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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Vincent dePaul Young

ENTITLED Purification and Partial Characterization of
Methylene-H_4MPT:Coenzyme F_{420} Dehydrogenase
from Methanobacterium thermoautotrophicum strain ΔH

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Purification and Partial Characterization of Methylene-H_4MPT:Coenzyme F_{420} Dehydrogenase from *Methanobacterium thermoautotrophicum* Strain ΔH

by

Vincent dePaul Young

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Table of Contents

Abbreviations.

A_{335}	absorbance at 335 nanometers
ATP	adenosine triphospate
BSA	bovine serum albumin
Buffer A	20mM potassium phosphate, 10mM 2-mercaptoethanol pH 7.0
CFE	cell free extract
EDC	N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride
FPLC	Fast Protein Liquid Chromatography by Pharmacia Inc.
F_{430}	nickel-tetrapyrrole
HS-CoM	2-mercaptoethanesulfonic acid
CH_3-S-CoM	2-(methylthio)ethanesulfonic acid
MFR	methanofuran
H_4MPT	tetrahydromethanopterin
µg	microgram
µl	microliter
µmol	micromole
mM	millimolar
M_r	molecular weight in daltons
NaCl	sodium chloride
pmol	picomole
pipes	piperazine-N,N'-bis(2-ethane sulfonic acid)
PAGE	polyacrylamide gel electrophoresis

F_{420}	coenzyme F_{420}, the N-(N-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin-5-phosphate.
SDS	sodium dodecyl sulfate

I. Introduction.

A. Methanogenesis.

Methanobacterium thermoautotrophicum strain ΔH is a thermophilic methanogen that utilizes CO_2 as its carbon scource and H_2 as its energy source by the reaction below:

$$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O$$

Much progress has been made towards understanding the complex biochemistry of methanogenesis since 1956 when Barker(2) first proposed a scheme in which CO_2 was fixed onto a C-1 carrier XH. The structures of six coenzymes unique to methanogens have been elucidated and an updated C-1 cycle from CO_2 fixation to CH_4 production has been proposed(fig 1)(14). The first stable intermediate of CO_2 fixation is formyl-MFR, which then transfers the formyl moiety to H_4MPT, a cofactor analagous to tetrahydrofolate. The formyl moiety is converted on the H_4MPT carrier through the methenyl, methylene, and methyl oxidation states of carbon. The methyl group is then transferred from H_4MPT to HS-CoM, the third and final carbon carrying coenzyme. In the terminal or methylreductase reaction, CH_3-S-CoM is reduced by HS-HTP to form methane and the heterodisulfide CoM-S-S-HTP. This terminal reaction is coupled to the production of ATP, presumably by the production of transmembrane proton motive force.

B. ATP Synthesis From a Proton Motive Force.

Cell membrane associated ATPases use the proton motive force created by a transmembrane proton gradient to make ATP, a conserved method of ATP production across species. In Methanogens, a group of archaebacteria, it was established that a transmembrane proton gradient is used to produce ATP(3), however the method by which the bacterium couples the production of a proton gradient with methane formation is unresolved. Evidence was obtained that in the course of the methylreductase reaction, a proton motive force is generated at the cytoplasmic membrane, which subsequently is used for ATP synthesis(13). Studies were done to discover if in fact enzymes of the terminal steps in methanogenesis were associated to the cell membrane. Muth established that the F_{420} reducing hydrogenase of *Methanococcus voltae* was membrane associated and Aldrich and Ossmer found that the methyl-coenzyme M reductase of *M. thermoautotrophicum* and *M. voltae* was also membrane associated.(12,1,13). Utilizing electron microscopy techniques to study the cell membrane of Methanogenic Bacterium Strain Göl, Mayer proposed the methanoreductosome, a multicomponent high-molecular-weight enzyme complex containing methyl coenzyme M methylreductase(11). The current hypothesis proposes that the methanoreductosome pumps protons into the periplasmic space as it catalyzes the terminal methylreductase reaction. More experimentation towards the elucidation of other enzymes attached to the cell membrane or associated with the methanoreductosome must be done to confirm this.

