previous light-scattering experiments had a low specific activity (6 U/mg protein) and contained the so-called fourth component (Bresters, 1975a; De Abreu, 1977b; Bosma, 1982). We have therefore repeated these measurements with the three-component, high specific activity preparations (15-19 U/mg protein) that were obtained with the revised purification procedure as described in chapter 2.

Although the PDC's from *A.vinelandii* and *E.coli* differ in molecular mass, a 17-20 S minor component has been observed in

some *E.coli* PDC preparations (Schmitt, 1975; Danson, 1979). Before the start of the present study, some observations had already indicated the existence of a 50-60 S form of the *A.vinelandii* PDC (G. Voordouw, personal communication). The complexes of the two organisms could therefore be much more similar than had previously been assumed. This hypothesis has been further investigated by sedimentation analysis and pressure-dependent light-scattering measurements on *A.vinelandii* PDC, the isolated E_2 component, and reconstituted subcomplexes.

MATERIALS AND METHODS

Pyruvate dehydrogenase complex was isolated from *Azotobacter vinelandii* as described in chapter 2. The complex was resolved into its component enzymes by the method of De Graaf and De Kok Kok (1982). Protein concentrations were measured by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Sedimentation analysis was performed with a MSE analytical ultracentrifuge (UV-absorption and schlieren optics) or with a MSE centriscan 75, equipped with an UV-monochromator. Corrections for the solvent density and viscosity were made according to standard procedures (Schachman, 1959); a partial specific volume of 0.74 was assumed for the complex. For the E_2 component, a partial specific volume of 0.749 ml/g was calculated from the amino acid composition (see chapter 5), according to the method of Cohn and Edsall (1943). The viscosity of the solutions containing polyethylene glycol 6000 were measured in comparison to the buffer solutions without PEG 6000, using an Ostwald viscosimeter.

The light-scattering measurements were performed with a Spectra-Physics model 120 He-Ne laser. Optical equipment was from Microcontrole (France). Measurements were performed at angles between 30° and 150°. A value of 0.191 ml/g was used for dn/dC to calculate the molecular mass (Tanford, 1961). Samples were filtered at least three times through 0.45 µm Acrodisc filters (Gelman, USA) to remove dust particles. Diffusion coefficients of the complex and the isolated E_2 component were determined by the quasi-elastic light-scattering method (Johnson,

1981), using a low-frequency spectrum-analyser, Hewlett Packard 3582A.

For the high-pressure experiments an octagonal glass cell (4 ml volume) was completely filled with protein solution. A thin rubber membrane was placed on top of this cell to isolate the protein solution from the distilled kerosin oil that was present as hydraulic fluid in the pressure-vessel. This membrane was tightly kept in place by a ring-shaped brass screw-on cap, and pressure was transferred to the protein solution through this thin rubber membrane.
The pressure vessel contained three windows: two opposite to each other, for the laser-beam, and one at 90^{o} for the detection of scattered light. Pressure was applied manually through a hydraulic system (Nova, Switzerland). In this experimental set-up, pressure could be raised and lowered reversibly up to 80 MPa (800 bar). In most cases, the protein sample could be recovered after the measurement, with almost no contamination with the kerosin.
Pressure-jump experiments were performed as described by Engelborghs (1983), the maximum pressure applied was 25 MPa.

RESULTS

The 18 S form of the complex

At a protein concentration of 1.5-2.0 mg/ml, the sedimentation coefficient of the *A.vinelandii* PDC is between 17.5 and 19.0 S, depending on the preparation. At protein concentrations below 1 mg/ml the sedimentation coefficient decreases (Fig. 4.1). This phenomenon was already observed by Bresters *et al.* (1975a), and interpreted as a dissociation of components from the complex. We determined the molecular mass of the complex from laser light-scattering measurements. At the wavelength used (633 nm), hardly any dissymmetry was observed (R_{45}/R_{135} <1.05). This is in contrast to the observations of Bresters *et al.* (1975a), who measured a dissymetry of about 1.4. Therefore, a high-molecular mass contaminant must have been present in their preparations. This could also explain the high value of 1 to 1.2 MDa that was found for the molecular mass of the complex at infinite dilution.

Fig. 4.2 shows the apparent molecular mass as a function

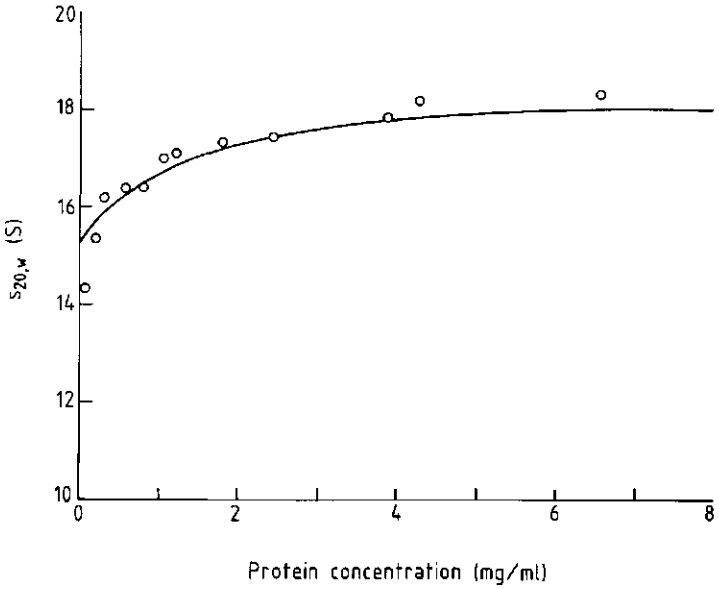


Fig. 4.1. Concentration-dependence of the sedimentation coefficient ($s_{20,w}$) of the pyruvate dehydrogenase complex of *Azotobacter vinelandii*; (0), experimentally determined values in 50 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM EDTA, temperature was 293 K. The drawn curve represents the sedimentation coefficient as a function of protein concentration, calculated for a monomer-dimer equilibrium. In this calculation, a dissociation constant of 6.86 μM was used (monomer molecular mass: 784 kDa). The sedimentation coefficient of the monomer was estimated at 15.3 S, that of the dimer at 22.0 S. Correction for the influence of the protein concentration was made according to $s_{20,w\ obs} = s_{20,w,0}(1-0.007 \times [\text{protein (mg/ml)}])$ (Schachman, 1959).

of the protein concentration; it parallels the concentration-dependence of the sedimentation coefficient. At very low protein concentrations a molecular mass of about 800 kDa was found. It is clear that an association-dissociation equilibrium must exist.

When association-dissociation phenomena are accompanied by volume changes, it is expected that pressure will influence the equilibrium, due to the $p\Delta V$ term in the Gibbs free energy (Weber, 1983). When all other factors remain constant, the intensity of scattered light will be linearly corellated with the

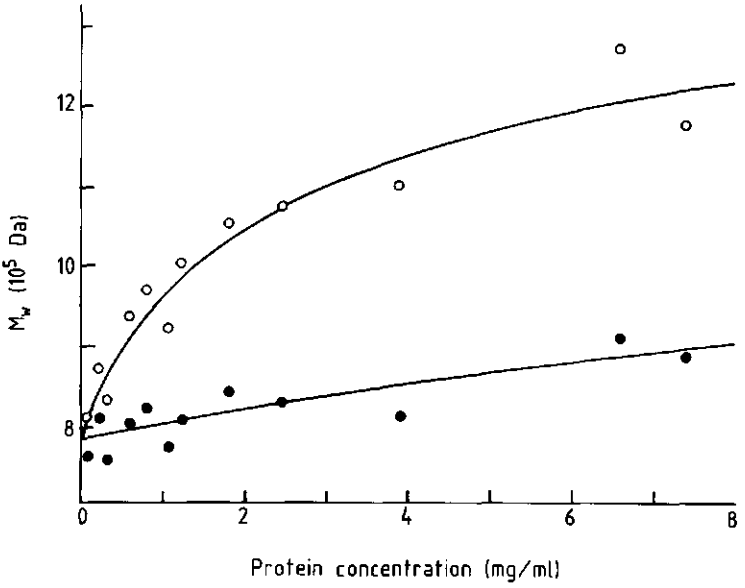


Fig. 4.2. The weight-average molecular mass of the pyruvate dehydrogenase complex of *Azotobacter vinelandii*, as determined at various protein concentrations in 50 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM EDTA; temperature was 293 K. The molecular mass was calculated from the intensity of scattered light using a value of 0.191 ml/g for dn/dC. (○), molecular mass at 0.1 MPa (room pressure); (●), molecular mass at 80 MPa (800 Bar). The drawn curves represent the calculated concentration dependence of the weight-average molecular mass of a monomer-dimer equilibrium, using the following data: monomer molecular mass, 784 kDa; dissociation constant at 0.1 MPa, 6.86 μM; dissociation constant at 80 MPa, 89.3 μM.

weight-average molecular mass of the protein particles, *i.e.* when the intensity of scattering of a solution of a dimeric protein is halved upon the application of pressure, this is a good indication that monomerization has occurred. We therefore investigated the *A.vinelandii* PDC equilibrium by light-scattering measurements at different pressure.
When pressure is applied on a sample of *A.vinelandii* PDC, the intensity of scattered light decreases; the effect being fully reversible (Fig. 4.3). The relative size of this effect increases with protein concentration, but the apparent molecular mass at 80 MPa (800 Bar) is almost independent of the protein concentra-

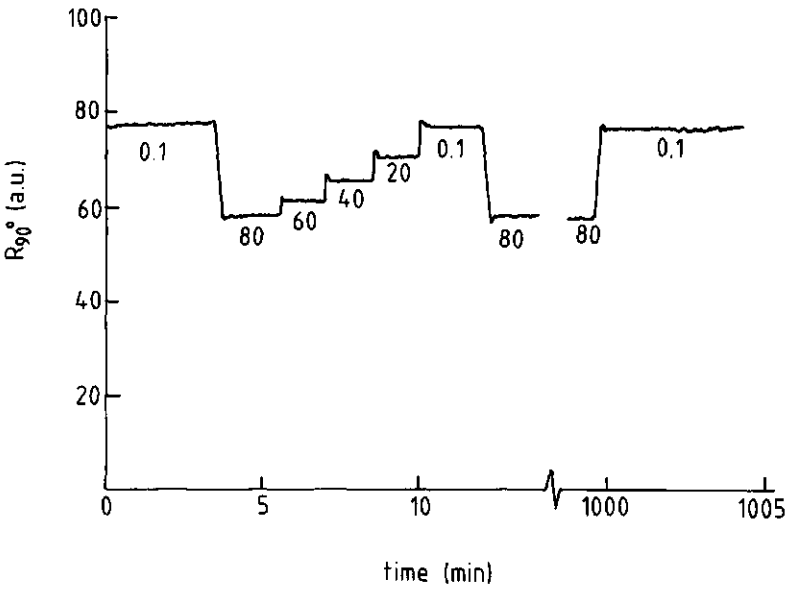


Fig. 4.3. Effect of pressure on the intensity of light-scattering of a sample of the pyruvate dehydrogenase complex of *Azotobacter vinelandii*. Protein concentration was 7.4 mg/ml in 50 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM EDTA; temperature was 293 K. Pressure is given in MPa.

tion, with values between 750 and 900 kDa (Fig. 4.2). We interpret these observations in terms of a monomer-dimer equilibrium, the monomer being a particle with a molecular mass of about 800 kDa. With this hypothesis, the experimental data can be approximated with a dissociation constant of 6.9 μM at 0.1 MPa and 89 μM at 80 MPa (drawn lines in Fig. 4.2).

Due to variations in the position of the cuvette in the high-pressure light-scattering apparatus, the absolute intensity of scattered light is a major source of error in the estimation of the molecular mass. Errors in the determination of protein concentration also contribute considerably to the "overall" error. Relative measurements of the intensity of scattered light at 0.1 MPa and 80 MPa circumvent these sources of error, and this ratio can therefore be determined more accurately. The experimental values of this ratio are shown in Fig. 4.4, together with the theoretically predicted concentration dependence as calculated with the dissociation constants given above. At

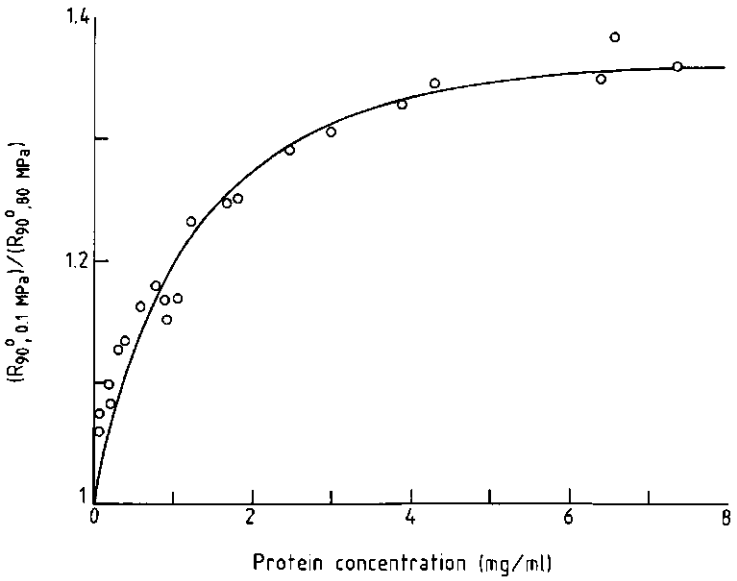


Fig. 4.4. Ratio of the intensity of scattered light at 0.1 MPa and 80 MPa of the pyruvate dehydrogenase complex of *Azotobacter vinelandii*, measured at various protein concentrations. The drawn curve represents the calculated ratio for a monomer-dimer equilibrium using the data given in the legend to Fig. 4.2.

protein concentrations above 1 mg/ml the fit is within experimental error, at very low protein concentrations the observed decrease in scattering after application of high pressure is however more pronounced than is calculated from the model.

The drawn curve in Fig. 4.1 represents the concentration-dependence of the sedimentation coefficient, assuming a monomer-dimer equilibrium with the given dissociation constant. At very low protein concentrations, the observed sedimentation coefficient is lower than is predicted by the model. Presumably at these very low protein concentrations, some dissociation of components occurs. This is also indicated by the observation that the complex is inactivated when it is stored at protein concentrations below 0.3 mg/ml. This inactivation occurs on a time-scale of fifteen minutes to a few hours.

For all samples that were used in the high-pressure experiments, the scattering intensity did not change during the observation

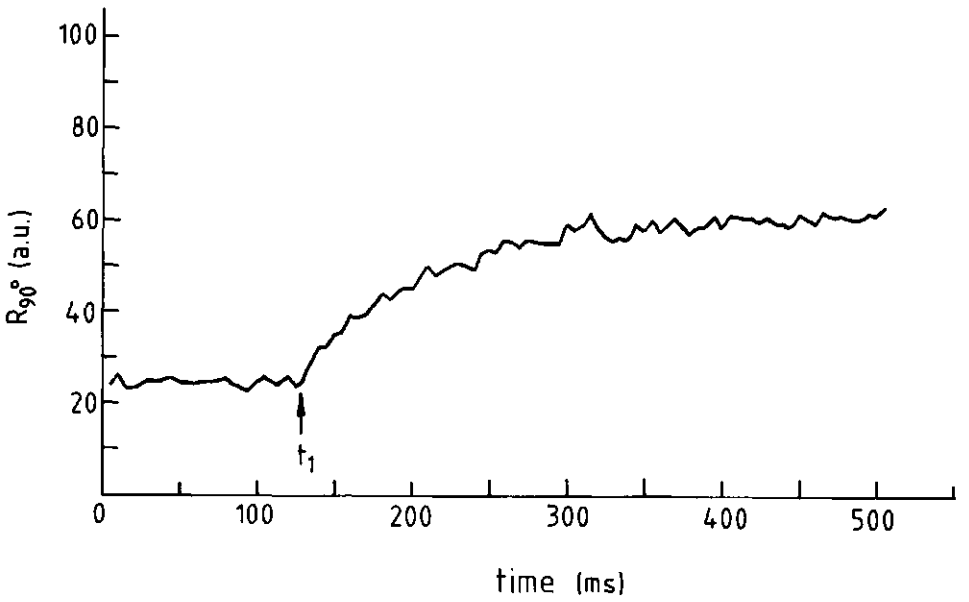


Fig. 4.5. Result of a pressure-jump experiment on the pyruvate dehydrogenase complex of *Azotobacter vinelandii*. A pressure of 25 MPa was applied manually by means of a hydraulic system on a sample of *A.vinelandii* PDC (protein concentration 6 mg/ml) in 50 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM EDTA. At the time indicated in the figure as t_1, pressure was released within a few milliseconds. The intensity of light-scattering was followed at 90°, temperature was 293 K.

time (30 min - 2 h), if the pressure remained constant. With one sample, we did not observe any change in scattering during a period of 16 hours at 80 MPa.

Pressure-induced changes in the intensity of scattered light always occurred within the time needed to change the pressure applied on the sample (a few seconds). To estimate the relaxation time of the equilibrium, we therefore performed some pressure-jump experiments. Due to experimental restrictions (maximal attainable pressure was 25 MPa), only a few observations at high protein concentrations (3-6 mg/ml) could be made. These measurements yielded an average relaxation time of 70 ms (Fig. 4.5).

The 56 S form of the complex

In some preparations of *A.vinelandii* pyruvate dehydrogenase complex, a faster sedimenting species was observed after prolonged storage in liquid nitrogen (longer than two months). Its sedimentation coefficient was between 52 and 60 S, and in this thesis it will be referred to as the 56 S form of the complex. The relative amount of this 56 S form varied between 0 and 50%, depending on the preparation that was used. The formation of the 56 S form was not accompanied by a detectable loss in enzyme activity. Thus, this large form of the complex is probably catalytically active.

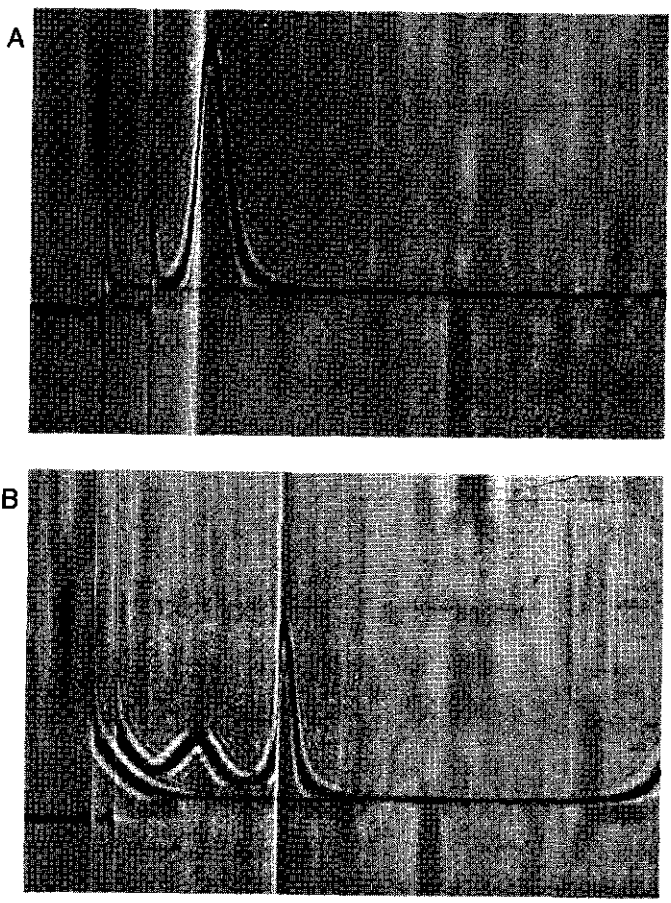


Fig. 4.6. Sedimentation velocity Schlieren patterns of the pyruvate dehydrogenase complex of *Azotobacter vinelandii*. Temperature was 293 K; protein concentration was 3 mg/ml in 50 mM potassium phosphate buffer (pH 7.0). (A), without PEG 6000; (B), with 3% (w/vol) PEG 6000 and 10 mM $MgCl_2$. Sedimentation is from left to right.

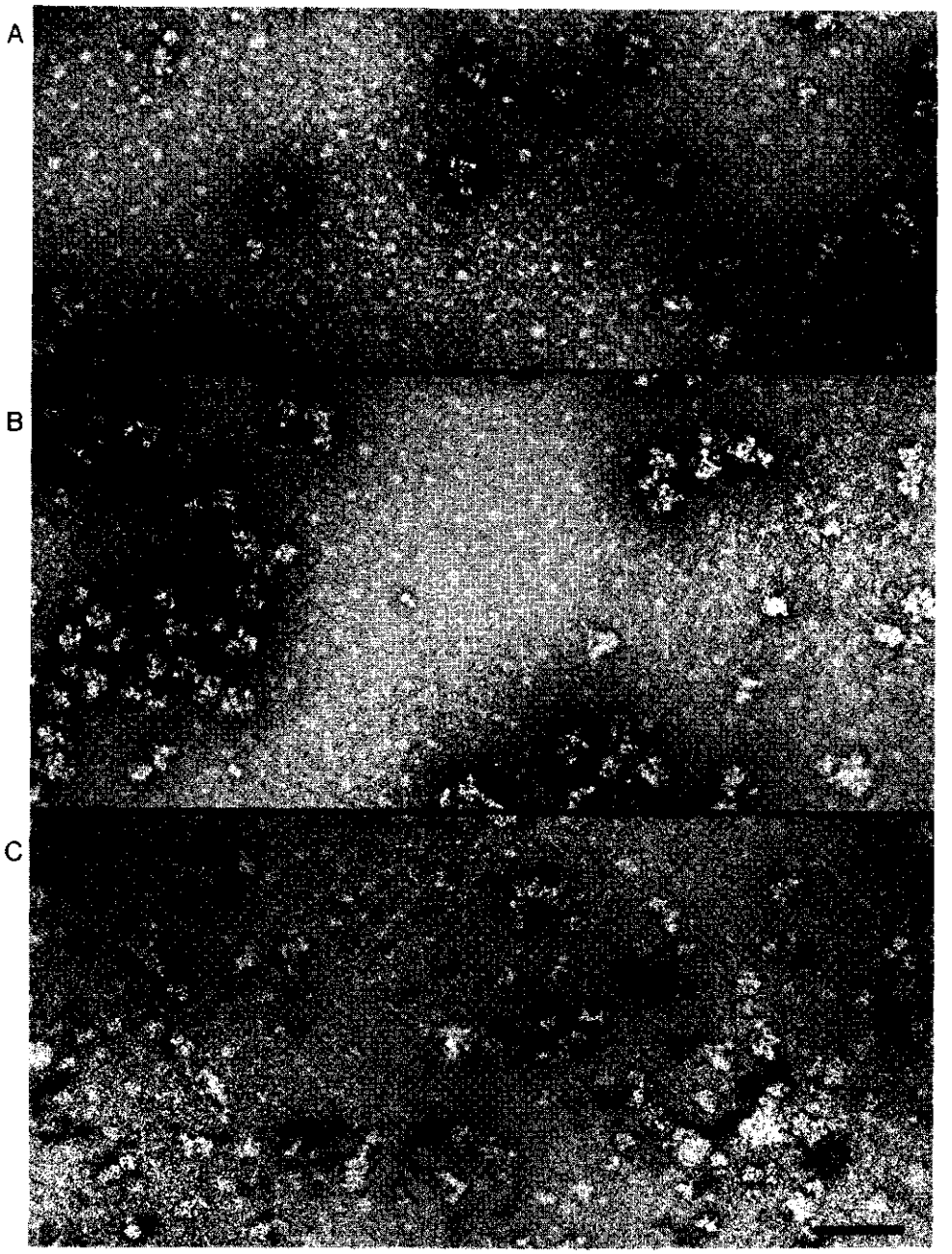


Fig. 4.7. Electron micrographs of the pyruvate dehydrogenase complex of *Azotobacter vinelandii*. Complex (5 mg/ml) in 50 mM potassium phosphate buffer (pH 7.0) with (A,B) or without (C) 3% (w/vol.) PEG 6000 and 10 mM $MgCl_2$ was incubated with 2% (w/vol.) glutaraldehyde for 15 minutes at room temperature. Uranyl acetate was used as a negative stain, bar represents 100 nm.

In freshly isolated *A.vinelandii* PDC preparations, we never detected the 56 S form. All material sedimented with a sedimentation coefficient of 17-19 S, as is shown in Fig. 4.6A. This form of the complex will be referred to as the 18 S form. However, upon addition of 3% (w/vol) polyethylene glycol 6000 (PEG 6000) and 10 mM $MgCl_2$, a large fraction of the 18 S material was converted into the 56 S form (Fig. 4.6B). Under the conditions as described in the legend to Fig. 4.6, 40-90% of the material was converted into the faster sedimenting species (depending on the preparation).

The formation of the 56 S species is favoured by an increase in temperature, in polyethylene glycol concentration, in ionic strength of the buffer solution, and in protein concentration. These observations indicate that hydrophobic interactions are important in the association process. The addition of 10 mM $MgCl_2$ shifts the equilibrium far more to the 56 S form than is expected from the increase in ionic strength. The value of the sedimentation coefficient of the faster sedimenting peak, as well as its sharpness, are not affected by these variations. This indicates that the addition of PEG causes self-association of the *A.vinelandii* pyruvate dehydrogenase complex to a particle of a similar size as the *E.coli* complex.

The distinct nature of this self-association follows from the fact that little material of intermediate sedimentation coefficient (30 S $< s_{20,w} <$ 50S) is observed. These results are confirmed by electron microscopy. Crosslinking of the complex in the presence of 3% PEG 6000 gives rise to a rather uniform population of spherical protein particles with a diameter of 30-40 nm (Fig. 4.7A). Although the electron micrographs of the 56 S form of the *A.vinelandii* PDC show little detail, the shape and overall dimensions of the 56 S particles are comparable to those of *E.coli* PDC (Reed 1968b).

The crosslinking procedure (see legend to Fig. 4.7) leads also to intermolecular crosslinking of the 30-40 nm protein particles (Fig. 4.7B). In the absence of PEG 6000 no distinct complex particles can be seen although some crosslinked protein aggregates are observed (Fig. 4.7C). We have not succeeded in obtaining satisfactory electron micrographs of the 18 S form of the *A.vinelandii* complex.

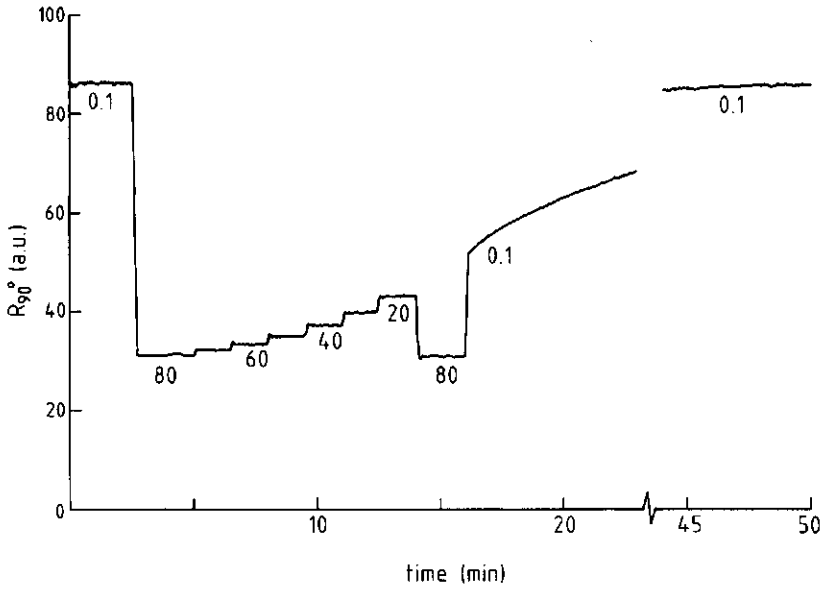


Fig. 4.8. The effect of pressure on the intensity of scattered light of a sample of *Azotobacter vinelandii* pyruvate dehydrogenase complex in the presence of 3% (w/vol) PEG 6000 and 10 mM $MgCl_2$. Protein concentration was 1.1 mg/ml in 50 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM EDTA; temperature was 293 K. The applied pressure is expressed in MPa.

In the presence of PEG 6000, the (corrected) sedimentation coefficient of the slower sedimenting boundary varied between 22 and 28 S; the higher value was observed when conditions were applied that favoured the formation of the 56 S form. The sedimentation coefficient was always higher than could be expected for the proposed dimeric form of the *A.vinelandii* PDC, and these observations indicate the existence of a rapid equilibrium between the dimer and another aggregation state of the complex.

The PEG-induced equilibria were also studied by light-scattering measurements at high pressure. The effect of pressure on the intensity of scattered light is much more pronounced in the presence of PEG 6000 and 10 mM $MgCl_2$, as compared to a sample without these additions (Fig. 4.8, Fig. 4.3). When the pressure is released, an increase in scattering intensity is observed that occurs within the "dead" time of the experimental set-up (a few seconds). The equilibrium level at 0.1 MPa is further reached

by a slower process with a relaxation time of the order of minutes (Fig. 4.8).

The determination of protein concentrations in the presence of PEG 6000 proved to be very inaccurate, and sometimes small amounts of protein aggregates were retained by the filter after the addition of PEG. Furthermore, the value of dn/dC may change upon addition of PEG, and the high-molecular weight forms of the complex may show more dissymetry. For these reasons, an accurate estimation of the weight-average molecular mass of the PEG-samples cannot be made.
Nevertheless, at 80 MPa the intensity of scattered light appeared to be at least 1.5 times as high as compared to samples without PEG 6000. This is a minimum value, based on the amount of protein present in the sample prior to filtering. Within experimental error, this ratio was independent of the protein concentration.

Relative changes in the intensity of scattered light can be determined more accurately. The ratio of the "fast" 0.1 MPa level and the 80 MPa level varied between 1.05 and 1.85. The ratio between the equilibrium levels at 0.1 and 80 MPa varied between 1.16 and 2.90. The lower values correspond to a sample without $MgCl_2$ and a protein concentration of 0.2 mg/ml, the higher values correspond to a sample with 10 mM $MgCl_2$ and a protein concentration of about 4 mg/ml. In this sample, 66% of the material was converted into the 56 S form, as was determined by sedimentation analysis. Due to the precipitation of magnesium phosphate, which occurred frequently at high pressure, only a few measurements could be performed in the presence of PEG and 10 mM $MgCl_2$.

Although the 56 S boundary always appeared well-separated from the smaller forms of the complex in analytical sedimentation experiments, we did not succeed in the isolation of this form by preparative ultracentrifugation or exclusion chromatography. This observation indicates that the slow increase in scattering intensity after the release of high pressure reflects the formation of 56 S material.

It should be emphasized at this point that the pressure effects described here seem to be unique for the *A.vinelandii* PDC. No effects were observed with the *E.coli* PDC in the range of pressures used (0.1 - 80 MPa).

Up to 40 MPa, little effect was observed on the 2-OGDC of *A.vinelandii*. At higher pressures some dissociation was observed, which was followed by the formation of large structures (500-600 S). The latter aggregation process was accompanied by a corresponding loss in enzyme activity. One should therefore be careful in the use of ultracentrifugal forces in the isolation of this complex.

The quaternary structure of the isolated E_2 component; Reconstitution of E_1E_2 and E_2E_3 subcomplexes

The isolated E_2 component of the *A.vinelandii* pyruvate dehydrogenase complex has a sedimentation coefficient of 20.8 ± 0.8 S (5 experiments with different preparations), this value is in good agreement with that reported for the isolated *E.coli* E_2 component (Koike, 1963). From sedimentation equilibrium experiments we calculated a molecular mass of 1.90 ± 0.14 MDa for the *A.vinelandii* E_2 component (12 experiments with 5 different preparations).

From a light-scattering measurement at a protein concentration of 0.5 mg/ml we calculated a molecular mass of 1.97 ± 0.1 MDa, using a value of 0.191 ml/g for dn/dC. The diffusion coefficient is 1.04 ± 0.04 x 10^{-7} cm^2/s, as determined with the dynamic light-scattering technique (Johnson, 1981). In combination with the sedimentation coefficient of 20.8 ± 0.8 S this yields a molecular mass of 2.0 ± 0.1 MDa (Schachman, 1959).

On SDS-gels the E_2 chains migrate with an apparent molecular mass of 83 kDa. No abnormal behaviour was detected as judged from relative migration on SDS-gels with acrylamide concentrations between 7.5 and 17.5% (w/vol.). The behaviour of the E_2 component of *A.vinelandii* PDC is in this respect similar to that of *E.coli* E_2 (Perham, 1971; Vogel, 1977). For the E_2 component of that organism, a subunit molecular mass of about 80 kDa was estimated from exclusion chromatography in the presence of 5 M guanidinium hydrochloride (Angelides, 1979b; Danson, 1979). A value of 65-70 kDa was however calculated by Eley *et al.* from sedimentation equilibrium experiments in 5 M guanidinium hydrochloride (Eley, 1972). The latter value has recently been confirmed by the elucidation of the DNA-sequence of *E.coli* E_2; a molecular mass of 66 kDa was calculated (Stephens, 1983b). We

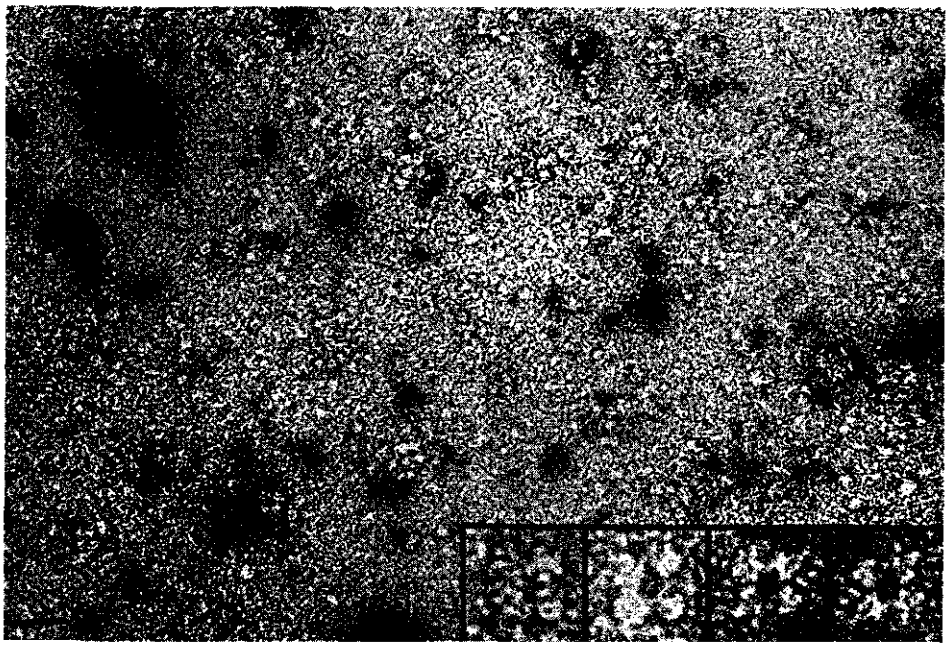


Fig. 4.9. Electron micrograph of the isolated E_2 component of the pyruvate dehydrogenase complex of *Azotobacter vinelandii*. The E_2 component (0.8 mg/ml) was crosslinked by reaction with 2% (w/vol.) glutaraldehyde in 50 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM EDTA at room temperature for 10 minutes.
The sample was applied on the grid by the spray-on method. Uranyl acetate was used as a negative stain; bar represents 50 nm. Inserts represent three-fold magnifications of selected structures.

have therefore repeated the sedimentation equilibrium experiment in 5 M guanidinium hydrochloride for the E_2 components of both organisms. This experiment yielded values of 61 kDa and 63 kDa for the E_2 components of *A.vinelandii* and *E.coli* respectively. This observation confirms the similarity between the E_2 components of the two organisms, allowing us to assume a subunit molecular mass of 66 kDa for the E_2 component of *A.vinelandii* PDC.

Fig. 4.9 shows an electron micrograph of the isolated E_2 component of *A.vinelandii* PDC. Its appearance closely resembles that of *E.coli* E_2: a cube of 13-15 nm with protein-dense structures at the corners (Reed, 1968b). From the sedimentation data,

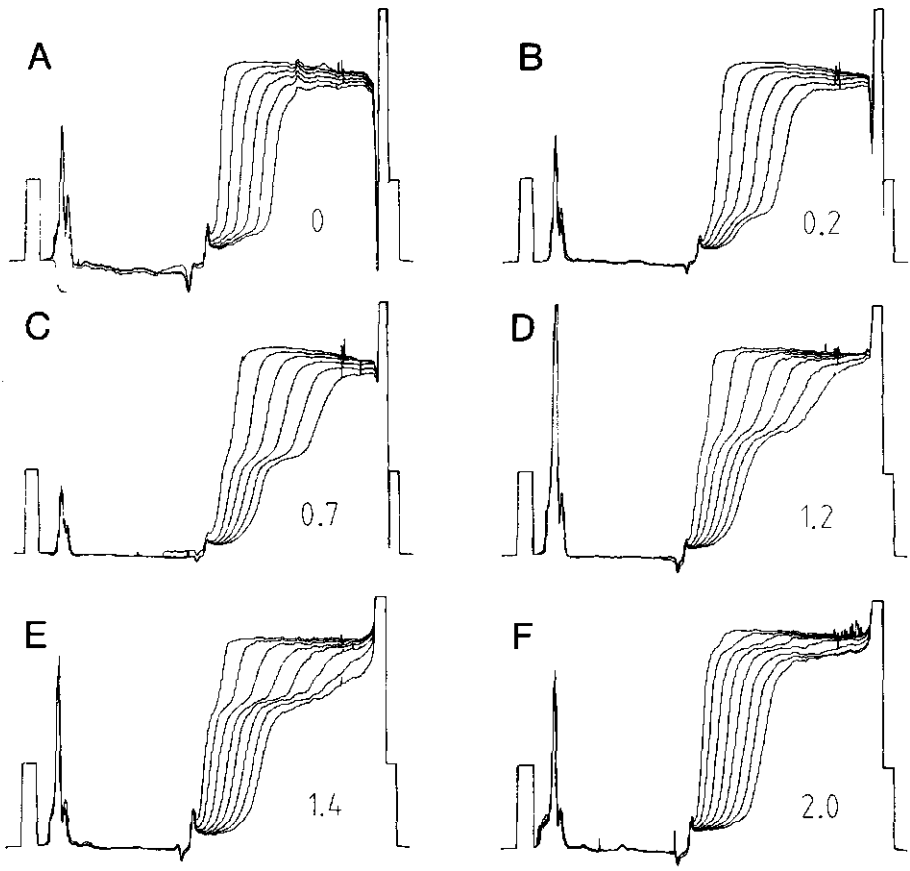


Fig. 4.10. Sedimentation velocity patterns of the isolated E_2 component of *Azotobacter vinelandii* pyruvate dehydrogenase complex, and of some E_1E_2 subcomplexes of varying composition (E_1:E_2 molar ratios are indicated in the figure). The concentration of E_2 was 0.27 mg/ml in 50 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM EDTA. Conditions: temperature, 288 K; 3 minutes interval between scans; rotor speed 45,000 rpm.

a frictional ratio (f/f_o) of 2.5 is calculated. This high ratio cannot be caused by the shape of the component, since a very symmetrical cubic structure is observed on the electron micrographs. From the sedimentation data we calculate a Stokes radius of 21 nm, and this indicates that the E_2 component must have a very thick hydration shell.

The similarities between the E_2 components from the two organisms are very surprising, since the intact *A.vinelandii* complex is

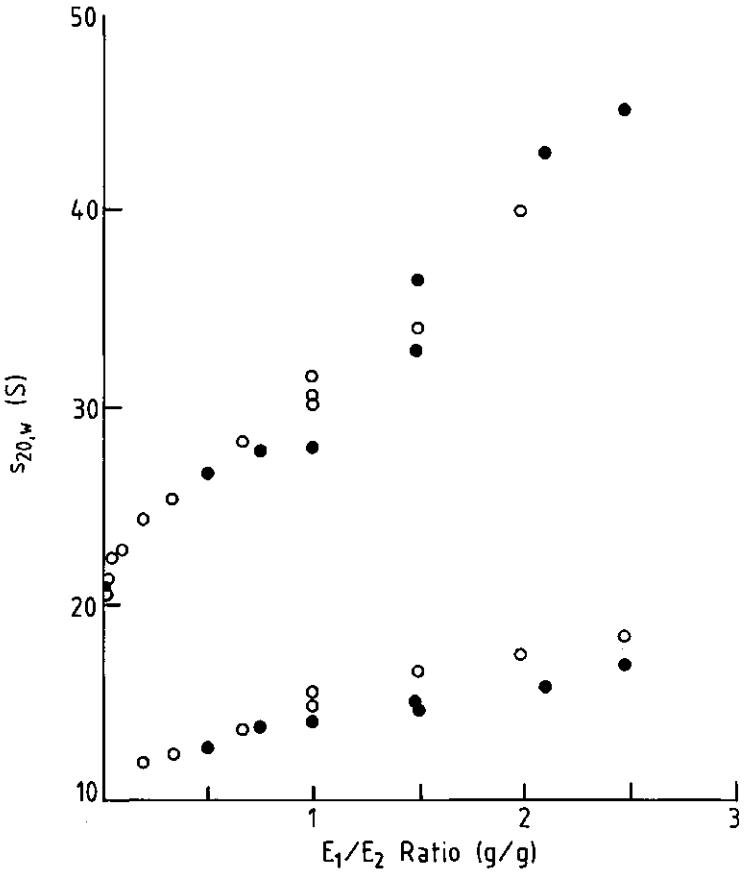


Fig. 4.11. Sedimentation coefficients ($s_{20,w}$) of the two species of the E_1E_2 subcomplexes of *A.vinelandii* PDC, with varying E_1:E_2 ratios. (○), E_2 concentration was 0.27 mg/ml; (●), E_2 concentration was 0.07 mg/ml. All other conditions as in the legend to Fig. 4.10.

about 6 to 8 times smaller than the PDC of *E.coli*. Therefore, upon recombination with the E_1 and E_3 components, the large *A.vinelandii* E_2 core must dissociate into the smaller units that are characteristic for the PDC of this bacterium.