C. Association Between Component C of the Methylreductase and the 5,10 methylene-H_4MPT:Coenzyme F_{420} Dehydrogenase.

Hartzell discovered in her studies of component C of the methylreductase that highly active preparations of the methylene-H_4MPT:coenzyme F_{420} dehydrogenase contained component C and there appeared to be a tight association between these enzymes(9). The methylene-H_4MPT:coenzyme F_{420} dehydrogenase reduces methenyl-H_4MPT to methylene-H_4MPT (reaction 3 in figure 1) and is completely dependent on coenzyme F_{420} as an electron donor(8). The reaction was specific for H_4MPT and 1.1 mol of coenzyme F_{420} was reduced per mole of methylene-H_4MPT oxidized; this is consistent with the role of F_{420} as a two electron acceptor(8). Hartzell also found that the dehydrogenase failed to bind F_{420} when it was resolved from component C(9). To understand the possible component C-dehydrogenase association and its relation to the methanoreductosome more completely, the dehydrogenase was purified by a modified method and antibodies to the purified enzyme were harvested for *in situ* localization. Determining if a physiological importance exists between the enzymes of the C-1 cylcle, such as the dehydrogenase, and component C is crucial to the understanding of energy production in methanogenic bacteria.

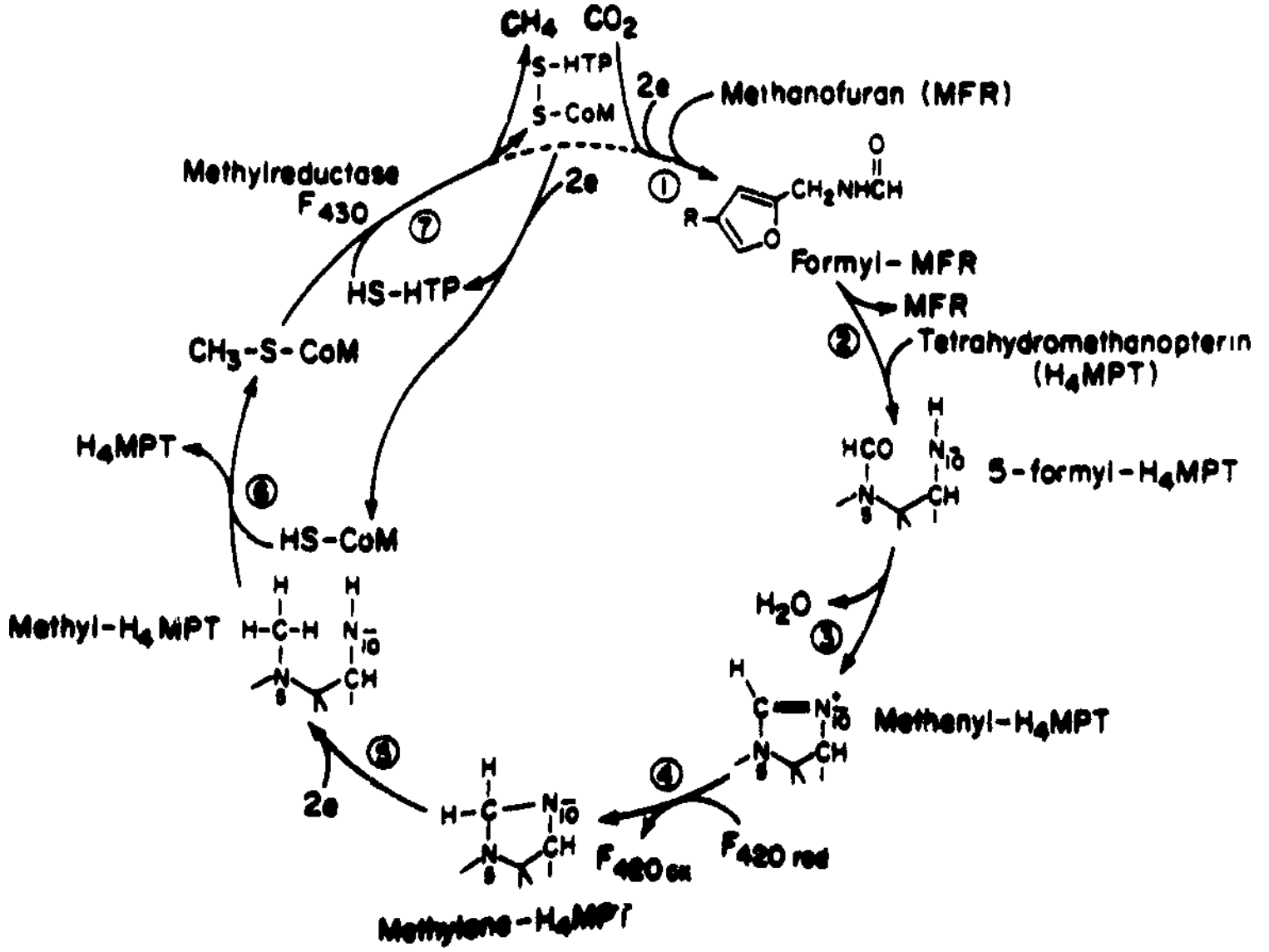


Figure 1. The C-1 cycle of the reduction of carbon dioxide to methane. F_{430}, nickel tetrapyrrole; B, HS-HTP; RPG, RPG effect or coupling of the methylreductase to CO_2 activation; from Rouvière and Wolfe (14).