Fig. 4.10 shows sedimentation scans of E_1E_2 subcomplexes of varying composition. With almost every E_1:E_2 ratio, two well-separated boundaries are observed. The sedimentation coefficient of the slowly sedimenting boundary increases from 11 to 18 S, while that of the faster sedimenting boundary changes from 21 to 45 S when E_1 is added in increasing amounts to the E_2 component (Fig. 4.11).

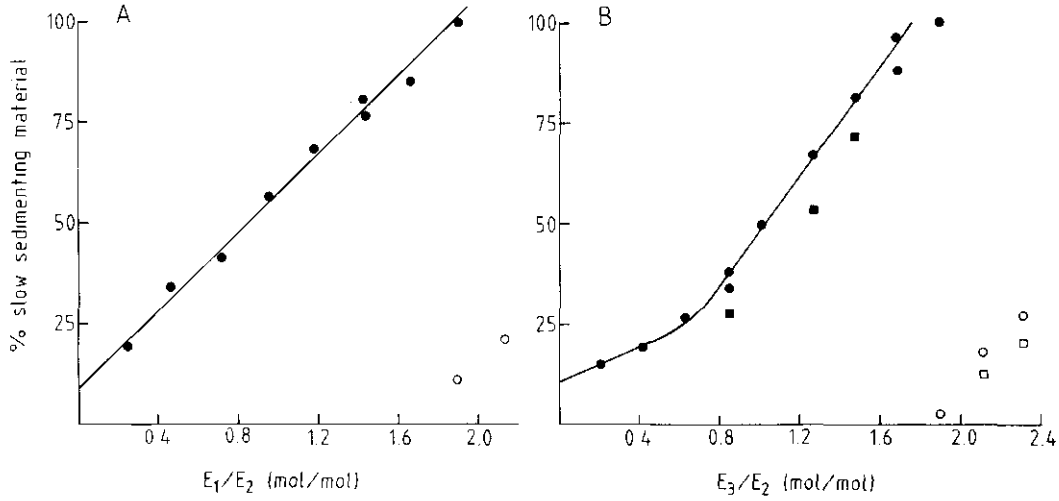


Fig. 4.12. Sedimentation analysis of E_1E_2 and E_2E_3 subcomplexes of the pyruvate dehydrogenase complex of *Azotobacter vinelandii*. Varying amounts of the periferal components were added to a fixed amount of E_2 in standard buffer. After 30 min. of incubation at room temperature, sedimentation runs were started (293 K, 45,000 rpm).

(A) Titration of E_2 with E_1. The concentration of E_2 varied between 0.34 and 0.61 mg/ml. Scans were performed at 280-290 nm, depending on the absorbance. (●-●), Percentage of slowly sedimenting subcomplex; (o-o), percentage of free E_1. Both are expressed as a percentage of the total ultraviolet absorption.

(B) Titration of E_2 with E_3. The concentration of E_2 varied between 0.45 and 0.61 mg/ml. (●-●), Percentage of slowly sedimenting subcomplex as calculated from the absorption at 280-290 nm. (■-■), percentage of slowly sedimenting subcomplex as calculated from the total absorption at 456 nm (flavin absorption); (o-o), percentage of free E_3 (290-290 nm); (□-□), percentage of free E_3 (456 nm).

Simultaneously the relative amount of the faster sedimenting species (as detected by UV-absorption) decreases when more E_1 is added. Under the conditions that were used in these experiments this decrease is linear with the amount of E_1 added (Fig. 4.12A). These observations indicate that E_1 binds to both

forms of the E_2 component. The larger form contains the large E_2 core, resembling that of the PDC from *E.coli*, the slower sedimenting species of the E_1E_2 subcomplexes contains the small E_2 core characteristic for *A.vinelandii* PDC. Apparently, the addition of E_1 results in the destabilization of E_2-E_2 interactions within the core, thus leading to dissociation. From the data of Fig. 4.12A it is clear that one dimer of E_1 can bind to each E_2 chain. Indeed, the boundary of free E_1 is observed at E_1:E_2 ratio's above 2:1.

No difference in sedimentation pattern is observed when E_1 is added slowly in small portions to E_2 or when E_2 is added in the same way to E_1, and the relative amount of the faster sedimenting species decreases when the total protein concentration is lowered. These observations indicate that the two forms of the E_1E_2 subcomplexes are in equilibrium. The two well-separated boundaries were only observed when the sedimentation experiments were performed at high speed; at a rotor speed of 15,000 rpm (instead of 45,000 rpm), only one, very broad sedimentation boundary was observed. From this observation we estimate that the equilibrium has a relaxation time of the order of 10 to 30 minutes (under the conditions of these experiments).
The equilibrium can be shifted towards the larger form by an increase in temperature or by the addition of PEG 6000 and $MgCl_2$. The equilibrium behaves in this respect similar to the PEG-induced equilibrium of the intact PDC of *A.vinelandii*. As in that case, the sedimentation coefficient of the faster sedimenting species does not change upon dilution. The sedimentation coefficient of the smaller form is however significantly lower in more dilute solutions (Fig. 4.11). This behaviour is comparable to that of the 18 S form of the intact PDC from *A.vinelandii*, indicating that also for the small form of the E_1E_2 subcomplexes a monomer-dimer equilibrium exists.

The addition of E_3 has similar effects on the association state of the E_2 core. In contrast to the E_1 component however, about 0.5 chain of E_3 per E_2 chain can be bound to the core with only little effect on the degree of dissociation (Fig. 4.12B). As in the case of E_1, a maximum of one E_3 dimer can be bound per E_2 chain, and the fully saturated E_2E_3 subcomplex is completely dissociated into its small form (Fig. 4.12B).

DISCUSSION

It is clear that the concentration dependence of the molecular mass of *A.vinelandii* PDC (18 S form) can be interpreted in terms of a monomer-dimer equilibrium.
The observed decrease in molecular mass upon dilution could also be explained by a dissociation of components from the E_2 core, but this would imply that a considerable fraction of the components (15% by weight) would dissociate from the complex at a protein concentration of 1 mg/ml. We have never observed a detectable amount of dissociated components in sedimentation experiments.
Furthermore, if the periferal components would dissociate at this extent from the core, an isolation procedure based on exclusion chromatography and ultracentrifugation would result in a very high loss of components, with a concomitant loss in overall enzyme activity, which is not observed. The activity of the complex only decreases significantly when PDC is stored at protein concentrations lower than 0.3 mg/ml. This loss in overall enzyme activity occurs at a time scale of fifteen minutes to several hours and can therefore not reflect the association-dissociation phenomenon, which is pressure-dependent with a relaxation time of less than a few seconds.
At protein concentrations below 0.5 mg/ml, the sedimentation coefficient is significantly lower than is predicted by the monomer-dimer model. The ratio of the intensity of scattered light at 0.1 MPa and 80 MPa is also too high at these low protein concentrations. Therefore, at protein concentrations below 0.5 mg/ml, an additional effect of the dissociation of components from the complex seems to occur. At higher protein concentrations this effect is negligible, for the reasons discussed above.

The observation of a 56 S form of the *A.vinelandii* PDC and the quaternary structure of its isolated E_2 component indicate that the complex is much more similar to the *E.coli* PDC than had been previously assumed. A 17-20 S form of the *E.coli* complex has also been observed by others (Danson, 1979; Schmitt, 1975). This small form is enzymatically active and it is present in small amounts in *E.coli* PDC preparations. The main difference

between the PDC's from both sources probably resides in the E_2 components. Clearly, some E_2-E_2 interactions in the large (*E.coli*-like) form of the E_2 core of *A.vinelandii* PDC are much weaker than in the *E.coli* E_2 core. As can be seen from Fig. 4.10, some dissociation of the large E_2 core of *A.vinelandii* can be observed in a sample of the isolated component, even in the absence of E_1 and E_3.
Upon addition of E_1 (or E_3), a fraction of the large form of the subcomplex is dissociated into the smaller form, which is characteristic for *A.vinelandii* PDC. The role of the E_1 component in this dissociation of the E_2 core is not clear at present, since our observations indicate that both forms of the E_2 core bind E_1 to a comparable extent. Apparently, binding of E_1 distorts E_2-E_2 interactions, either by direct effects such as steric hindrance or indirectly by E_1-mediated structural changes in E_2. The E_3 component shows the same behaviour, although the binding of the first E_3 dimers (up to 0.5 E_3 per E_2 chain) has less effect on the dissociation equilibrium than the binding of following E_3 dimers. The implications of this observations will be further discussed in chapter 7.

When polyethylene glycol is added to a sample of *A.vinelandii* PDC, the formation of the 56 S species is accompanied by the appearance of a new aggregation state of the complex.
The corrected sedimentation coefficient of the slower sedimenting peak varies between 22 and 28 S, depending on the conditions. This could be the result of the formation of a species with a sedimentation coefficient of about 30 S. This is confirmed by the observations from the high-pressure light-scattering experiments. Although, for the reasons given in the results section, the absolute value for the weight-average molecular mass cannot be estimated accurately in the presence of PEG, the level of light-scattering at 80 MPa is at least 1.5 times as high as compared to that of a sample without PEG. At high protein concentrations, this level of scattered light increases almost two-fold within the dead time of the experimental set-up, when pressure is released. If we assume that, due to the addition of PEG and $MgCl_2$, the 22 S dimeric form is the predominant species at 80 MPa, this increase in light-scattering intensity could represent a dimer-tetramer equilibrium. The tetramer would

be the 30 S species that was observed in the sedimentation experiments.

The 56 S form of the *A.vinelandii* PDC is formed at a much lower rate after the release of pressure. From the few data that are available, only a rough estimate of the molecular mass of this species can be made. A sample, in which about 66% of the material was converted into the 56 S form, showed about 2.9 times as much scattering at 0.1 MPa as compared to the level of scattering at 80 MPa. This observation indicates that the 56 S form of *A.vinelandii* could be about four times as large as the predominant species at 80 MPa (in the presence of PEG); it could therefore be an octamer of the 15 S monomer form of the complex. This can be summarized in the following scheme:

monomer		dimer		tetramer		octamer
15 S	$\rightleftarrows$	22 S	$\rightleftarrows$	30 S (?)	$\rightleftarrows$	56 S
	fast		fast (PEG)		slow (PEG)	

In this model the monomers could represent the protein-dense corners of the E2 cube, as observed on electron micrographs (Fig. 4.9).

The main factor of uncertainty in this model is the value of the weight average molecular mass at 80 MPa in the presence of PEG 6000. The value of 1.5 times the mass of the monomeric species could be an indication for a 1:1 mixture of monomers and dimers. The pressure-dependent light-scattering data would then be consistent with the formation of hexamers upon the relieve of pressure. However, no concentration dependence of the apparent molecular mass was found at high pressure, as would be expected for such a mixture. Moreover, the effect of pressure seems to level off at 80 MPa (Fig. 4.8). This indicates the presence of mainly one species. Because some protein may have been retained on the filter in the presence of PEG and $MgCl_2$, the value of 1.5 is considered as a lowest estimate. Taking these considerations into account, the association scheme presented seems to be the most likely one.

From three independent measurements (combination of sedimentation velocity with diffusion coefficient, sedimentation

equilibrium, light-scattering) we calculate a molecular mass of the E_2 core of 1.95 ± 0.1 MDa. Eley *et al.* (1972) estimated the mass of the *E.coli* E_2 core at 1,65 MDa. Thus, while our data lead to about 30 E_2 chains in the E_2 core, the data on the *E.coli* E_2 core yield 24 chains, based on a subunit molecular mass of 66 kDa.
This difference may be caused by the considerable difference in isolation procedure, or an error in the estimation of the molecular weight of the E_2 core. It could however also reflect a real difference in the subunit organization of the E_2 core of both complexes and thus explain the effects described in this chapter.

Our observations on the aggregation states of the *A.vinelandii* PDC show that a high degree of resemblance exists between the PDC's of *A.vinelandii* and *E.coli*. The question about the quaternary structure of the *A.vinelandii* complex *in vivo* still remains unanswered. Even when all precautions were taken (no high pressure, no ultracentrifugation, no ammonium sulfate precipitations), the (partially) isolated *A.vinelandii* PDC still sedimented with a sedimentation coefficient of about 18 S. As suggested by Nichol *et al.* (1981), polymers that are present in the cell could induce the formation of the 56 S species *in vivo*, like the process we have observed *in vitro* upon addition of PEG.

5. CHAIN-STOICHIOMETRY OF THE PYRUVATE DEHYDROGENASE COMPLEX OF *AZOTOBACTER VINELANDII*

INTRODUCTION

Knowledge of the chain-stoichiometry of a multienzyme complex is essential for the understanding of its working mechanism and for the construction of a three-dimensional model. In combination with restrictions of symmetry, some estimation can then be made of the number of interactions between the component enzymes. The determination of the chain stoichiometry however proves to be difficult. It can be divided into two parts: the determination of the molecular mass of the complex, and the estimation of its chain-ratio.

Three different approaches for the estimation of the chain-ratio can be used. Firstly, the chain-ratio can be estimated from the content of prosthetic groups that are characteristic for the several protein components (TPP, lipoyl groups, FAD). The chain-ratio can also be calculated from the weight-ratio of the peptide chains, as determined by staining with dyes or by modification with a chromophore or a radioactively labelled compound. Finally, the chain-ratio can be estimated from recombination of the isolated enzymes into an active enzyme complex.

Since the E_2 chains form the core to which the E_1 and E_3 components are non-covalently bound, the chain-ratios are normalized on the E_2 component. The chain-ratio determinations are complicated by heterogeinity of the complex preparations, *i.e.* not all complex particles have the same composition. The experimentally determined chain-ratio will therefore always represent an average of the ensemble of complex particles that are present in the preparation. Furthermore, the chain-ratio can vary *in vivo*, depending on the growing conditions (Vogel, 1972a), and during the isolation procedure loss of components may occur (Bosma, 1982; chapter 2).

Nevertheless, the E_2 component has an inborn capacity for the binding of the E_1 and E_3 components, but to obtain this "optimal"

chain-ratio, the experimentally determined chain-ratios have to be interpreted into integer values. For the well-studied PDC of *Escherichia coli* this has led to a long-lasting discussion, chain-ratios of 1:1:1, 2:1:1 and 1:1:0.5 have been proposed (Vogel, 1972b; Bates, 1975b; Eley, 1972).
The determination of the molecular mass of protein particles in the range of a few million dalton is very difficult, and for the reasons already mentioned, this is even further complicated for these multi-enzyme complexes. The estimations of the molecular mass of the *E.coli* PDC therefore range from 3.0 to 6.1 MDa (Dennert, 1970a; Eley, 1972; Vogel, 1972b; Danson, 1979).

Previously, the chain-stoichiometry of the PDC of *Azotobacter vinelandii* was estimated at 6-8:4:2 from the lipoyl and flavin content of the complex preparations (Grande, 1975; De Abreu, 1977a). These preparations often also contained the so-called fourth component (see chapter 2).
In the present study, we have estimated the chain-ratio of the three components in the PDC of *A.vinelandii* by the three approaches mentioned above.

MATERIALS AND METHODS

Materials

Trinitrobenzene sulfonic acid (TNBS) was obtained from Eastman Kodak (USA). 2-^{14}C-pyruvate and N-Ethyl-(2,3-^{14}C-maleimide were from the radiochemical centre Amersham (UK). ^{3}H-methylacetimidate was synthesized from ^{3}H-acetonitril (NEN, USA) as described by Bates (1975a). Biochemicals were from Boehringer (FRG), all other chemicals were analytical grade.

Methods

Enzyme activities were assayed as described previously (Bresters, 1975a; chapter 2). Protein concentrations were measured according to the method of Lowry *et al.* (1951). The flavin content of the complex was determined by the method of Wassink and Mayhew, using FMN standards (Wassink, 1975).
The complex was resolved into its individual components by the thiol-Sepharose method of de Graaf-Hess and de Kok (1982). SDS-gelelectrophoresis was performed according to a modification

of the procedure of Laemmli (1970), as described by Dorssers *et al.* (1982).
Sedimentation analysis was performed with a MSE centriscan 75, equipped with an UV-vis monochromator. Partial specific volumes were calculated from the amino acid compositions of the proteins, according to Cohn and Edsall (1943). The sedimentation equilibrium experiments of the isolated components were performed at 277 K in 50 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM EDTA. Rotor speeds between 2500 and 4300 rpm were used for the E_2 component, and 9000 to 14500 rpm was used for the E_1 component. The molecular mass calculated from the UV-absorption scans according to standard procedures (Schachman, 1959).
Amino acid analysis was carried out on a JEOL-JLC 5AH amino acid analyzer according to standard procedures (Moore, 1963). Protein samples were hydrolyzed for 24 and 96 hours in 6 M HCl at 383 K in sealed evacuated tubes. L-Norleucine and α-amino-β-guanidino propionic acid were included as internal standards for the long and the short column respectively; the former standard was added prior to and the latter immediately after the hydrolysis step. The analytical results with the hydrolysates were averaged with the following exceptions. Threonine, serine and tyrosine contents were derived from extrapolation to zero hydrolysis time, and the measures of the 96 h hydrolysates were taken for valine and isoleucine. Tryptophane content was determined fluorimetrically as described by Pajot (1976). For the lipoamide dehydrogenase component, determination of cysteine and cystine by an acid ninhydrin method (De Koning, 1971) gave essentially the same results.

Trinitro benzene sulfonic acid-modification of the complex
The complex (0.2-1.5 mg/ml) was modified with 3 mM trinitrobenzene sulfonic acid (TNBS) in 6 M Urea, 1% (w/vol.) $NaHCO_3$ (pH 8.5) for 16 hours at room temperature. All steps were performed in the dark to prevent photolysis of TNBS and its products. The reaction was stopped by addition of an equal volume of SDS-incubation buffer (Dorssers, 1982), containing 15 mM glycin. The sample was heated for 5 minutes at 373 K, followed by electrophoresis on tubular gels (5 x 80 mm). On each gel

Table 5.1

Amino acid composition of the three components of the pyruvate dehydrogenase complexes of *Azotobacter vinelandii* (A.v.) and *Escherichia coli* (E.c.)*. The amino composition is repressed as a molar percentage of the total number of amino acids.

Amino acid	E_1		E_2		E_3	
	A.v.	E.c.	A.v.	E.c.	A.v.	E.c.
	mol/100 mol					
Asx	10.0	10.2	5.6	7.9	7.5	8.1
Thr	4.1	4.7	3.3	4.3	4.9	5.5
Ser	4.4	4.9	6.0	4.6	4.9	2.9
Glx	12.5	12.7	11.2	11.6	9.5	9.9
Pro	4.5	4.0	7.5	5.9	3.5	4.4
Gly	8.7	8.9	8.5	8.1	11.0	10.7
Ala	8.4	8.1	19.5	15.3	13.9	10.5
½Cys	0.5	0.7	0.6	0.2	0.6	1.0
Val	6.2	5.8	9.3	10.8	11.6	9.5
Met	3.0	2.4	1.8	2.5	1.6	2.3
Ile	5.1	6.3	5.1	7.2	5.9	8.2
Leu	8.4	7.9	8.9	5.2	8.3	7.3
Tyr	4.0	4.7	0.8	0.5	1.5	1.7
Phe	4.3	3.8	1.6	3.0	2.9	2.9
Lys	5.2	5.4	5.5	8.4	7.3	8.2
His	2.6	2.6	1.0	0.8	2.2	2.7
Arg	6.9	5.6	3.4	3.2	2.4	3.2
Trp	1.4	1.2	0.4	0.5	0.4	0.8
Mr	94.000	99,474	66.000	65.959	56.000	50.554

*The amino acid compositions of the *E.coli* enzymes are from Stephens *et al.* (1983a, b and c).

20-80 µg of protein was applied, and each sample was analyzed on 4 to 8 gels. To remove background absorption due to non-protein TNBS-products, the gels were washed for several hours in fixation solution.
The gels were scanned at 345 nm with a Gilford spectrophotometer, equipped with a linear transporter. Area of the peaks was determined by cutting and weighing of the peaks on the graphpaper.