D. Molecular Biology of Methanogens.

Despite recent advances in genetic and molecular techniques, the study of the methanogenic genome has progressed slowly. Some archaebacterial genes contain introns and families of repeat sequence DNA, characteristics not found in eubacteria(16). Recently the genes of the methyl coenzyme M reductase from *M. thermoautotrophicum* were cloned and found to be encoded by a gene cluster(4). In addition the gene encoding the formyl-methanofuran:H_4MPT formyltransferase (reaction 2 of figure 1) has been cloned and sequenced (personal communication, A. DiMarco). The amino-terminal amino acid sequence of the dehydrogenase was determined for future cloning experiments which may help to understand the relationship between the C-1 cycle, the methanoreductosome, and energy production in methanogens. Furthermore, genetic linkage among the cloned enzymes of the C-1 pathway will be investigated using this sequence as an additional probe.

II. Materials and Methods.

A. Materials and Chemicals.

Amicon filters and Centricons from Amicon Corp., Lexington, MA.; 1.5 ml plastic semimicro cuvettes purchased from Markson Science Inc. Phoenix, AZ; 20 mm red rubber serum stoppers from Wheaton Scientific, Millville, NJ; anerobic chamber from Coy Laboratories, Ann Arbor, MI; RC-5 Superspeed Refrigerated Centrifuge from Sorvall; DuPont Co., Wilmington, DE; Perkins-Elmer Lambda 3B Spectrophotometer from Perkins-Elmer, St. Louis, MO; 6-aminohexanoic acid-Sepharose 4B, EDC, phenyl-Sepharose CL-4B, betaine, complete Freunds adjuvant, Blue Dextran and protein molecular weight standards were purchased from Sigma Co , St. Louis, MO; FPLC Mono Q anion exchange HR 5/5 column, FPLC Superose 12 10/30 gel filtration column from Pharmacia Inc., Piscataway, NJ; electrophoresis purity acrylamide from Bio-Rad Laboratories, Richmond, CA; Immobilized Methotrexate, Coomassie Blue G-250 protein assay reagent from Pierce Chemical Co., Rockford, IL. F_{420} was a gift from Fritz Widdel purified from cells by a previously described method(6). All other chemicals were of reagent grade or better.

B. Growth of Cells and Extract Preparations.

M. thermoautotrophicum ΔH was grown in a 200-liter fermentor as described previously(5). CFE was prepared from a cell slurry as described previously(5).

C. Synthesis of F_{420} Affinity Chromatography Resin.

Two grams of 6-aminohexanoic acid-Sepharose 4B was reswelled in 15 mls of 0.5 M NaCl for fifteen minutes at room temperature. The gel was washed with 400 mls of 0.5 M NaCl on a scintered glass filter. The gel was then washed with 100 mls dH_2O at pH 4.5. 16 µmol (14.2 mg) of F_{420} was dissolved in 16 mls of dH_2O pH 4.5. The F_{420} solution was added to the swollen gel and the pH adjusted between 4.5-6. 100 mg of EDC was added as a free flowing powder to the F_{420}-gel mixture. The reaction mixture was shaken gently and the pH was monitored and adjusted between 4.5-6 during the first hour. The reaction proceeded overnight shaking gently at room temperature.

The synthesized F_{420}-resin was washed with 50 mls of 0.5 M NaCl followed by 50 mls of bufffer A. 150µl of desalted 70% ammonium sulfate suprnatant was loaded and washed with 10 mls of Buffer A. The protein was then eluted stepwise with 3 mls of 0.1, 0.2, 0.3,...., 1 M potassium acetate in buffer A. The 3 ml fractions were concentrated in a Centricon. Specific elution was done with 3 mls of 4mM F_{420} plus 0.5 M NaCl in Buffer A.