RESULTS

Amino acid composition and quaternary structure of the three components

The amino acid compositions of the three components of *A.vinelandii* PDC have to be known for the calculation of chain-ratios from the results of lysine modification by TNBS or radioactive methylacetimidate (Bates, 1975b). Table 5.1 lists the amino acid compositions of the components of *A.vinelandii* PDC, those of the corresponding enzymes from *E.coli* (Stephens, 1983a, 1983b and 1983c) are given for comparison.
The compositions compare quite well, especially those of the E_1 components. The largest deviations are found in the E_2 components where the difference in the number of small side-chain residues and of proline may indicate differences in the secondary structure of these components.
From these amino acid compositions we calculated a partial specific volume of 0.734, 0.749 and 0.750 for the E_1, E_2 and E_3 component respectively, according to the method of Cohn and Edsall (1943). These values were used to calculate the molecular mass of the components and to correct the sedimentation coefficients to standard conditions.

The E_1 component has a subunit molecular mass of 94 kDa as estimated by SDS-gelelectrophoresis; its mobility is slightly higher than that of the *E.coli* E_1 component. From sedimentation equilibrium measurements at pH 7.0 we calculated a molecular mass of 190 ± 5 kDa. At this pH the component has a sedimentation coefficient of 9.8 ± 0.3 S, this decreases to 6.3 ± 0.3 S at pH 10. These observations are in good agreement with the reports on *E.coli* E_1 (Dennert, 1970b; Perham, 1977; Vogel, 1977)

and show that the dimer of E_1 dissociates into its monomers at higher pH values.
The E_3 component has a subunit molecular mass of 56 kDa as estimated from SDS-gelectrophoresis and its flavin content. Its mobility on SDS-gels is slightly lower than that of the *E.coli* E_3. The E_3 component has a sedimentation coefficient of 5.4 S. From sedimentation equilibrium experiments a molecular mass of 100 kDa was calculated, indicating that E_3 is a dimer.

Comparison of the subunit molecular masses of the E_2 components from *E.coli* and *A.vinelandii* PDC showed no significant differences (chapter 4). Because the DNA-sequence indicates a mass of 66 kDa for the *E.coli* E_2 component (Stephens, 1983b), we have used the same value for the E_2 component of *A.vinelandii*. The isolated E_2 component of *A.vinelandii* PDC associates into a cubical structure composed of 32 E_2 chains (chapter 4; Bosma, 1984a).

Chain-ratio determination by lysine-modification with trinitrobenzene sulfonic acid

Bates and Perham (1975b) introduced a method for the determination of chain-ratios based on the covalent modification of lysine residues in the three components. They used radioactively labelled methylacetimidate as a reagent, and the reaction was performed under denaturing conditions to assure the modification of all lysine residues. After SDS-gelectrophoresis of the modified preparation, the chain-ratio was calculated from the amount of radioactivity detected in the three protein bands; corrections were made for the amount of reactive amino groups in each chain.
In our hands, however, the radioamidination method was not reproducible. For one *A.vinelandii* PDC preparation for instance, we found 0.92(±0.09): 1 :0.88 (±0.88) and 1.91(±0.23): 1 :1.58 (±0.06) for E_1:E_2:E_3 in two different experiments.
In another experiment, a PDC preparation that was almost identical in FAD content, lipoyl content and specific activity yielded a 1.52(±0.16): 1 :0.59(±0.05) ratio (prep. 1, Table 5.2). The gels sometimes showed appreciable degradation of protein bands, especially of E_2. Possibly the E_2 component of *A.vinelandii* PDC is very sensitive to proteolysis during the long dialysis

step at room temperature that is required in this procedure. We sometimes also observed material on top of the gel, possibly due to crosslinking.

We therefore used trinitrobenzene sulfonic acid (TNBS) as a reagent for the lysine residues. TNBS reacts specifically with the ε-amino group of lysine residues and the amino-terminal end of the peptide chain, yielding a trinitrophenylamino acid residue with a strong absorbance at 345 nm (Okuyama, 1960). The chain-ratio can be calculated from 345 nm scans of SDS-gels of TNBS-modified PDC preparations.

The lysine contents of the components of *A.vinelandii* PDC as calculated from the amino acid composition are: 43.1 per 94 kDa for E_1, 35.3 per 66 kDa for E_2, and 39.2 per 56 kDa for E_3. These values must be corrected for the terminal amino group and two lipoyl groups per E_2 chain that are covalently bound to lysine residues. Rounded off to the nearest integer, the values for the amount of reactive groups per peptide chain become 44 for E_1, 34 for E_2 and 40 for E_3, and these values were used in the calculations of the chain-ratio of *A.vinelandii* PDC. Possibly three lipoyl groups are bound to each E_2 chain (Hale, 1979a; Stephens, 1983b; chapter 7), but this would only cause an error of 3% in the estimation of the E_2 content.

The results from these chain-ratio determinations did not vary significantly when the protein concentration (during modification) was varied between 0.1 and 1.5 mg/ml, or when reaction times were varied between 30 minutes (lower than 25% modification) and 30 hours (over 95% modification). At room temperature, more than 90% of the lysine residues was modified within 8 hours.

The modification can also be performed at 313 K for 2 hours as described by Habeeb (1966), however under these conditions more background due to reaction of TNBS with urea was observed. Omission of urea leads to erroneously low estimates of the E_2 component at protein concentrations below 0.5 mg/ml. Above 0.75 mg/ml no significant difference was found between modification in 6 M urea, buffer without any addition, and 5 M guanidinium hydrochloride. We therefore routinely used a 16 h modification at room temperature in 6 M urea.

It is very surprising that the apparent chain-ratio is not in-

Table 5.2.

Chain-ratios of some preparations of pyruvate dehydrogenase complex from *Azotobacter vinelandii*

Preparation	chain-ratio measured			FAD-content		lipoyl-content	
				measured	calculated	measured	calculated
	E_1	E_2	E_3	(nmol/mg)	(nmol/mg)	(nmol/mg)	mol/mol E_2
1	1.52(±0.16)	1	0.59(±0.05)*	2.0(±0.1)	2.5(±0.3)	7.2(±0.2)***	1.7*** - 2.4**** (±0.2)
1	1.43(±0.08)	1	0.50(±0.05)**	2.0(±0.1)	2.2(±0.2)	9.8(±0.3)****	1.6*** - 2.2**** (±0.1)
2	1.37(±0.08)	1	0.58(±0.05)**	2.3(±0.1)	2.6(±0.3)	n.d.	
3	1.27(±0.04)	1	0.48(±0.02)**	2.4(±0.1)	2.3(±0.1)	11.7(±0.3)***	2.5(±0.2)
4	1.27(±0.04)	1	0.51(±0.03)**	2.2(±0.1)	2.4(±0.2)	n.d.	
5	1.30(±0.12)	1	0.47(±0.05)**	2.5(±0.1)	2.2(±0.2)	8.4(±0.2)***	1.8(±0.2)

* As determined by the radioamination method of Bates *et al.* (1975b).

** As determined by the TNBS method.

*** As determined from incorporation of 2-^{14}C-pyruvate (de Abreu, 1977a).

**** As determined from modification with N-ethyl(2,3-^{14}C)maleimide in the presence of NADH (de Kok, 1982).

Standard deviations are given between brackets. n.d. means not determined.

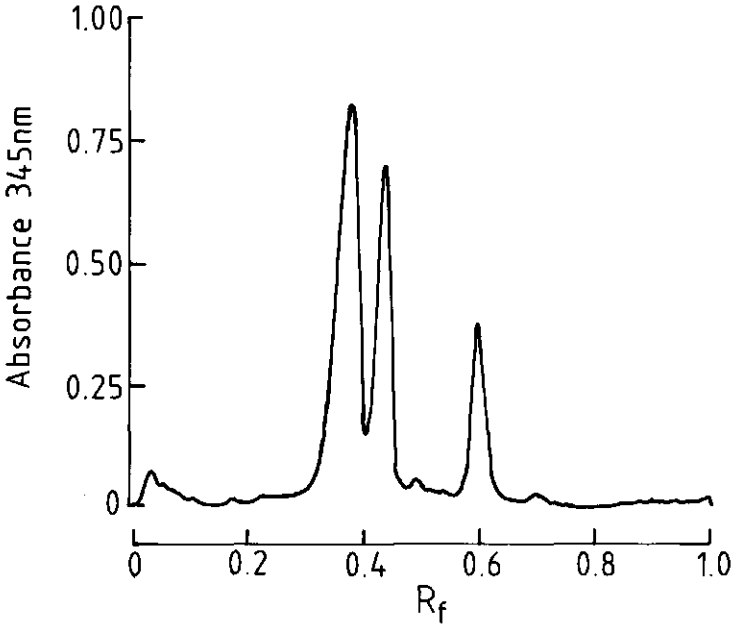


Fig. 5.1. Densitogram of a tubular SDS-gel of a recombined *A.vinelandii* pyruvate dehydrogenase complex. E_1, E_2 and E_3 were mixed in a 1.45:1:0.51 ratio and were modified with TNBS. The chain-ratio as estimated from this densitogram is 1.42:1:0.51. The small peaks at Rf = 0.47 and 0.68 probably originate from the E_1 component and represent less than 3% of its trinitrophenyl content.

fluenced by the extent of the modification, or by the presence of denaturing agents. It has been shown that the lysine residues do not react at the same rate with TNBS (Goldfarb, 1966). If there is a difference in reaction rate of the various lysine residues in the *A.vinelandii* PDC, their relative contribution must be comparable in each of the three components.
We omitted 2-mercaptoethanol from our electrophoresis buffers since this compound can reduce the trinitrophenyl group, resulting in a decrease in absorption at 345 nm (Means, 1971).

As a test, we mixed the isolated three components in a 1.45(±0.04): 1 :0.51(±0.03) ratio and analyzed this mixture with the TNBS method. It resulted in a 1.42(±0.05): 1 :0.51(±0.03) ratio, indicating that the method is reliable, at least under our conditions. A densitogram of one of the gels of this experiment is shown in Fig. 5.1.

The TNBS method is relatively cheap and fast. The protein sample is transferred onto the gel directly after modification, thus preventing proteolysis as observed with the radioamidination method, or other possible artefacts. In reproducability and accuracy it is at least comparable to the latter method.

Stoichiometry of the isolated A.vinelandii *PDC*

The chain-ratios of some preparations of *A.vinelandii* PDC are given in Table 5.2. The preparations were obtained by the isolation method as described in chapter 2. For preparation no. 1 chain ratios as determined by the TNBS method and the radioamidination method are given; it illustrates the underestimation of E_2 by the radioamidination method as described in the previous section.

The TNBS-chain-ratios do not seem to vary more than 10% between the complex preparations and they are comparable to those as obtained by Perham's group for the *E.coli* complex (when appropriate corrections are made for the subunit molecular mass of the E_2 component).

Lipoyl and FAD contents of some of the complex preparations are also given, since these values have been used for the estimation of the stoichiometry of PDC (Eley, 1972; Speckhard, 1977; Collins, 1977; De Abreu, 1977a). The flavin contents, as calculated from our chain-ratios, are within 10% of the measured values, which are in good agreement with the reported flavin content of *E.coli* PDC (Eley, 1972; Speckhard, 1975).

The lipoyl content determined by incorporation of ^{14}C-acetyl groups varied considerably between different preparations; however, we always found values of about 10 nmol/mg when fresh samples were used. For some unknown reasons, the amount of acetylatable groups decreased upon aging, with no apparent effect on overall enzyme activity. For preparation 1 of Table 5.2, we were able to detect the higher amount of lipoic acid residues by modification with N-ethyl-(2,3-^{14}C)-maleimide after reduction with NADH, under conditions where no modification of E_3 took place, as described by De Kok *et al.* (1982).

Preparation 5 of Table 5.2 was used in the light-scattering experiments that are described in chapter 4. These experiments yielded a value of 750-850 kDa for the complex molecular mass.

From the chain-ratio of this preparation (1.30 : 1 : 0.47), a molecular mass per E_2 chain of 215,000 is calculated. We therefore conclude that the PDC of *A.vinelandii* is based on a tetramer of E_2.

Reconstitution of the complex from isolated enzymes

The isolated components of the *A.vinelandii* PDC as obtained by the thiol-Sepharose method of de Graaf and de Kok (1982) can be recombined to give a fully active complex.

Fig. 5.2A shows the effect of recombination of E_2 with varying amounts of E_1; a fixed amount of E_3 was added to these E_1E_2 subcomplexes to restore complex activity. Interpolation of the initial increase in activity and the plateau value at saturating E1 amounts gives a 1.59(±0.16): 1 ratio for E_1:E_2 (5 experiments with different preparations, E_2 concentrations between 20 and 80 µg/ml (0.3 - 1.2 µM)). This interpolation is justified since inactivation studies with thiamine thiothiazolone pyrophosphate, a transition state analog of TPP (Gutowski, 1976), have indicated that the overall enzyme activity is linearly correlated with the amount of active E_1 bound to the complex (chapter 6; Stanley, 1981). The same equivalence point was found when smaller amounts of E_3 were added, only the plateau value and the slope of the initial curve decreased (not shown).

Fig. 5.2B (filled circles) shows reconstitution of complex activity with varying amounts of E_3. With the same range of E_2 concentrations as used above in the titration with E_1, the titration curve is linear only at low E_3:E_2 ratios and no plateau value is reached. When the reconstitution is performed at E_2 concentrations between 10 and 50 nM (open circles), the initial increase in overall activity is almost linear until an optimal value is reached, whereafter a decrease in activity is observed. Extrapolation of both parts of the titration curve yields an E_2:E_3 chain-ratio of 1:0.5(±0.05) (5 experiments with different preparations). From these data it is concluded that in the optimal stoichiometry an E_2 tetramer binds one E_3 dimer and three E_1 dimers.

When the E_1 titration is performed at E_2 concentrations between 10 and 50 nM (open circles in Fig. 5.2A) no sharp equivalence point is observed. It is concluded that the affinity of E_1

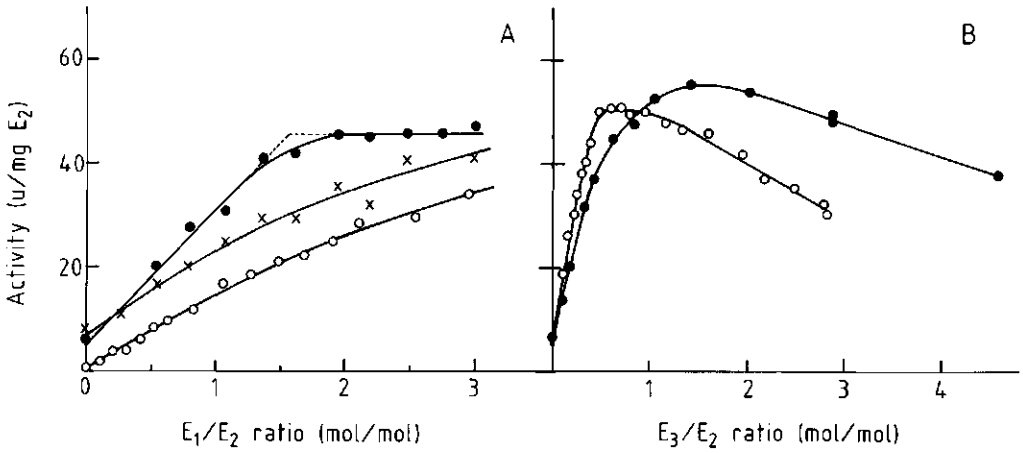


Fig. 5.2. Reconstitution of *Azotobacter vinelandii* PDC from its individual components.

(A) Titration of E_2 with E_1; varying amounts of E_1 were added to a fixed amount of E_2. After 20 minutes of incubation at room temperature, a fixed amount of E_3 was added to restore overall enzyme activity. After 20 minutes incubation, 10-25 µl of the incubation mixture was assayed for complex activity in a volume of 1 ml (●-●, x-x), or recombination was performed at low protein concentrations in the cuvette containing all the components of the assay mixture and the reaction was started by the addition of pyruvate (o-o). Enzyme activity is expressed as µmol NADH produced min^{-1} $(mg\ E_2)^{-1}$. Enzyme preparations were in 50 mM potassium phosphate, pH 7.0. Concentrations of components during recombination: (●-●), 0.49 µM E_2, 0.53 µM E_3; (x-x), 0.49 µM E_2, 1.74 µM E_3; (O-O), 50 nM E_2, 53 nM E_3.

(B) Titration of E_2 with E_3; same conditions as in A. 10-25 µl of the incubation mixture was assayed for complex activity in a volume of 1 ml (●-●), or recombination was performed in the cuvette and the reaction was started by the addition of pyruvate (O-O). Concentration of components during recombination was: (●-●), 0.49 µM E_2, 1.74 µM E_1; (O-O), 50 nM E_2, 152 nM E_1. For the calculation of concentrations subunit molecular masses of 94, 66 and 56.5 kDa were used for the E_1, E_2 and E_3 components respectively.

for E_2 is considerably lower than the affinity of E_3. Assuming three E_1 binding sites, a Scatchard plot can be constructed using points that deviate from the stoichiometric titration curve. From this linear plot a dissociation constant of 0.5 nM for the binding of E_1 to E_2 was calculated (Bosma, 1984a). Due to the limited range over which the binding could be studied, no conclusion can be drawn about possible interactions between binding sites.

From the shape of the E_3 titration curves it is clear that this component competes with E_1 for the E_1 binding sites on the E_2 core. From the E_1 titration curve in the presence of excess E_3 (crosses in Fig. 5.2A) or the decrease in activity in the presence of excess E_3 in the E_3 titration curve (open and closed circles of Fig. 5.2B) it can be concluded that the affinities of E_1 and E_3 for E_1 binding sites are of comparable magnitude. Less competition is observed between E_1 and E_3 for the E_3 binding site, as a significant decrease in the overall activity is only observed when a very large excess of E_1 is added (E_1:E_2 ratio higher than 5, not shown). From the degree of inhibition and the concentration ratio of the free periferal components, it was calculated that the affinity of E_3 for the E_3 binding site is two orders of magnitude higher than the affinity of E_1 for this site (Bosma, 1984a). No absolute affinities could be estimated and it may well be that the affinity of E_1 for this site is lower than for the three other sites. The mutual binding-inhibition has also been observed for the *E.coli* E_1 and E_3 components (Reed, 1975).

Thus it may be concluded that the E_2-tetramer contains four binding sites for the periferal components. The reciprocal binding-inhibition relation leads to an optimal stoichiometry of three E_1 dimers and one E_3 dimer.

In these experiments the binding of one component is studied in the presence of a saturating amount of the other component. Thus the observed difference in affinity may result from mutual interaction between the periferal components and need not reflect the presence of intrinsically different binding sites on the E_2 tetramer.

DISCUSSION

From the data presented in this chapter we conclude that the pyruvate dehydrogenase complex from *Azotobacter vinelandii* is based on a tetramer of E_2. The isolated components can be recombined to yield a fully active complex, and from these recombination experiments we deduce an "optimal" E_1:E_2:E_3 chain-ratio of about 1.6:1:0.5. Since the pyruvate dehydrogenase complex from *Azotobacter vinelandii* is smaller than the complex of *E.coli*, interpretation of the experimental data into integer stoichiometries is much easier, and we conclude that in the optimal composition one dimer of E_3 and three dimers of E_1 are bound to the E_2 tetramer.

The sedimentation experiments described in chapter 4 show a binding capacity of either one E_1 or one E_3 dimer per E_2 chain. Thus fully assembled complex based on an E_2 tetramer will contain a total of four E_1 and E_3 dimers. Due to the high turn-over number of the E_3 component, maximal overall activity is expected for a complex particle containing three E_1 dimers and one E_3 dimer, which is experimentally confirmed by the reconstitution experiments.

In the case of a trimeric E_2 core, maximal activity would be expected upon binding of two E_1 dimers and one E_3 dimer. Such a composition is in conflict with the E_1:E_3 ratio as obtained from the chain-ratio determinations and reconstitution experiments. The molecular mass and the flavin content of such a complex would also not agree with experimentally determined values. Therefore a trimeric E_2 core, by some groups considered as the morphological subunit of the *E.coli* complex (Reed, 1968b; Danson, 1979), seems highly unlikely for the *A.vinelandii* complex.

The average chain-ratio of the complex preparations as obtained with the revised purification method as described in chapter 2 is 1.3:1:0.5. Addition of E_1 causes an increase of up to 20% in overall PDC activity, whereas we have never detected a measurable increase in activity upon addition of small amounts of extra E_3. This observation strongly supports our 6:4:2 model, and it also indicates that the isolation procedure as described in chapter 2 yields PDC preparations that are almost maximally active. This is also reflected in the very small loss in complex activity during the purification (less than 20%).