D. Enzyme Assays.

Oxidoreductase activity was measured in 1.5 ml plastic, semimicro cuvettes capped with a 20 mm red rubber serum stopper. 5 µl of 1 M HCHO, 5 µl of 0.1 mM F_{420} , and 5 µl of 7.5 mM H_4MPT (purified as described previously [7]) were added to 800 µls of anoxic pipes buffer pH 6.1, in an anerobic chamber. The reaction mixture was heated to 60°C for 10 minutes in a Perkin-Elmer model Lambda 3B Spectrophotometer. The reaction was initiated by injecting 2 to 30 µl of enzyme preparation bringing the total volume in the

cuvette to 817 to 845 µls. The reaction was monitored by the appearance of methenyl-H_4MPT and an increase in A_{335}. Acrylamide gel slices were tested for activity by incubating the crushed gel slice in 500 µls of buffer A overnight at 4°C. Supernatant liquid was injected to start the reaction.

E. Enzyme Purification.

All enzyme steps were carried out in an anerobic chamber unless otherwise stated. The dehydrogenase was first purified as done previously(8) and this enzyme is refered to as the first purification throughout the thesis. The modified method follows and is referred to as the second purification throughout the thesis. Solid ammonium sulfate (6.4g) was slowly added with stirring to a volume of 15.0 mls of CFE to a final concentration of 70% saturation. This solution was stirred 24 hours at 4°C, transferred to a plastic centrifuge tube, and centrifuged at 27,000 x g for 30 minutes at 4°C. The supernatant was then applied to a column (2.54 cm radius by 6.35 cm) of phenyl Sepharose CL-4B equilibrated in buffer A with 2.0 M NaCl. The column was developed with a linear gradient of 840mls from 2.0 to 0 M NaCl in buffer A. The pool of active fractions was then loaded onto a 20 ml column of immobilized methotrexate (methopterin linked to Sepharose by a six-carbon spacer) equilibrated in buffer A plus 5% betaine. The column was washed with two bed volumes of equilibration buffer and developed in 160mls with a linear gradient of 0 to 1 M potassium acetate in buffer A. Further purification was performed using the Pharmacia FPLC system. 350µg of concentrated methotrexate column eluate protein was loaded onto a Mono Q column and developed with a 40 min linear gradient from 0 to 1 M KCl in buffer A at a flow rate of

1 ml/min. The peak of activity was collected and concentrated in a centricon placed in an anerobic plastic centrifuge tube and spun at 12,000 x g. 100µl samples of Mono Q eluate were injected onto a Superose 12 10/30 column equilibrated with buffer A plus 0.1 M KCl. The flow rate was 0.2 mls/min. Protein determinations were performed by the method of Bradford(3a) using BSA as the standard.

F. Analytical PAGE.

Denaturing and nondenaturing analytical discontinuous PAGE was performed in slab gels with a pH system of 6.8 to 8.8(10) as described previously(5). The coomassie brilliant blue R-250 solution described by Fairbanks et. al.(7a) was used to stain the proteins.

G. Molecular weight determinations.

The apparent molecular mass of the native dehydrogenase was determined by gel filtration chromatography on a Superose 12 10/30 column equilibrated in buffer A plus 0.1 M KCl at a flow rate of 0.2 mls/min. Samples of the following standards(20µl, 2 mg/ml)were used: blue dextran(M_r 2,000,000), apoferritin(M_r 460,000), ß-amylase(M_r 200,000), alcohol dehydrogenase from Yeast(M_r 150,000), BSA (M_r 67,000), ß-lactoglobulin(M_r 18,400), F_{420} (M_r 860). 100µg of dehydrogenase was injected in 150µl and its molecular size was calculated from its relative elution volume.

The subunit molecular mass of the denatured protein was determined by SDS-PAGE at 15% acrylamide. The following standards were used: fructose 6-PO_4 kinase(M_r 84,000), pyruvate kinase(M_r 58,000), fumarase(M_r 48,500), lactic

dehydrogenase(M_r 36,500), triosephosphate isomerase(M_r 26,600), lysozyme(M_r 14,300). Subunit size was determined by calculating the relative mobility of the denatured dehydrogenase.

H. Preparation of Antibody.

200 to 400µg of purified enzyme or enzyme extracted from a PAGE slice in a volume of 0.5 mls was added to an equal volume of Freunds complete adjuvant and injected intrascapularly every 28 days. Serum was treated as described previously(12). The specificity of the antibody was tested by using WESTERN blots(15) of purified dehydrogenase or of 70% ammonium sulfate extract of *M. thermoautotrophicum* separated on a 10% PAGE. The antibodies were tested in activity assays by adding 10µl of purified dehydrogenase enzyme to 50µl of purified serum and incubating at 4°C overnight. This mixture was injected into a reaction cuvette and the relative rates of normal rabbit serum and anti-dehydrogenase serum were compared.