The question how a 3:1 ratio for E_1:E_3 is obtained on the basis of affinities is indicated by the competition experiments. Apparently, when three dimers of E_1 are bound to the E_2 core, the binding of an E_3 dimer is preferred over the binding of a fourth E_1 dimer. This could be due to the presence of a single high-affinity binding site for the E_3 dimer on the E_2 core but it is also possible that the binding sites for E_1 and E_3 are identical and that the binding of three E_1 dimers leads to an increased affinity of the unoccupied site for an E_3 dimer. Because we can only deduce relative affinities, nothing can be said at present about cooperativity in the binding of the E_1 and E_3 dimers.

As was already shown in chapter 4, the PDC's of *A.vinelandii* and *E.coli* are much more similar than had previously been assumed. A comparison between our experimental results on the chain-ratio of *A.vinelandii* PDC and results obtained by others on the *E.coli* complex therefore seems interesting. The reported values for the chain-ratio of *E.coli* PDC are contradictory; "optimal" chain-ratios of 2:1:1, 1:1:0.5, and 1:1:1 have been proposed (Bates, 1975b; Eley, 1972; Vogel, 1972b). Our measurements concerning the chain-ratio of the *A.vinelandii* complex are in good agreement with the majority of those obtained by others on the PDC from *E.coli*. The lipoic acid and flavin contents do not differ significantly between the complexes of the two organisms (Collins 1977; Speckhard, 1975 and 1977; De Abreu, 1977a; Table 5.1). The "optimal" E_1:E_2 chain-ratio as determined from reconstitution experiments is also in good agreement (Bates, 1977). It is therefore interesting to discuss the interpretation of the experimental data, since essentially the same results have been translated into these conflicting models.

The 1:1:1 "core" complex as described by Vogel probably is an isolation artefact; the author describes the removal of "excess" E_1 on a calcium phosphate column (Vogel, 1972a and 1972b). We have also been able to produce a similar "core" complex of the PDC from *A.vinelandii* using a blue-Sepharose column (De Abreu, 1977b; Bosma, 1982).

The 1:1:0.5 ratio has been proposed by Reed and co-workers and is supported by other American groups (Reed, 1968b; Eley,

1972; Speckhard, 1975 and 1977; Angelides, 1979b). The model is based on the determination of flavin and lipoic acid contents of *E.coli* PDC preparations. The number of E_1 chains was calculated indirectly from the molecular mass of the complex. The estimation of the molecular mass of molecules in the range of several million dalton is very difficult and it is even further complicated by the heterogeinity of the PDC preparations (Schmitt, 1980; Gilbert, 1980). In sedimentation equilibrium experiments this heterogeinity will lead to an underestimation of the molecular mass of the complex, and the E_1 content of *E.coli* PDC could therefore be underestimated in the 1:1:0.5 model. An optimal E_1:E_2 ratio of 1.5:1 was calculated from reconstitution experiments (Bates, 1977; Graupe, 1982), and more direct determinations of the E_1 content of the isolated *E.coli* complex also yield an E_1:E_2 ratio above 1:1 (Bates, 1975b; Vogel, 1972b; Hale, 1979a). We do not have an explanation for the results of the experiments of Angelides and Hammes that confirm the 1:1:0.5 model; these authors estimated the chain-ratio from Coomassie Brilliant Blue staining of the components on SDS-gels (Angelides, 1979b).

The 2:1:1 model of Perham's group is based on the chain-ratios as determined for isolated *E.coli* PDC by chemical modification of the three component enzymes (Bates, 1975b; Hale, 1979a), and from reconstitution experiments (Perham, 1977; Bates, 1977; Graupe, 1982). However, a subunit molecular mass of 83 kDa was used for the E_2 component in these calculations, and since this value has been unequivocally determined at 66 kDa (Stephens, 1983b), this model needs correction. This results in a E_1:E_2 chain-ratio of 1.5:1, in agreement with our results on *A.vinelandii* PDC.

For the E_2:E_3 ratio, reports from Perham's laboratory range from 1:0.6 to 1:1.0; Bates, 1975b and 1977; Hale, 1979a; Danson, 1976 and 1979). With correction for the subunit molecular mass of E_2 this results in 1:0.5 to 1:0.8. Without further experimental proof this was interpreted in terms of a maximal binding capacity of 12 dimers of E_3 per E_2 core, *i.e.* an E_2:E_3 ratio of 1:1 (Bates, 1975b).

Unfortunately, this group has not reported on the flavin content of their complex preparations; if their chain-ratio determina-

tions are correct, the flavin content of their preparations would be significantly higher (above 3 nmol/mg PDC) than the values of 1.8-2.6 mol/mg PDC that are routinely found (Eley, 1972; Speckhard, 1975; De Abreu, 1977a).
In general, the E_3 content as determined for the *E.coli* PDC by the radioamidination method is 10-30% higher than we calculated from the TNBS-modification of *A.vinelandii* PDC. We have some indications that the radioamidination method may result in underestimation of the E_2 content (see results section).

Although we do not have direct evidence that the "optimal" chain-ratio for the *E.coli* PDC also is 1.5:1:0.5, it is clear that our model meets the experimental data obtained by others at least equally well as other proposed chain-ratios (*i.e.* our model is a sort of compromise).
In the case of *A.vinelandii* PDC the interpretation of chain-ratios into "optimal" chain-stoichiometries is relatively easy, due to its small size. For the PDC of *E.coli* our model would result in a chain-stoichiometry of 36:24:12, when an E_2 core consisting of 24 chains is assumed (as is generally accepted). However, if the E_2 core of *E.coli* PDC resembles that of the isolated E_2 component of *A.vinelandii* PDC and therefore is composed of 32 E_2 chains, a 48:32:16 would be optimal. The implications of these proposals are discussed in chapter 7.

6. ACETYLATION REACTIONS OF THE PYRUVATE DEHYDROGENASE COMPLEX OF *AZOTOBACTER VINELANDII*

INTRODUCTION

The lipoic acid residues that are covalently bound to the E_2 component are a characteristic feature of the 2-oxoacid dehydrogenase multienzyme complexes. In the case of *A.vinelandii* and *E.coli* PDC, each E_2 chain probably carries two lipoic acid residues (Collins, 1977; De Abreu, 1977a; White, 1980), although the possibility of three lipoyl groups per E_2 chain however cannot be excluded (Hale, 1979a; Stephens, 1983b). Based on the assumption that each E_2 chain carries two lipoyl groups, chain-ratios were estimated from the lipoic acid content of PDC preparations (Collins, 1977; De Abreu, 1977a; White, 1980).

The lipoyl groups are very mobile (Grande, 1975; Ambrose, 1976), and they are bound to very flexible regions of the E_2 chains (Perham, 1981b and 1983; Roberts, 1983). These flexible protein "arms" protrude from the surface of the E_2 component, resulting in a cloudy appearance on electron micrographs (Bleile, 1979). Such a structural organization enables the lipoyl groups to shuttle the covalently bound acetyl group and reducing equivalents between each other, and in this way a large network of interacting lipoyl groups is formed. As a result, a large part of the E_2 core can be acetylated by a single E_1 dimer, a phenomenon called servicing (Bates, 1977; Collins, 1977).
In so-called "servicing experiments" the degree of acetylation is measured as a function of the amount of active E_1 that is bound to the E_2 core. The extent of the E_2-E_2 acetyl exchange reactions can then be expressed in a "servicing number". This number reflects the amount of acetyl-entry sites on the E_2 core that are coupled through the system of interacting lipoyl groups. The acetyl transfer reactions are thought to be rapid enough to be of physiological significance (Danson, 1978), and it has been speculated that these reactions can increase the efficiency of the complex (Bates, 1977; Perham, 1983).

In the study of acetylation reactions within the *A.vine-*

landii PDC, three different reactions must be considered. The first reaction is the reductive acetylation of a lipoic acid residue by a neighbouring E_1 molecule. We have studied the kinetics of this reaction with rapid mixing techniques.
The second process is the transacetylation between lipoic acid residues within an E_2-core (intra-core transacetylation). This phenomenon can be studied with the servicing experiments.
Since the *A.vinelandii* PDC has a tendency to aggregate, a third type of reaction cannot be excluded: transacetylation between lipoic acid residues situated on different E_2 cores, so-called inter-core transacetylation. This process can also be studied by the servicing experiments.
In addition, these servicing experiments can yield information on the question whether the 56 S form of the *A.vinelandii* PDC is either a mere aggregate of independently functioning 18 S particles or a large interacting structure like the PDC of *E.coli* (*cf.* chapter 4).

MATERIALS AND METHODS

Materials

2-^{14}C-Pyruvate and N-ethyl(2,3-^{14}C)maleimide were obtained from the Radiochemical Centre, Amersham (UK). Thiamine thiothiazolone pyrophosphate (TTTPP) was a kind gift from Drs. C.J. Stanley and R.N. Perham, University of Cambridge (UK). Biochemicals were from Boehringer (FRG), all other chemicals were analytical grade.

Methods

Enzyme activities were assayed as described by Bresters *et al.* (1975a). Protein concentrations were measured by the method of Lowry *et al.* (1951). The flavin content of the enzyme preparations was determined by the method of Wassink and Mayhew (1975), using FMN standards.
The *A.vinelandii* PDC was isolated as described in chapter 2. The complex was resolved into its components by the thiol-Sepharose method of De Graaf and De Kok (1982).

Acetyl incorporation

50-150 µg of the complex in 100 µl potassium phosphate buffer pH 7.0, containing 2 mM $MgCl_2$ and 0.2 mM TPP, was incubated with 5 mM 2-^{14}C-pyruvate at 273 K for 1 minute. The protein was then precipitated by addition of 2 ml of 10% (w/vol.) ice-cold trichloroacetic acid. This suspension was kept at 273 K for 5-15 minutes, the protein was then collected on two stacked Whatman GF/C filters (2.5 cm diameter). The filters were washed with 25 ml of the ice-cold trichloroacetic acid solution, followed by 3 ml ice-cold acetone. The filters were dried under vacuum for 5 minutes, prior to the addition of 4 ml of Instafluor (Packard).

The samples were counted with a Packard model 3375 scintillation counter.

In the rapid-mixing chemical-quench experiments, an Update model 1000 instrument was used (Update, Madison, USA). The instrument was calibrated according to standard procedures, using DCPIP-ascorbate as a model reaction.

In the experiments, one syringe was filled with 50 mM potassium phosphate buffer pH 7.0, containing 2 mM ^{14}C-pyruvate, 1.25 mM $MgCl_2$, and 0.5 mM TPP. The other syringe (of identical dimensions) was filled with a 2 mg/ml solution of *A.vinelandii* PDC in the same buffer, but without pyruvate. The solutions were mixed at flow rates between 0.5 and 4 ml/s. A control sample of PDC passed through this system at the highest flow rate did not show a detectable decrease in overall activity, indicating that the complex was not inactivated during the passage through the rapid-mixing system.

To stop the reaction, the end of the delay hose was held into 2 ml of ice-cold 10% (w/vol.) trichloroacetic acid solution in a small reaction tube. The resulting protein precipitate was further treated as described above. To correct for variations in the volume delivered by the syringe ram, the receiving reaction tube was weighed just before and after the push. Between the pushes, the mixing chamber and aging hose were flushed with potassium phosphate buffer, to remove enzyme and radioactive material.

Servicing experiments

The servicing experiments were essentially performed as described by Stanley *et al.* (1981). Stock pyruvate dehydrogenase complex (10-20 mg/ml) was diluted into 100 µl potassium phosphate buffer pH 7.0, containing 1.5 mM NAD^+, to give a protein concentration of about 1 mg/ml. Thiamine thiothiazolone pyrophosphate (TTTPP) was added to the sample in amounts varying between 0 and 3 nmol (volume 0-15 µl), followed by a 30 minutes incubation at 273 K to ensure effective binding of the inhibitor.

After this period, 5 µl of a solution containing 20 mM $MgCl_2$ and 4 mM TPP was added and the sample was incubated for 1.5 minutes at the desired temperature.

A sample of 5 µl was then withdrawn for the assay for overall enzyme activity. Two minutes after the addition of TPP and $MgCl_2$, the assay for enzyme activity was performed and 1 µl of a 26 mM 2-^{14}C-pyruvate solution was added to the sample. Exactly 30 seconds later, the incorporation of radioactive label was stopped by the addition of 2 ml ice-cold 10% (w/vol.) trichloroacetic acid. The samples were further treated as described under acetyl incorporation. Levels of acetylation were expressed relative to that of an uninhibited sample.

In the acetylation experiments with partially reconstituted *A.vinelandii* PDC, TPP and $MgCl_2$ were added at least 30 minutes before the addition of pyruvate. The enzyme assays were also performed at that time. Apart from the omission of TTTPP, all other conditions were kept identical to those of the TTTPP experiments.

RESULTS

Acetylation stoichiometry

The lipoic acid content of the pyruvate dehydrogenase complex of *A.vinelandii* was determined by incubation with ^{14}C-labeled pyruvate, in the absence of coenzyme A. Under these conditions, no deacetylation of the lipoyl groups occurs, and the amount of incorporated acetyl groups can be measured in the trichloroacetic acid-precipitated protein sample (De Abreu, 1977a).

The lipoic acid content of freshly isolated *A.vinelandii* PDC as determined by this method varied between 9 and 11 nmol/mg

PDC, and the lipoyl:FAD ratio was always between 4 and 5. The addition of NAD^+ did not have a detectable effect on these values.

For unknown reasons, the amount of acetylatable groups decreased upon aging, even when the complex preparations were stored in liquid nitrogen. This decrease in detectable amount of lipoic acid residues was not accompanied by a significant decrease in overall enzyme activity. Loss of lipoyl groups from the E_2 component without a detectable loss in overall complex activity has also been observed for the *E.coli* complex (Stepp, 1981; Berman, 1981).
For one of those "old" preparations, a lipoyl content of about 10 nmol/mg could however still be detected by modification with N-ethyl-(2,3-^{14}C)-maleimide after reduction with NADH, described by De Kok *et al.* (1982).

Table 6.1

Lipoyl content of some preparations of *A.vinelandii* PDC, and of some (partially) reconstituted complex preparations.

Sample	chain -ratio E1:E2:E3	lipoyl content measured nmol/mg protein	lipoyl content calculated mol/mol E2
1. PDC as isolated	1.43:1:0.50*	7.2(±0.2)***	2.2(±0.2)
		9.8(±0.3)****	2.4(±0.3)
2. PDC as isolated	1.27:1:0.48*	11.7(±0.3)***	2.5(±0.2)
3. PDC as isolated	1.30:1:0.47*	8.4(±0.2)***	1.8(±0.2)
4. Partially reconstituted	2.11:1:0.92**	7.0(±0.2)***	2.2(±0.1)
5. Partially reconstituted	2.00:1:0.04**	9.6(±0.2)***	2.4(±0.1)

* As determined with the TNBS-method (see chapter 5).

** Relative quantities of components in the recombination mixture.

*** Determined from ^{14}C-acetyl incorporation as described in the materials and methods section.

**** Determined from modification with N-ethyl-(2,3-^{14}14C)maleimide after reduction by NADH, as described by De Kok *et al.* (1982).

These results are in excellent agreement with the results as previously obtained by De Abreu *et al.* (1977) for the so-called four- and three-component PDC preparations from *A.vinelandii*. In combination with the measured chain-ratio of the complex preparations (see chapter 5) it can be calculated that usually two lipoyl groups per E_2 chain (or slightly more) are reductively acetylated.

The thiol-Sepharose method of de Graaf and de Kok (1982) yields an isolated E_2 component with high enzymatic activity (see also chapter 5). After recombination with varying amounts of E_1, we detected a lipoyl content of 35.5 (±2) nmol/mg E_2. This corresponds to an average value of 2.3 (±0.2) lipoyl groups per E_2 chain. The results of these experiments are summarized in Table 6.1.

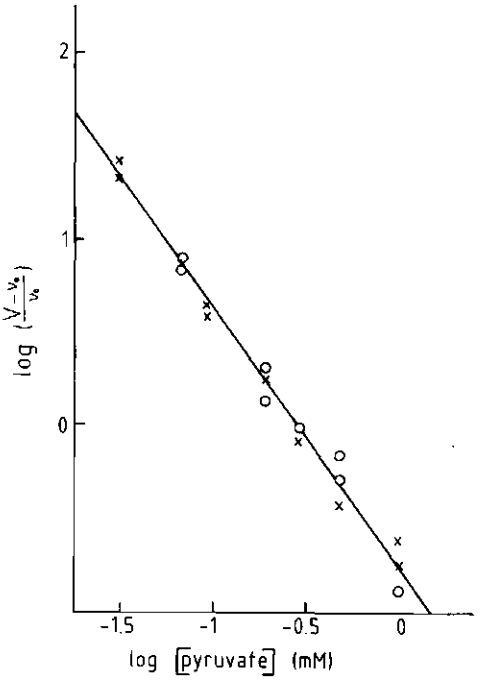


Fig. 6.1. Hill-plot of the influence of the pyruvate concentration on the overall activity of the *A.vinelandii* PDC (x), and of the DCPIP-dependent reaction of the complex-bound E_1 component (o). Measurements were performed in 50 mM potassium phosphate buffer, pH 7.0, temperature was 298 K.

Kinetics

Bresters *et al.* (1975a) previously demonstrated that the *A.vinelandii* PDC overall reaction shows cooperativity towards pyruvate; a Hill-coefficient of 2.3-2.7 was reported. In the present study however, we routinely obtained a value of 1.4-1.6 for the Hill-coefficient; the pyruvate concentration giving half-maximal velocity ($S_{0.5}$) was 0.3 mM. The same values were found for the partial reaction of the complex-bound E_1 component, using DCPIP as an artificial electron acceptor (Fig. 6.1).
The values for the Hill-coefficient and $S_{0.5}$ are hardly influenced by temperature. The rate of the overall reaction at saturating levels of substrates is however strongly dependent on the assay temperature (Fig. 6.2); an activation energy of 47 kJ/mol can be calculated.

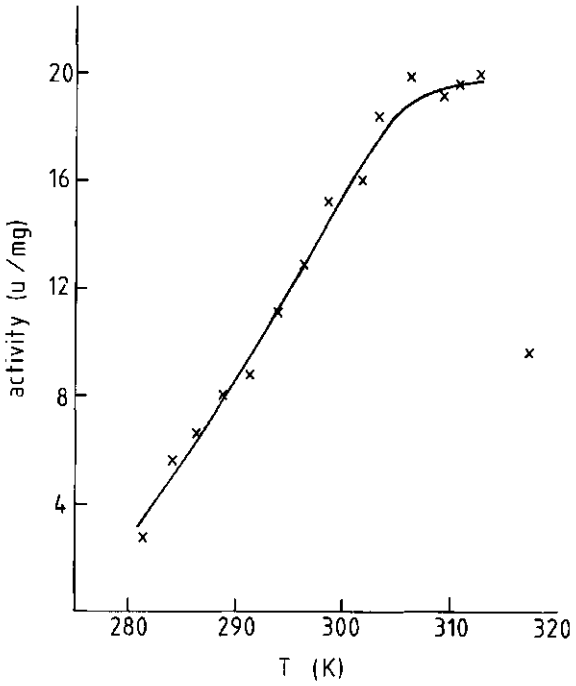


Fig. 6.2. Influence of the assay temperature on the overall activity of *A.vinelandii* PDC. The enzyme stock solution (1 mg/ml) and the assay cuvette were equilibrated at the indicated temperature for about 3 minutes prior to the assay.

The disagreement between our present results and those of Bresters is likely due to the treatment of the enzyme preparations prior to kinetic analysis. In order to be able to study the effect of several effectors, Bresters dialyzed the PDC preparations against Tris-Cl buffer. We have however always performed the kinetic measurements in potassium phosphate buffer, since the complex shows a higher activity and is more stabile in this buffer. We also observed Hill-coefficients of 2.1-2.7, when the PDC preparations were treated according to the method of Bresters. If such a preparation was brought in phosphate buffer again, a Hill coefficient of 1.4-1.6 was found.
An overnight dialysis step against Tris-Cl buffer results in an irreversible loss in overall enzyme activity and an irreproducible loss of TPP groups from the complex (F.G.H. van Wijk, unpublished results). Possibly, this loss of TPP from the complex could explain the apparent increase of the Hill-coefficient. Bisswanger has shown for the *E.coli* complex, that the binding of TPP to the E_1 component is relatively slow, resulting in a lag-phase in the production of NADH (Bisswanger, 1974). The binding of TPP is accelerated by pyruvate (Sümegi, 1983), which will result in an apparently higher overall activity at higher pyruvate concentrations; and thus in an apparent increase in the Hill-coefficient.

There is increasing evidence that for the PDC of other organisms, the reductive acetylation of the lipoyl groups is the rate-limiting step in the turnover cycle of the complex (Bates, 1977; Danson, 1978; Cate, 1980).
To determine whether this step is also rate-limiting in the *A.vinelandii* PDC, we have performed chemical-quench rapid-mixing experiments. As can be calculated from Fig. 6.3, the initial rate of acetylation under the applied conditions (293 K; 1 mM pyruvate) was 180(±30) $nmol.s^{-1}.(mgPDC)^{-1}$, which corresponds to a specific activity of 10.8(±1.7) U/mg PDC. Corrected to standard conditions (298 K, 5 mM pyruvate), this would result in an overall enzyme activity of about 15 U/mg PDC (see also Figs. 6.1 and 6.2); the specific activity of the complex preparation that was used in this experiment was 16 U/mg PDC. In addition to this evidence that the acetylation reaction is rate-limiting, the involvement of the E_1 component in the rate-

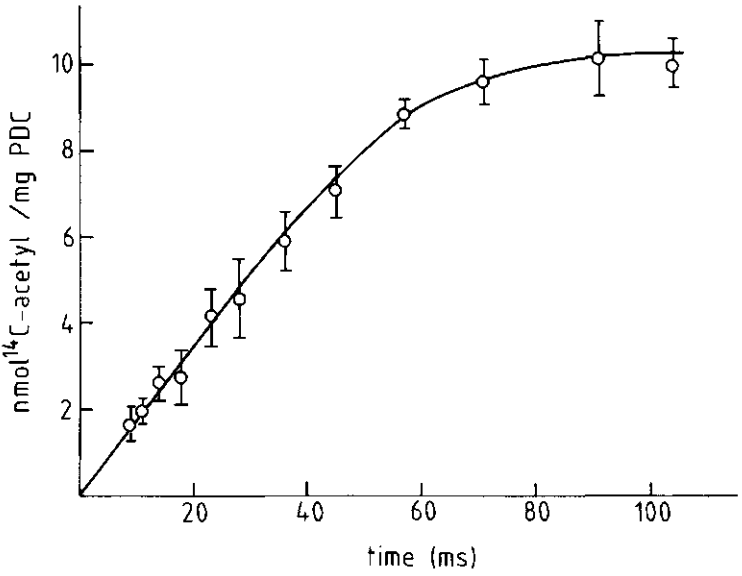


Fig. 6.3. Time-course of ^{14}C-acetyl incorporation in *A.vinelandii* PDC, as determined by rapid-mixing chemical-quench experiments. PDC concentration was 1 mg/ml in 50 mM potassium phosphate buffer, pH 7.0, containing 1.25 mM $MgCl_2$ and 0.5 mM TPP. ^{14}C-Pyruvate concentration was 1 mM, temperature was 293 K.

determining step can also be derived from the observation that the overall complex activity is linearly correlated with the amount of active E_1 bound to the E_2 core (Bates, 1977; chapter 5; next paragraph, Fig. 6.4).

The E_1 component catalyzes two partial reactions: the decarboxylation of pyruvate, and the reductive acetylation of the E_2 component. To determine which of these two steps is rate-limiting, we performed a pulse-chase experiment. Due to experimental complications, this experiment could not be performed with the rapid-mixing apparatus. The rate of acetylation was therefore lowered considerably by changing the temperature to 273 K, and the pyruvate concentration was lowered to 0.15 mM. Under these conditions, about 50% of the lipoyl groups was acetylated after 10 seconds. A subsequent 60 second chase with a 70-fold excess of non-radioactive pyruvate still resulted in a 100%(±10%) incorporation of radioactive acetyl groups. The

following scheme shows the steps leading to the reductive acetylation of the lipoyl group:

$$TPP\text{-}E_1 + pyr \rightleftarrows TPP\text{-}E_1\text{-}Pyr \rightarrow HE\text{-}TPP\text{-}E_1 \rightarrow acetyl\text{-}S\text{-}E_2 + E_1\text{-}TPP$$

The first step represents the binding equilibrium between substrate and enzyme; it is supposed to have a very short relaxation time. The next step is the decarboxylation of pyruvate to yield CO_2 and hydroxyethyl-TPP (HE-TPP), this step is irreversible and proceeds at a lower rate than the initial binding of pyruvate. Since the HE-TPP group is not covalently bound to the protein, addition of trichloroacetic acid at this stage is not expected to result in coprecipitation of radioactive label and protein. The last step in the scheme represents the reductive acetylation of the lipoyl groups, resulting in a covalent linkage between label and protein.
After 10 seconds of incubation, the chase with an excess of "cold" pyruvate does not influence the incorporation of the radioactive label. This indicates that most of the acetyl groups that are eventually incorporated into the E_2 component already must have undergone the decarboxylation step. The actual reductive acetylation of the lipoyl group must therefore be rate-determining, at least under the applied conditions, as will be discussed in the following section.

At 273 K, 95% of the lipoyl groups is rapidly deacetylated by 0.2 mM coenzyme A (within the "dead" time of 10 seconds). This fraction however decreases upon prolonged incubation of the complex with pyruvate, *e.g.* after 5 minutes preincubation with 0.15 mM pyruvate at 273 K, only 75% of the lipoyl groups is rapidly deacetylated by CoA. The total amount of incorporated acetyl groups does not increase during this prolonged incubation with pyruvate. The presence of NAD^+ has no effect on these results. A transfer of acetyl groups from the lipoic acid residues to other (sulfhydryl?) groups in the protein therefore does not seem very likely, since such a transfer would render the lipoyl group available again for acetylation (Akiyama, 1980). The observation can be explained by the hypothesis that the acetyl group is transferred from the sulfhydryl group at the 6-position of the lipoyl group to that at the 8-position, as

has already been suggested by Hale and Dixon (Hale, 1981). Such a transfer would also explain the observed inactivation of the complex during prolonged incubation with pyruvate (De Abreu, 1977a).
Under turn-over conditions, the fraction of acetylated lipoyl groups is lower than 5%. This was measured by trichloroacetic acid-precipitation of a preparation of the complex that was incubated with radioactive pyruvate and all other components involved in the overall reaction. Concentrations of the substrates were the same as routinely used in the overall enzyme assay (Bresters, 1975a; chapter 2). This observation again indicates that the deacetylation reaction proceeds at a much higher rate than the acetylation of the lipoyl groups.
The deacetylation reaction is not only very fast, but also very specific. We did not observe any deacetylation after a 10 minutes incubation with either 0.2 mM dithiotreitol, oxidized lipoamide or reduced lipoamide.

Servicing experiments

Due to the transacetylation reactions between E_2 chains, a single E_1 monomer can acetylate several lipoyl groups. Conversely, a lipoyl group can be "serviced" by a certain number (n) of E_1's, and n is called the servicing number.
If a fraction p of the E_1's is active, the probability that n E_1's are inactive is $(1-p)^n$. Thus, the probability that a given lipoyl group will be acetylated is $1-(1-p)^n$; *i.e.* when the servicing number is high, full acetylation of the E_2 core can still be accomplished by only a few active E_1's. In servicing experiments, the degree of acetylation is measured as a function of the amount of active E_1 that is bound to the E_2 core; the servicing number can then be estimated from the experimental results. The servicing number n can be most clearly interpreted as the number of acetyl-entry sites on the E_2 core that are coupled through the system of interacting lipoyl groups. The servicing number can therefore never exceed the number of E_1 chains that are maximally bound to the complex. Thus, for the small form of the *A.vinelandii* complex, which is based on a tetramer of E_2, a maximum servicing number of 6 is expected (see chapter 5).

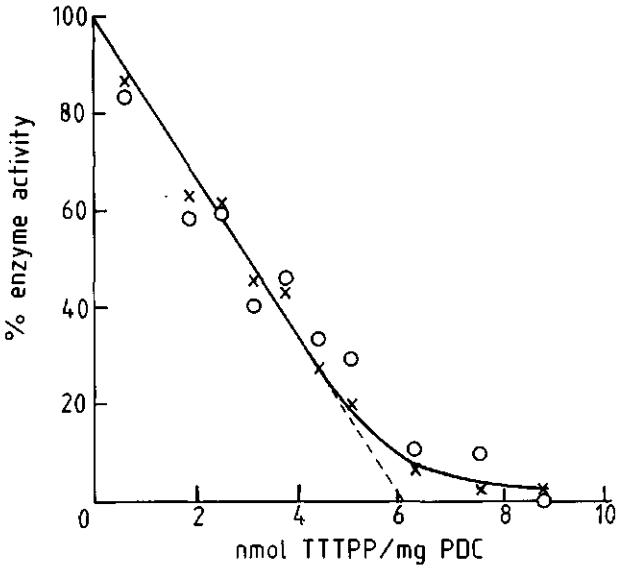


Fig. 6.4. Inhibition of *A.vinelandii* PDC with TTTPP. Varying amounts of TTTPP (in 0-15 µl) were added to 150 µl aliquots of *A.vinelandii* PDC (1.05 mg/ml), as described for the servicing experiments in the materials and methods section.
Both the overall enzyme activity (x) and the DCPIP-dependent activity of the E_1 component (o) were measured at 303 K relative to the activities of an uninhibited sample.

In order to be able to "service" its maximum number of lipoyl groups, the E_1 monomer must have the opportunity to proceed through several reaction cycles. Since the turnover time of an E_1 monomer is of the order of 35 ms (see Fig. 6.3), this means that the incubation time with pyruvate in the servicing experiments should not be taken shorter than a few seconds.

The servicing experiments can be performed in two ways: in one method, the E_1 component in a PDC preparation is at varying levels inhibited by a specific inhibitor (Collins, 1977; Stanley, 1981). In the other approach, isolated E_1 is in varying amounts added to an E_2E_3 subcomplex and the degree of acetylation is subsequently measured (Bates, 1977).
We have used both methods to study the acetyl transfer reactions in the *A.vinelandii* PDC. Since we were not able to isolate the

component enzymes in an active form at the start of this study, most of the servicing experiments were performed by the E_1-inhibition method, in which we have used thiamine thiothiazolone pyrophosphate (TTTPP), a transition state analogue of TPP (Gutowski, 1976). Titration of *A.vinelandii* PDC with TTTPP shows that the inhibitor very strongly binds to the E_1 component (Fig. 6.4). Interpolation of the linear part of this curve to zero activity yields a value of 6.1 nmol TTTPP bound per mg PDC. Assuming a chain ratio of 1.3:1:0.5 (see chapter 5), this means the binding of 1.0 TTTPP molecule per E_1 chain.
The linearity of the titration curve indicates that the E_1 chains act independently in the acetylation of E_2, *i.e.* an E_1 dimer is only inhibited for 50% upon the binding of one TTTPP molecule to one of the dimer chains. It can also be seen from Fig. 6.4 that the overall complex activity is linearly correlated with the amount of active E_1. This observation indicates that the E_1 component is involved in the rate-determining step of the reaction sequence (see also preceeding section). In the practice of the servicing experiments it means that the (relative) amount of active E_1 can be monitored by the overall enzyme activity, which can be determined more accurately than the E_1 partial activity measured by DCPIP reduction.

At temperatures of 293 K or higher, a servicing number of 12-24 was found (Fig. 6.5). The servicing number gradually decreased to 3 when the temperature was lowered to 273 K. Since servicing numbers higher than 6 are improbable for the small, E_2-tetramer-based complex of *A.vinelandii*, these observations were originally interpreted in terms of the formation of the 56 S species of *A.vinelandii* PDC. The temperature dependence of the servicing number seemed to agree with this conclusion, since the formation of the 56 S species of the complex is favoured by an increase in temperature (see chapter 4).
There was however no difference between the servicing number of a pure 18 S complex preparation and that of a preparation containing a 50%-50% mixture of 18 S and 56 S species (in standard potassium phosphate buffer). Addition of PEG 6000 to either one of these samples also did not have an effect on the servicing number. At 303 K, the servicing number is also unaffected by a five-fold variation in pyruvate concentration, a five-fold va-

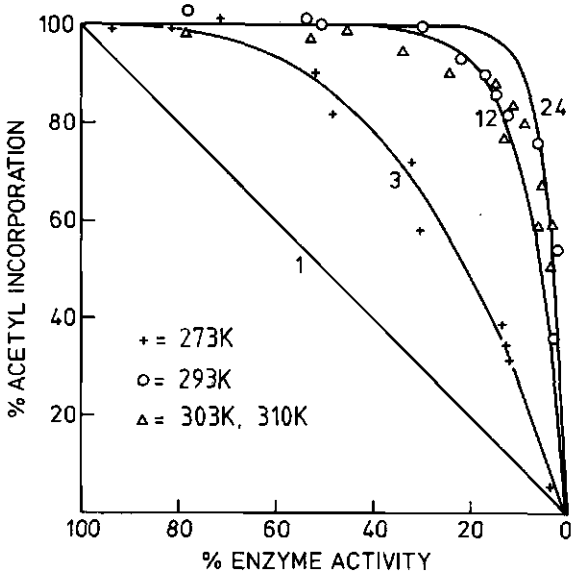


Fig. 6.5. Servicing curves of the *A.vinelandii* PDC, as obtained with TTTPP-inhibition at several temperatures. The solid lines represent the theoretical curves for servicing numbers (n) 1, 3, 12 and 24. Conditions: 1 mg/ml PDC, 0.15 mM pyruvate, 0.5 mM NAD^+, 0.2 mM TPP, and 2 mM $MgCl_2$. The reaction was stopped 30 seconds after the addition of ^{14}C-pyruvate with a 10% (w/vol.) solution of ice-cold trichloroacetic acid.

riation in protein concentration, and a ten-fold variation in solvent viscosity. Clearly, there is no correlation between the amount of 56 S species that is present in a complex preparation and the observed servicing number.

As discussed before, the apparent servicing number will be time-dependent at very short pyruvate-incubation times. The E_1 component will then not have had the opportunity to proceed through several reaction cycles, and the maximum level of acetylation will not have been reached. This process takes place at the time-scale of milliseconds to a few seconds, and therefore, at longer incubation times, the level of acetylation is expected to be constant. Indeed, for the complexes of *E.coli* and of beef heart, no difference in servicing number was found after 20 seconds or 2 minutes incubation with pyruvate (Bates, 1977; Stanley, 1981; Packman, 1983).

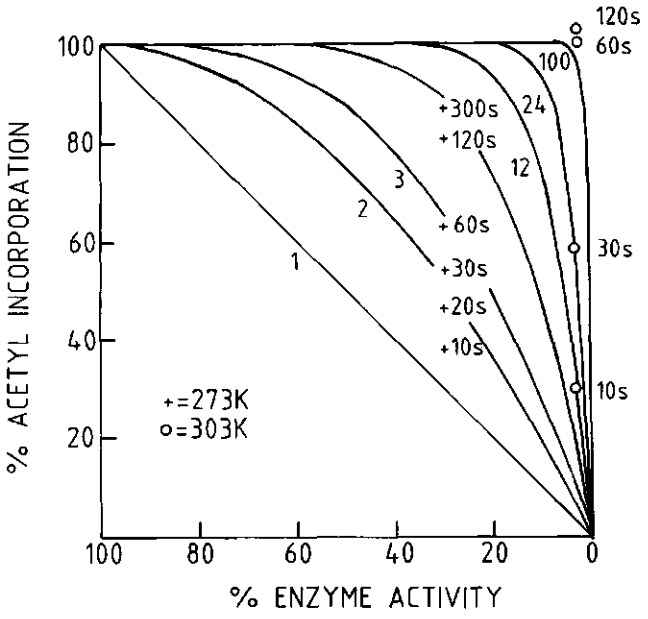


Fig. 6.6. Time-dependence of the servicing number of TTTPP-inhibited *A.vinelandii* PDC. The pyruvate incubation time was varied at a fixed degree of inhibition of the complex activity. Theoretical servicing curves for n = 1, 3, 6, 12, 24 and 100 are drawn.
Other conditions as in the legend to Fig. 6.5.

For the PDC of *A.vinelandii*, the servicing number however proved to be time-dependent on this time-scale. After 60 seconds incubation at 303 K with ^{14}C-pyruvate (instead of 30 s), a servicing number higher than 100 was found. At 273 K the servicing number varied between 1 and 6, dependent on the duration of the incubation (Fig. 6.6).

A possible explanation for our observations would be a rapid association-dissociation of active E_1 chains from the PDC particles. In this way, a single E_1 molecule could interact with a large number of E_2 chains, resulting in a very high servicing number. For the beef heart complex, a rapid E_1 exchange was observed by Cate and Roche (1979, 1980). An exchange of TTTPP molecules between E_1 chains would have a similar effect. To determine whether one of these two processes occurs, we inhibited a sample of *A.vinelandii* PDC with TTTPP to a level of 5% residual activity. Another PDC sample was specifically inhibited

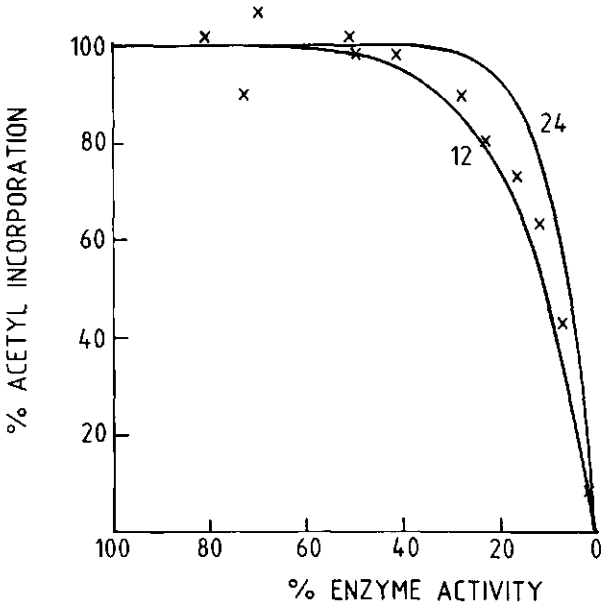


Fig. 6.7. Servicing curve of an *A.vinelandii* E_2E_3 subcomplex (1:0.8 chain-ratio), recombined with varying amounts of E_1. The 100% values for the overall enzyme activity and acetyl incorporation were obtained from a reconstituted complex with an estimated chain-ratio of 1.6:1:0.8. E_2 concentration was 0.21 mg/ml, temperature was 293 K, other conditions as described in the legend to Fig. 6.5.

on the E_2 component for 90% by reaction with N-ethyl-maleimide after reduction with NADH (De Kok, 1982).
Mixing of these two samples did not result in detectable restoration of PDC activity within a few hours, which indicates that exchange of E_1 or TTTPP molecules does not occur on this time-scale, as was also shown for the *E.coli* complex (Hale, 1979b).

It has been shown that the *A.vinelandii* PDC particles that are based on a tetramer of E_2 (so-called monomeric PDC particles) have a tendency to dimerize. This dimerization is very fast: with a relaxation time of 70 ms (chapter 4, Fig. 4.5). To explain our results of the servicing experiments, we suggest that transacetylation reactions occur between the E_2 cores of the monomeric PDC particles during the dimer-stage (inter-core transacetylation).

The result of another type of servicing experiment is shown

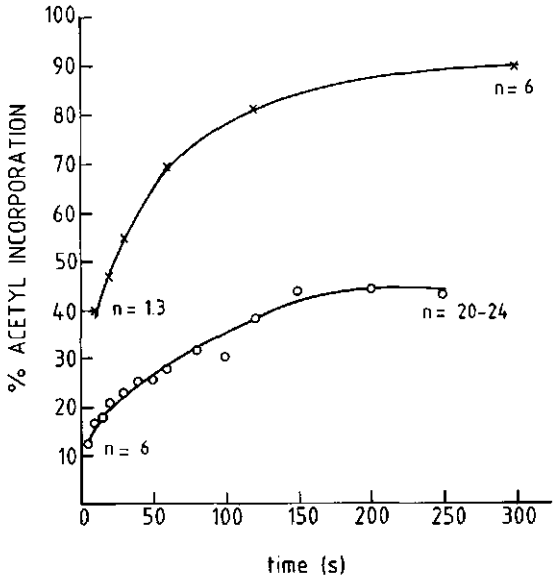


Fig. 6.8. Time-dependence of the acetyl incorporation in a partially reconstituted *A.vinelandii* PDC, with a chain-ratio of 0.08:1:0.04 and 5% residual activity (0). The 100% acetyl incorporation value was obtained from a sample with a recombination chain-ratio of 2.0:1:0.04. Conditions: E_2 concentration tion was 0.17 mg/ml, temperature 298 K, all other conditions as in the legend to Fig. 6.5.
(x), Time-dependence of the acetyl incorporation in an *A.vinelandii* PDC preparation that was inhibited to a residual activity of 30% by TTTPP. Temperature was 273 K, the same experimental results are plotted in Fig. 6.6.

in Fig. 6.7. In this experiment, an E_2E_3 subcomplex was recombined with varying amounts of E_1. The experimental conditions are comparable to those of the TTTPP experiments. There is however an important difference between the two types of servicing experiments. In the TTTPP experiments, each E_1 chain is independently inactivated and a single E_1 chain is the smallest active unit that can be bound to an E_2 core. In the reconstitution experiments however, the E_1 component binds to the E_2 core in the form of dimers (chapter 4). The servicing number will then represent the amount of E_1-dimer binding sites on the E_2 core that are coupled through the system of interacting lipoyl groups. The observed servicing number will therefore be half of

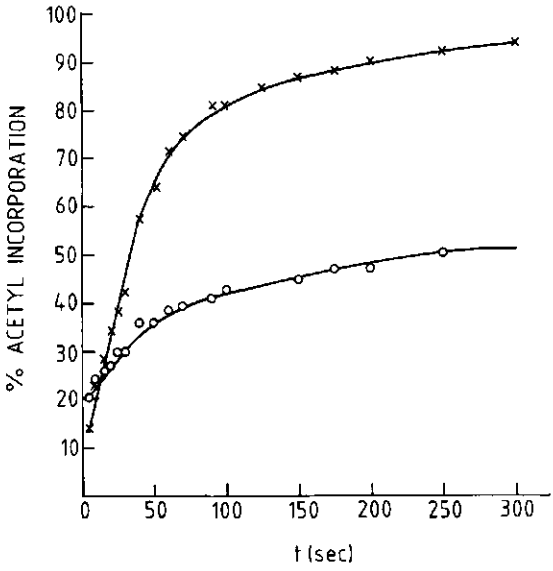


Fig. 6.9. Time-dependence of the incorporation of acetyl groups into an E_1E_2 subcomplex of *A.vinelandii* PDC; (o), E_1:E_2 chain-ratio of 0.05:1; (x), the same subcomplex with E_3 added, chain ratio of 0.05:1:2.04. E_2 concentration was 0.17 mg/ml, temperature was 303 K, all other conditions as in the legend to Fig. 6.7.

that found in the TTTPP experiments, when all other factors remain constant. Thus, to obtain a good comparison with the TTTPP experiments, we have doubled the servicing numbers from the recombination experiments. Fig. 6.7 shows that the (corrected) servicing number of this type of experiment is the same as that of the TTTPP experiments.

Fig. 6.8 shows the time-dependence of acetylation of the E_2 core under conditions where only small amounts of E_1 and E_3 are added to the E_2 component (chain-ratio 0.08:1:0.04). Initially the amount of acetylation corresponds to a servicing number of 6, which can be interpreted as transacetylation within an E_2 tetramer, since a maximum number of three E_1 dimers can be bound to such an E_2 tetramer (chapter 5). On a longer timescale of 10 seconds to 2 minutes the degree of acetylation increases, levelling off at a value corresponding to a servicing number of about 20 to 24.

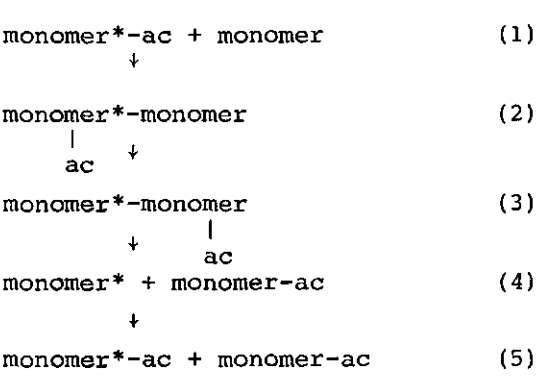
Unlike the TTTPP-treated complex preparations, the extent of transacetylation is restricted to a certain level in this partially reconstituted complex. Since only very low amounts of E_1 and E_3 are present, about 85% of the subcomplex will be associated into a large *E.coli*-like form (chapter 4; Bosma, 1984a). This large form is probably composed of 32 E_2 chains and is therefore an octamer of the small form of *A.vinelandii* PDC, based on a tetramer of E_2 (so-called monomeric PDC). The octameric form of *A.vinelandii* PDC is only in slow equilibrium with the PDC monomers and dimers (small, 18 S form of *A.vinelandii* PDC).
On the timescale of this experiment, transacetylation reactions can therefore only occur within the large *E.coli*-like octamers. This hypothesis is supported by the results of the experiment given in Fig. 6.9. Addition of large amounts of E_3 to an E_1E_2 subcomplex with a low E_1:E_2 ratio will cause the dissociation of the large "*E.coli*"-like octamers into the small structures characteristic for *A.vinelandii* PDC (chapter 4; Bosma, 1984a). As predicted by the model, the addition of E_3 results in a higher degree of acetylation after prolonged incubation with pyruvate. This observation is another indication that inter-core transacetylation occurs, when individual PDC particles are involved in a functional, rapid association-dissociation equilibrium.

DISCUSSION

The acetylation of the E_2 core of the *A.vinelandii* PDC occurs in a complex series of events, and in the interpretation of the experimental results at least three different processes must be considered.
The first step is the reductive acetylation of a lipoic acid residue by a neighbouring E_1 molecule. This step can be followed by a transfer of the acetyl group and the reducing equivalents to other lipoyl groups on the E_2 core (so-called intra-core transacetylation). In this way, a relay system of interacting lipoyl groups is formed. Finally, a transfer of acetyl groups and reducing equivalents between individual E_2 cores could occur, resulting in the extension of the relay system

beyond the limits of a single E_2 core (inter-core transacetylation). Eventually, this could lead to the reductive acetylation of all E_2 cores present in the assay mixture by only one active E_1 molecule (infinitely large servicing number). From the experiments described in this chapter it is clear that both the intra-core and the inter-core transacetylation reactions occur within the PDC of *A.vinelandii*.

The phenomenon of inter-core transacetylation has until now only been observed for *A.vinelandii* PDC. Another typical feature of the PDC of this organism is its association behaviour; we have previously shown that the *A.vinelandii* PDC particles that are based on a tetrameric E_2 core (so-called PDC monomers) have a tendency to dimerize (chapter 4). This combination of observations suggests that inter-core transacetylation can only occur when individual PDC particles become functionally associated. The sequence of events in the inter-core transacetylation process is represented in the following scheme. Monomer* represents a monomeric *A.vinelandii* PDC particle that contains one or more active E_1 chains, and its E_2 core can therefore be acetylated(-ac). In the monomer without asterisk, all E_1 chains are inhibited by TTTPP.



The actual inter-core transacetylation step occurs when the two monomeric PDC particles are associated into a dimer (step 2-3). This hypothesis has been confirmed by acetylation experiments with partially reconstituted *A.vinelandii* PDC. When very low amounts of E_1 and E_3 are bound to the E_2 core, almost all of the (incomplete) PDC will be in the large, "*E.coli*" PDC-like

octameric form, based on 32 E_2 chains. These large structures are in slow equilibrium with the E_2-tetramer-based PDC-monomers that are characteristic for *A.vinelandii* PDC, and the octamers show no tendency to form even larger polymers (chapter 4).
The degree of acetylation of such a preparation is restricted to a maximum level. This level corresponds to a servicing number that is theoretically compatible with a structure based on 32 E_2 chains. These results indicate that no inter-core transacetylation occurs between octameric particles.
When the octameric structures are dissociated into the monomers (that are in rapid equilibrium with dimers) by addition of a high amount of E_3, inter-core transacetylation is again observed, at a rate comparable to that in the TTTPP-experiments (Fig. 6.9). Since it is generally accepted that E_3 is not involved in the acetylation or transacetylation reactions, this observation indicates that the process of inter-core transacetylation requires a rapid association-dissociation equilibrium, leading to close functional contact between the individual PDC particles.

Since the time-constants of the transacetylation reactions are much higher than the relaxation time of the dimerization equilibrium, other reactions must be rate-limiting. This also is evident from the servicing experiments: the observed servicing number of TTTPP-inhibited *A.vinelandii* PDC is strongly temperature-dependent between 273 and 293 K, whereas no change in servicing number is observed between 293 and 310 K.
Apparently, in the two temperature domains, different processes are rate-determining. Because the acetyltransfer is a catalyzed process, a functional contact is required for the inter-core transacetylation. A lipoyl group located on one core must be able to interact with the catalytic site of an E_2 chain on the other core. To obtain the proper geometry, some reorganization is necessary after formation of the dimer, and this step could be rate-limiting under certain conditions.

The kinetics of the (intra- and inter-core) transacetylation reactions can only be studied when the preceeding step is not rate-limiting, *i.e.* the "input" of acetyl groups into the E_2 core by E_1 must proceed fast enough. This could cause a problem when experiments are performed at low temperature and low pyruvate concentrations.

It can be calculated, that under the conditions that were used in the experiments described in this chapter, the E_1 reaction is not rate-limiting (see Figs. 6.1, 6.2 and 6.3). This can also be seen from Fig. 6.8: there is an initial 'jump' in the acetyl incorporation, followed by a slower increase that occurs on a timescale of ten seconds to several minutes. At 273 K, the initial jump corresponds to a servicing number of about 1, which means that no transacetylation reactions have occurred within the few seconds that have elapsed from the addition of pyruvate. The acetyl incorporation then slowly increases with time, levelling off at a value corresponding with a servicing number of about 6. This is the theoretically predicted maximum value for the *A.vinelandii* PDC, when no inter-core transacetylation would occur.

The time-curve of the acetyl incorporation of a partially reconstituted PDC sample shows an initial jump corresponding with a servicing number of 6 (Fig. 6.8). At a time-scale of minutes the incorporation of acetyl groups gradually increases and it clearly levels off at a much higher servicing number (20 to 24). Since only very low amounts of E_1 and E_3 are bound to the E_2 core, about 85% of the subcomplex preparation will be in the large, "*E.coli*-like" octameric form which is in slow equilibrium with the E_2-tetramer based "monomeric" form. This experiment shows that the transacetylation reactions proceed faster within the E_2 tetramers, even when these tetramers are associated in distinct, larger structures (octameric form). As discussed above, this experiment also shows that in these large structures the transacetylation reactions are restricted to the 32-meric E_2 core, no inter-core transacetylation reactions occur.
It is tempting to conclude that in this PDC octamer, only part of the E_2 core can be acetylated by a single E_1 dimer (n=24 instead of 48). One should however keep in mind that it is practically very difficult to distinguish between these large servicing numbers.

The results of the servicing experiments can be summarized as follows: the inter-core transacetylation reactions are relatively slow, with time-constants of the order of seconds to several minutes. The transacetylation reactions are strongly temperature-dependent: at 273 K no inter-core transacetylation

is observed after 5 minutes, whereas at 293 K (or higher) this process is already detectable after 10 seconds.
Experiments with partially reconstituted *A.vinelandii* PDC show that the transacetylation reactions inside the E_2 tetramers proceed at a higher rate than those within the large core based on 32 E_2 chains. This large structure therefore seems to be composed of eight E_2 tetramers that function rather independently.

Due to its slowness, the physiological importance of the inter-core transacetylation process between the E_2 tetramers remains questionable, although *in vivo* conditions can occur like high acetyl-CoA and NADH levels, under which the rate of the overall-reaction of the complex will be very low (Bresters, 1975a). It has been suggested by Perham's group (Danson, 1978; Packman, 1983) that the transacetylation reactions are not rate-limiting in the *E.coli* PDC. Danson has performed rapid-quench experiments, but these measurements were done at very low pyruvate concentrations (to slow down the reaction rate). Our results show that care has to be taken to maintain the E_1 activity as high as possible in order to be able to study the kinetics of the inter-core and intra-core transacetylation. Therefore these measurements do not seem to be conclusive to us. Packman *et al.* (1983) concluded from the work of Akiyama and Hammes (Akiyama, 1980) that the transacetylation reactions are not rate-limiting. In rapid-mixing experiments at 277 K, two distinct phases in the acetylation of (uninhibited) *E.coli* PDC were observed. The fast process is fast enough to be of catalytic importance and corresponds with about half of the lipoic acid residues that are present on the E_2 core. The second process proceeds at a time-scale of seconds and, although Akiyama and Hammes reach a different conclusion, the rate of this process is not dependent on the pyruvate concentration.
In view of our observations on *A.vinelandii* PDC, we suggest that this slow process could be the transacetylation reaction. One lipoic acid residue per E_1 monomer is rapidly acetylated. Apparently, the E_1 molecule can only proceed through a second complete reaction cycle when the slower process has taken place, *i.e.* when the acetyl group and the reducing equivalents have been transferred to another lipoyl group. If this hypothesis is correct, each E_1 molecule would have its "own" lipoic acid

residue. It would also mean that the transacetylation reaction is not merely a by-pass for the complex activity under certain conditions; intra-core transacetylation (or the exchange of reducing equivalents) would be an essential step in the reaction cycle.

However, on the basis of the experimental results presently available, no unequivocal conclusions can be drawn on the role of the transacetylation reactions in the reaction mechanism of the pyruvate dehydrogenase complexes of *A.vinelandii* and *E.coli*.

7. THE QUATERNARY STRUCTURE OF THE PYRUVATE DEHYDROGENASE COMPLEX OF *AZOTOBACTER VINELANDII*

From the data presented in the preceeding chapters we conclude that the pyruvate dehydrogenase complex of *Azotobacter vinelandii* is based on a tetrameric E_2 core, to which a total of four E_1 and E_3 dimers can be bound in a non-covalent, mutually exclusive way. Maximal complex activity is obtained upon binding of three E_1 dimers and one E_3 dimer. This optimal chain-stoichiometry is achieved by the existence of one high-affinity binding site of E_3; this site is either intrinsically present, or it is formed upon the binding of three E_1 dimers (chapter 5).

The PDC of *A.vinelandii* has a tendency to dimerize, and therefore the E_2-tetramer-based form of the complex is referred to as the PDC-monomer. Upon addition of polyethylene glycol 6000 (PEG 6000) and $MgCl_2$, a large form of the complex is observed, which is probably composed of eight monomers (chapter 4). Its sedimentation coefficient and its appearance on electron micrographs resembles that of the PDC of *Escherichia coli*. This octamer (the so-called 56 S form) is in slow equilibrium with the monomeric and dimeric species of the complex (the 18 S form), and a tetrameric species is probably also present under these conditions (chapter 4).

When the E_1 and E_3 dimers are dissociated from the E_2 component, a large E_2 core is formed, composed of about 30 E_2 chains. On electron micrographs this structure has the appearance of a cube, and it closely resembles the isolated E_2 component of *E.coli* PDC. For reasons of symmetry we conclude that it is composed of 32 E_2 chains, *i.e.* eight E_2 tetramers. Thus, the association of the isolated E_2 component and the formation of the octameric PDC form upon addition of PEG 6000 seem to be related processes.

A model for the quaternary structure of *A.vinelandii* PDC must therefore take into account:

1. The mutual exclusive binding of E_1- and E_3 dimers.
2. The existence of one high-affinity binding site for E_3 or its formation upon the binding of three E_1 dimers.
3. Symmetry requirements
4. The association behaviour of the complex and of the isolated E_2 component.

For a tetrameric E_2 core, two quaternary structures need to be considered: a square plane and a tetrahedron. The square planar model can be excluded on the basis of the considerations given above. Such a structure cannot associate into an octameric cube without major rearrangements. Furthermore, the existance or formation of a single high-affinity binding site for E_3 is not evident, and in the optimal stoichiometry a highly asymmetrical structure would be formed.

For the tetrahedron model some arrangements seem possible; two of those are pictured in Fig. 7.1, as part of the proposed octameric structure. Within the E_2 tetramer, the positions of E_1 and E_3 are considered topologically equivalent, which explains the observed mutually exclusive binding characteristics. For the 6:4:2 chain-stoichiometry, these arrangements yield the highest obtainable degree of symmetry. The existence of a single high-affinity site for E_3 in the E_2 tetramer is improbable, and according to the model it should therefore be formed upon the binding of three E_1 dimers, through E_1-induced (minor?) rearrangements in the E_2 component.

In contrast to the situation in the monomeric PDC particle, the positions of E_1 and E_3 are not equivalent in the large octameric structure. E_3 is positioned at the corners of the cube and E_1 is placed either on the edges (not shown) or in the planes. The E_3 component is far removed from the interfaces between E_2 tetramers and it is expected that the binding of E_3 at this position has no influence on the dissociation of the octameric structure. We have indeed experimentally shown that one E_3 dimer per E_2 tetramer can be bound with almost no effect on the dissociation of the large E_2 core (Fig. 4.12B). As predicted by the model, binding of small amounts of E_1 to the large E_2 component already promotes its dissociation into the E_2 tetramers (Fig. 4.12A).

It is clear that the positioning of E_1 and E_3 dimers on the

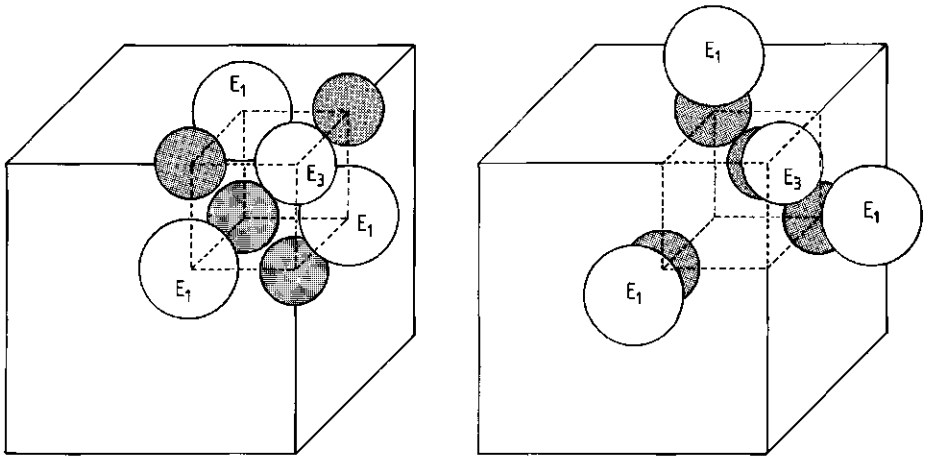


Fig. 7.1. Two possible arrangements of the components of the pyruvate dehydrogenase complex from *Azotobacter vinelandii*, that fit into the cubic structure of the isolated E_2 component and the aggregated form of the complex. E_2 is pictured as a monomer (shaded), E_1 and E_3 are pictured as dimers.

trigonal axis of the E_2 tetrahedron presents symmetry problems. In model A each E_1 or E_3 dimer interacts with three E_2 subunits, *i.e.* the E_1 and E_3 dimers must contain three trigonal symmetric E_2 binding domains, which seems unlikely. Furthermore, one of the E_2 subunits is positioned in the centre of the large E_2 cube, with no interaction with the E_3 component. Due to this position, its function is obscure and we therefore prefer model B in which each E_2 chain is supposed to interact with one E_1 or E_3 dimer, through a single, probably two-fold symmetric binding domain.

In model B, the E_2 chain positioned on the trigonal cubic axis (the corner) fulfills a special function, *e.g.* the transfer of reducing equivalents from dihydrolipoyl groups on other E_2 chains to the E_3 component.
The lipoyl groups located on the "E_1-dedicated" E_2 chains will accept the acetyl group and reducing equivalents from the E_1 component. The deacetylation by coenzyme A is very rapid (chapter 6; Akiyama, 1980 and 1981) and could occur before transace-

tylation between adjacent lipoyl groups takes place. The reducing equivalents are then transferred to the lipoyl groups on the E_2 subunit to which the E_3 component is bound. This proposed sequence of events can also explain the acetylation and deacetylation kinetics observed for the PDC of *E.coli* (Akiyama, 1980 and 1981).

This brings us to the question on the amount of lipoic acid residues per E_2 chain. Although it is generally accepted for the E_2 component of *E.coli* PDC that each E_2 chain carries two lipoic acid residues (Collins, 1977; Danson, 1976 and 1981a; White, 1980), it follows from the DNA sequence of this protein that three lipoyl-binding regions could exist (Stephens, 1983b). In freshly isolated *A.vinelandii* PDC we have routinely found values between 2 and 2.5 lipoyl groups per E_2 chain (*i.e.* 8-10 per E_2 tetramer). Assuming that this deviation from 2 is significant, some explanations are possible.

In the first place, the amount of lipoic acid residues per E_2 chain may be variable, since these groups are enzymatically coupled to the E_2 chains after transcription (Leach, 1970), and they can also be removed by lipoamidase (Suzuki, 1963).

Secondly, due to the special function of one of the four E_2 subunits in the transfer of reducing equivalents to E_3, this E_2 chain could contain three lipoyl groups, since it would have to accept the reducing equivalents from the three other E_2 chains. The asymmetric position of the E_3 dimer with respect to these three lipoyl groups could be averaged out in time, due to the high mobility of the E_3 dimer within the complex, and the high flexibility of the lipoyl-containing regions of the E_2 chains (Grande, 1976 and 1980; Perham, 1981b; Bosma, 1982; Duckworth, 1982; De Kok, to be published).

The third possibility is that all E_2 chains contain three lipoyl groups, but that those "dedicated" to E_3 cannot be acetylated. With our experimental results, it is impossible to distinguish between the three possibilities.

In the PDC of *E.coli*, about 50% of the lipoyl groups can be excised from the E_2 core with almost no decrease in overall activity (Bleile, 1979; Stepp, 1981; Berman, 1981; Perham, 1983). From computer simulations of these experimental results it was concluded that each E_1 dimer must be able to interact with at

least three, and possibly even four E_2 subunits (Hackert, 1983b). We have not performed this type of experiment on the PDC of *A.vinelandii*, but if the complexes would behave similarly in this respect, model B of Fig. 7.1 would be invalid; model A would be the most probable arrangement of *A.vinelandii* PDC, in spite of the objections against this model given above.

The main feature of our 32-E_2 model is that it is composed of morphological subunits. The small form of the complex is supposed to associate into the octameric form without major reorientation or redistribution of component enzymes. Indeed we have no indications from sedimentation velocity experiments that components would dissociate from the E_2 core when association into the large "*E.coli* PDC-like" structure occurs. The association equilibria in polyethylene glycol and those of (partially) reconstituted subcomplexes are, however, slow processes with relaxation times in the order of 10-30 minutes. This is indicative for a high energy of activation which could be caused by changes in the quaternary structure. Therefore the extrapolation from the small tetrameric form to the large, cubic structure of E_2 and *vice versa* may not be allowed.
This touches to the more general problem: why would a tetramer in the form of a tetrahedron associate into a cubic structure? This is only possible when the symmetry is somewhat distorted upon association, resulting in a cubical structure composed of two classes of E_2 chains. 24 of these E_2 chains would have identical environments, different from the 8 chains located on the corners of the cube (Fig. 7.1B). Our measurements however indicate that the isolated *A.vinelandii* E_2 component is composed of significantly more than 24 E_2 chains, as was assessed by three independent techniques (chapter 4). Nevertheless, the symmetry requirements within the large (isolated) E_2 component of *A.vinelandii* PDC remains the main argument against the 32-mer model.

As is shown in chapters 4, 5 and 6, the PDC of *A.vinelandii* is much more similar to the complex of *E.coli* than previously had been assumed. For the *E.coli* complex, dissociation into enzymatically active 19 S particles with conservation of chain-stoichiometry and complex activity has been observed (Schmitt, 1975; Danson, 1979). Furthermore, we have discussed that the optimal chain-ratio probably is 1.5:1:0.5 for both complexes:

the values we have obtained for the flavin and lipoyl content of *A.vinelandii* PDC do not differ significantly from those reported for the *E.coli* complex and the optimal E_1:E_2 reconstitution ratio also seems to be equal (chapter 5; Bates, 1977). Because of the high degree of similarity between the complexes of *A.vinelandii* and *E.coli*, it seems attractive to propose a unifying model in which the *A.vinelandii* complex may represent the morphological subunit of the larger structure present in *E.coli* and perhaps in other gram-negative bacteria.

For the PDC of *E.coli* it is however generally accepted that the E_2 core consists of 24 chains, arranged in a cubic structure with 432 symmetry. Fig. 7.2 shows a schematic representation of the model as proposed by the group of Reed (Reed, 1968b; Hackert, 1983b). The model is based on the appearance of the E_2 core on electron micrographs (Reed, 1968b), on sedimentation equilibrium experiments (Eley, 1972), and on X-ray diffraction studies on an E_2E_3 subcomplex (Fuller, 1980).
In this model, all E_2 chains have identical environments, in contrast to the E_2 subunits in our 32-mer model. Another essential difference between the two models is the position of the E_3 dimer; in the *E.coli* model this component is placed in the centre of the planes of the cube, whereas in our model it is placed at the corners.
The E_1 dimers are placed on the edges of the cube, and it has been calculated (Hackert, 1983b) that they can interact with four E_2 chains, which is well explained by the 24-mer model. The mutual exclusive binding characteristics are however not evident from the *E.coli* model, this phenomenon is better explained by our 32-mer model.

The model for the *E.coli* PDC is based on a single large entity and does not take into account that dissociation into smaller structures can occur. Nevertheless, these smaller structures have been observed for the *E.coli* PDC, albeit in minor quantities (Schmitt, 1975; Danson, 1979). It is clear from Fig. 7.2 that a fractionation of the large core into eight corner structures based on an E_2 trimer would lead to asymmetric and incompletely assembled particles. Another possibility would be the dissociation of the large core into E_2 tetramers (*i.e.* the planes of the cube), to which two E_1 dimers and one E_3 dimer

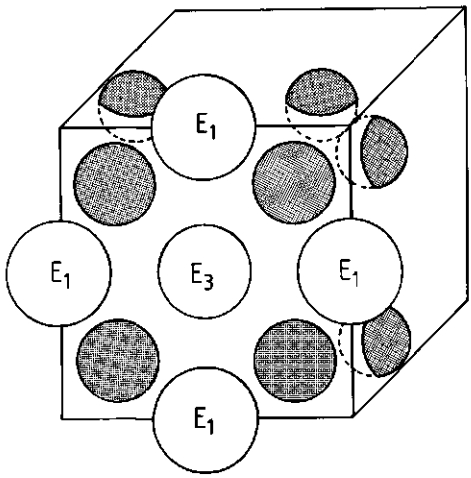


Fig. 7.2. Simplified representation of the arrangements of the components in the pyruvate dehydrogenase complex of *Escherichia coli* (Reed, 1968b; Perham, 1971; Eley, 1972; Hackert, 1983b). E_2 is pictured as monomer (shaded), E_1 and E_3 are pictured as dimers. For simplicity only part of the cubic E_2 core and the periferal components are drawn.

are bound. Such a dissociation also seems unlikely, since the E_1 dimers are supposed to be located on the edges of the cube, *i.e.* each E_1 dimer is "shared" by two planes of the E_2 cube. Upon fractionation, these E_1 dimers must be properly divided between the tetramers, which seems improbable.

Another argument against the 24 E_2 model comes from the amount of E_1 dimers that can be bound to the E_2 core. Direct determination of chain stoichiometries of isolated complexes or recombination experiments of E_1 with E_2E_3 subcomplexes performed by the group of Perham (Bates, 1975 and 1977, Danson, 1979) are very close to the values we have obtained for the *A.vinelandii* PDC, when they are corrected for the molecular mass of the E_2 chain of 66 kDa. This would mean that about 18 E_1 dimers must be bound to the E_2 component (36:24:12 chain stoichiometry). It is clearly impossible to arrange this number of E_1 dimers in a symmetrical way around the cubic E_2 core. In our model, with

its 48:32:16 chain-stoichiometry, the symmetrical arrangement of the components poses no problems.

Neither model can explain all observations in a satisfactory way, and unfortunately, the results of the electron microscopic and X-ray diffraction studies on the *E.coli* E_2 are not detailed enough to discriminate between the two models. Nevertheless, due to the high degree of similarity between the *A.vinelandii* and *E.coli* PDC, a unifying model seems attractive. On the other hand, the unique association behaviour of the *A.vinelandii* PDC could be caused by a unique quaternary structure.

From the transacetylation experiments described in chapter 5, it followed that the large *A.vinelandii* E_2 core is composed of eight more or less independently functioning E_2 tetramers. Similar experiments with the *E.coli* E_2 have shown that the E_2 component of that complex functions as a large entity (Bates, 1977; Collins, 1977; Stanley, 1981; Packman, 1983).
Our model is based on experimental results obtained on the *A.vinelandii* PDC; for the *E.coli* complex we have to rely on the data published in available literature. Therefore, to establish whether a unifying model does exist, further experiments will have to be performed in which the complexes of the two organisms are directly compared.

It is not clear at present whether the *A.vinelandii* complex occurs *in vivo* in the 18 S or the 56 S form; it has been suggested by Nichol *et al.* (1981) that macromolecules present in the cytoplasma could have effects comparable to those we have observed for polyethylene glycol *in vitro*, *i.e.* the 56 S form could be the predominant species in the cell, whereas the freshly isolated complex is in the 18 S form.

The question whether association into large agglomerates only has a structural function or may also lead to an improvement in catalytic power remains as yet unanswered. Possibly the larger structures are more efficient under physiological conditions or can be more finely tuned by their regulators.
The *A.vinelandii* complex offers an interesting object to study these questions.

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SUMMARY

In this thesis, some studies on the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase multienzyme complexes of *Azotobacter vinelandii* are described; the emphasis strongly lies on the pyruvate dehydrogenase complex.

A survey of the literature on 2-oxoacid dehydrogenase complexes is given in chapter 1. It appears that the *A.vinelandii* pyruvate dehydrogenase complex resembles the complexes from other gram-negative bacteria with respect to its composition and working mechanism. The *A.vinelandii* complex is however much smaller than the pyruvate dehydrogenase complexes isolated from other sources.

Chapter 2 describes the procedure that has been optimized for the isolation of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase multienzyme complexes (PDC and OGDC respectively) from *A.vinelandii*. In comparison to the previous isolation procedure, several advantages exist. The *A.vinelandii* PDC is obtained as an essentially pure three-component complex, in a high yield (40-50%). 80% of the losses can be accounted for by discarded side-fractions, which indicates that the complex is hardly inactivated during its purification. The specific activity of the final preparation is about two times higher (15-19 U/mg) than previously could be obtained.
From these observations we conclude that the formerly observed "fourth component" of *A.vinelandii* PDC was a mere contaminant. With the revised procedure, the 2-oxoglutarate dehydrogenase complex (OGDC) is obtained in a high yield (40-50%), free from contaminants. In the "old" procedure this complex was irreversibly inactivated by the action of protamine sulfate.

In chapter 3 some observations on the *A.vinelandii* OGDC are reported. The molecular mass of this complex is of the order of 2.4 to 3.2 MDa, as determined by laser light-scattering measurements. The three component enzymes have the same molec-

ular masses as have been reported for the OGDC's of *Escherichia coli* and pig-heart. The activity of the complex is regulated by its substrates in an analogues way as has been reported for the *E.coli* complex, and we therefore conclude that the *A.vinelandii* complex probably strongly resembles the OGDC of *E.coli*. In this chapter, an isolation procedure for the lipoamide dehydrogenase component is described, and it is shown that the lipoamide dehydrogenase components of the *A.vinelandii* PDC and OGDC probably are identical.

The association behaviour of the *A.vinelandii* pyruvate dehydrogenase complex is described in chapter 4. From sedimentation and light-scattering studies we conclude that a monomer-dimer equilibrium exists for this complex; the molecular mass of the monomer has been estimated that 800 kDa. In this thesis, this monomer-dimer mixture is referred to as the 18 S form of the complex. Upon addition of polyethylene glycol 6000 and $MgCl_2$, the 18 S form of the complex aggregates into a large structure, resembling the pyruvate dehydrogenase complex of *E.coli* with respect to its sedimentation coefficient (56 S) and its appearance on electron micrographs.
The isolated dihydrolipoyl transacetylase component of *A.vinelandii* PDC has a molecular mass of 2 MDa, and on electron micrographs it resembles the dihydrolipoyl acetyltransferase component of *E.coli*. It is concluded that this large structure probably is composed of 32 subunits. Upon the binding of the pyruvate dehydrogenase and lipoamide dehydrogenase components, this large particle dissociates into the smaller structures that are characteristic for the intact *A.vinelandii* complex.
The small (18 S) and the large (56 S) forms of the (sub)complexes are in slow equilibrium, and this equilibrium can be perturbed by high hydrostatic pressure. From light-scattering measurements at varying pressures it is concluded that the 56 S form of the complex probably is an octamer of the 800 kDa monomers.

The measurements concerning the chain-stoichiometry of *A.vinelandii* PDC are described in chapter 5. A novel method for the determination of chain-ratios was developed, based on the covalent modification of lysine residues in the three component enzymes with trinitrobenzene sulfonic acid. With this technique, an average chain ratio of 1.3:1:0.5 (pyruvate hydrogenase: di-

hydrolipoyl acetyl transferase:lipoamide dehydrogenase) was found for the isolated *A.vinelandii* PDC. In combination with the results of chapter 4, it is concluded that *A.vinelandii* PDC is based on a tetrameric dihydrolipoyl acetyltransferase core, to which the periferal components are bound in a non-covalent way. The complex can be reconstituted from its individual components, and from these reconstitution experiments it follows that the complex has maximal activity when three pyruvate dehydrogenase dimers and one lipoamide dehydrogenase dimer are bound to the dihydrolipoyl transacetylase tetramer.

In chapter 6, the results of acetylation experiments are given. It is shown that the reductive acetylation of the lipoyl groups probably is the rate-limiting step in the reaction sequence of the *A.vinelandii* pyruvate dehydrogenase complex. In so-called servicing experiments, an extensive exchange of acetyl groups between individual (monomeric) pyruvate dehydrogenase complex particles is found. This phenomenon (inter-core transacetylation) has until now only been observed for the *A.vinelandii* complex. It is shown that the inter-core transacetylation occurs when two monomeric particles are associated. Although the transacetylation reactions show large effects in the servicing experiments, these reactions are however too slow to be of physiological importance. The servicing experiments also show that the large "*E.coli*-like" isolated dihydrolipoyl acetyltransferase component is composed of rather independently operating tetramers, *i.e.* the large form of the *A.vinelandii* PDC does not function as a large entity.

In chapter 7, the results of the three preceding chapters are summarized and translated into a three-dimensional model of the molecular organisation of the *A.vinelandii* PDC. The merits of this model are discussed in relation to the generally accepted model for the pyruvate dehydrogenase complex of *E.coli*. It is suggested that the pyruvate of *Azotobacter vinelandii* could represent the morphological subunit of the larger structure that is found in *Escherichia coli* and perhaps in other gram-negative bacteria.
It is concluded that further experiments have to be performed, in which the complexes of the two organisms are directly compared, to establish whether such a unifying model does exist.

SAMENVATTING

Dit proefschrift handelt over studies die verricht zijn aan het pyruvaatdehydrogenase en het α-ketoglutaraatdehydrogenase multienzymcomplex van *Azotobacter vinelandii*. De nadruk ligt vrijwel volledig op het pyruvaatdehydrogenase complex.

In hoofdstuk 1 wordt een overzicht gegeven van de literatuur over α-ketozuurdehydrogenase complexen. Hieruit blijkt dat het pyruvaatdehydrogenase complex van *A.vinelandii* zich vooral onderscheidt door de relatief geringe grootte; de andere eigenschappen van het complex verschillen niet opvallend van die van de complexen uit andere gram-negatieve bacteriën.

In hoofdstuk 2 wordt een geoptimaliseerde procedure beschreven voor de zuivering van het pyruvaatdehydrogenase complex (PDC) en het α-ketoglutaraatdehydrogenase complex (KGDC) uit *Azotobacter vinelandii*. Beide complexen worden in hoge opbrengst verkregen (40-50%). Het uiteindelijke PDC preparaat heeft een specifieke activiteit (15-19 U/mg) die bijna tweemaal zo hoog is als met de oorspronkelijke procedure kon worden bereikt. Het KGDC kon met de oude procedure zelfs geheel niet gezuiverd worden, het werd irreversibel geïnactiveerd door protaminesulfaat. Beide complexen zijn samengesteld uit drie componenten, en de vroeger voor het *A.vinelandii* PDC waargenomen "vierde component" is daarom waarschijnlijk een verontreiniging geweest.

In hoofdstuk 3 worden enkele eigenschappen van het KGDC van *A.vinelandii* beschreven. De molecuulmassa van het complex is met behulp van laser-lichtverstrooiing bepaald op 2.4-3.2 MDa. Deze waarde komt goed overeen met de in de literatuur vermelde molecuulmassa's van KGDC's uit andere organismen. Dit is ook het geval voor de molecuulmassa's van de componenten waaruit het complex is opgebouwd. Bovendien zijn de kinetische eigenschappen van het *A.vinelandii* complex vergelijkbaar met die van het KGDC uit *E.coli*. In dit hoofdstuk wordt tevens een procedure

beschreven voor de isolatie van de lipoamide dehydrogenase component van het *A.vinelandii* KGDC. Uit een immunologisch experiment blijkt dat de lipoamide dehydrogenase componenten in *A.vinelandii* PDC en KGDC waarschijnlijk identiek zijn.

In hoofdstuk 4 wordt het associatiegedrag van het PDC van *A.vinelandii* beschreven. Het geïsoleerde complex blijkt onderworpen te zijn aan een snel relaxerend monomeer-dimeer evenwicht. Met behulp van lichtverstrooiingsmetingen (ook bij verhoogde druk) is de molecuulmassa van het monomeer vastgesteld op 800 kDa. In dit proefschrift wordt het mengsel van monomeer en dimeer aangeduid als de 18 S species van het complex.
Deze 18 S species wordt door toevoeging van polyethyleenglycol gedeeltelijk omgezet in een grotere structuur met een sedimentatiecoëfficient van 56 S; deze nieuwe species van het complex lijkt op het PDC uit *E.coli*. De 18 S en 56 S species zijn met elkaar in evenwicht, en dit evenwicht kan in de richting van de 18 S species worden verschoven door het aanleggen van hoge hydrostatische druk. Lichtverstrooiingsmetingen bij variable druk tonen aan dat de 56 S species waarschijnlijk is opgebouwd uit acht PDC-monomeren.
De geïsoleerde dihydrolipoyl transacetylase component van *A.vilandii* heeft een molecuulmassa van 2 MDa, en waarschijnlijk is deze structuur opgebouwd uit 32 ketens. Op electronenmicroscopische opnamen lijkt de *A.vinelandii* transacetylase component sterk op de transacetylase "kern" van *E.coli* PDC. Als de andere (perifere) componenten binden aan de transacetylase component, dissocieert deze weer in de kleinere structuren die karakteristiek zijn voor het compleet geassembleerde *A.vinelandii* PDC.

In hoofdstuk 5 worden de studies naar de samenstelling van het PDC van *A.vinelandii* beschreven. Voor de bepaling van de verhouding waarin de verschillende componenten voorkomen in het complex is een nieuwe techniek ontwikkeld. Deze is gebaseerd op de covalente modificatie van de lysine residuen in de eiwitketens met trinitrobenzeen sulfonzuur. Met behulp van deze techniek is de gemiddelde keten-ratio voor *A.vinelandii* PDC bepaald op 1.3:1:0.5 (pyruvaatdehydrogenase:acetyltransferase:lipoamide dehydrogenase).
Rekening houdend met het in hoofdstuk 4 bepaalde molecuulmassa van 800 kDa, volgt hieruit dat het PDC van *A.vinelandii* geba-

seerd is op een uit vier ketens gevormde acetyltransferase "kern". Het PDC kan gereconstitueerd worden uit de geïsoleerde componenten, en deze reconstitutie-experimenten geven aan dat het complex maximale activiteit vertoont als drie pyruvaatdehydrogenase dimeren en één lipoamide dehydrogenase dimeer zijn gebonden aan de tetramere transacetylase component.

In hoofdstuk 6 worden de resultaten van acetyleringsexperimenten weergegeven. De reductieve acetylering van de lipoylgroepen vormt waarschijnlijk de snelheidsbepalende stap in het door het complex gekatalyseerde proces.
Uit zogenaamde "servicing" experimenten blijkt dat de PDC monomeren onderling acetylgroepen kunnen uitwisselen. Dit proces vindt waarschijnlijk plaats als een PDC-dimeer is gevormd. Een uitwisseling van acetylgroepen tussen afzonderlijke PDC deeltjes is tot nu toe alleen nog maar waargenomen voor het *A.vinelandii* complex. Alhoewel de transacetyleringsreacties opvallende effecten te zien geven in de "servicing" experimenten, verlopen ze waarschijnlijk te langzaam om van fysiologische betekenis te zijn.
De "servicing" experimenten hebben tevens aangetoond dat de geïsoleerde acetyltransferase component feitelijk is samengesteld uit min of meer onafhankelijk functionerende tetrameren, m.a.w. er is geen grote functionele eenheid gevormd.

In hoofdstuk 7 worden de resultaten van de voorgaande drie hoofdstukken met elkaar in verband gebracht, resulterend in een drie-dimensionaal model van de moleculaire organisatie van het PDC van *A.vinelandii*. Het model is vergeleken met het algemeen aanvaarde model voor *E.coli* PDC. Het blijkt dat geen van beide modellen alle experimentele resultaten bevredigend kan verklaren. Gezien de overeenkomsten tussen het PDC van *A.vinelandii* en dat uit *E.coli* lijkt het mogelijk dat het *A.vinelandii* complex de morfologische subeenheid vormt van het grotere PDC zoals dat in *E.coli*, en wellicht in andere gram-negatieve bacteriën wordt gevonden.
Om vast te stellen of een dergelijk uniform model bestaat, zullen er uitgebreide experimenten moeten worden verricht waarin de complexen van beide organismen direct met elkaar vergeleken worden.