III. Results

A. Purification of the 5,10 Methylene-H_4MPT:Coenzyme F_{420} Dehydrogenase.

A modified purification was performed to purify the dehydrogenase enzyme. After 70% ammonium sulfate fractionation, the resulting supernatant was passed over a phenyl-sepharose chromatography column which completely removed the methyl-coenzyme M methylreductase component C from the resulting pool of activity as determined by native PAGE(figure 2, lanes 3 and 4). There were two peaks of activity from the phenyl-sepharose column; one eluted at 1.56M NaCl and one peak eluted during the gradient at 0.45 M NaCl(figure 3). The active fractions were pooled and concentrated. The concentrated pool was loaded onto a methotrexate affinity column and the dehydrogenase eluted at 0.7 M KAc. The active fractions were pooled, desalted, and concentrated. The two remaining purification steps were performed on a Pharmacia FPLC system. 350µg of protein was loaded onto a Mono-Q HR 5/5 anion exchange column and eluted with a linear gradient. The enzyme eluted at 0.56 M KCl in buffer A. Gel filtration was performed with a Superose 12 10/30 and the protein eluted 56 minutes after injection. Figure 2 is a 10% PAGE which shows the different levels of purification of the dehydrogenase The native M_r as determined by gel filtration was 217,000(figure 4) and the M_r of the subunit determined by SDS-PAGE was 32,800(figures 5 and 6), suggesting some type of aggregation. The specific activity of the purified enzyme was 15,983 nmol/min/mg of protein (table 1).

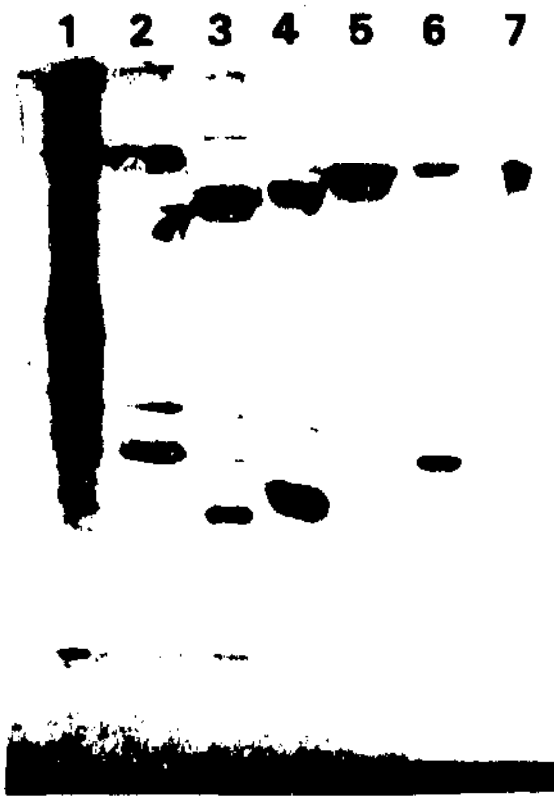


Figure 2. Ten percent native PAGE of methylene-H_4MPT: coenzyme F_{420} dehydrogenase at six levels of purification. Lane 1, 50µg of CFE protein; lane 2, 30µg of 70% ammonium sulfate; lane 3, 30µg first peak of activity from phenyl-sepharose (with component C +); lane 4, 30µg second peak of activity from phenyl-sepharose (component C separated); lane 5, 20µg methotrexate pool; lane 6, 10µg Mono Q; lane 7, 5µg Superose.

Table 1.

Purification of the 5,10 methylene-H_4MPT:coenzyme F_{420} dehydrogenase.

Purification Step	Protein (mg/ml)	Specific Activity (nmol/min/ mg protein)	Purification
Dialyzed 70% $(NH_4)_2SO_4$ Supernatant	20	71,520	1
Phenyl-Sepharose	2.4	4,027	.056
Methotrexate	7.8	327	4.57×10^{-3}
FPLC Mono Q 5/5	.09	9,450	.132
FPLC Superose	.026	15,983	.223

Figure 3. Elution profile of phenyl-Sepharose 4B chromatography as described in Materials and Methods.

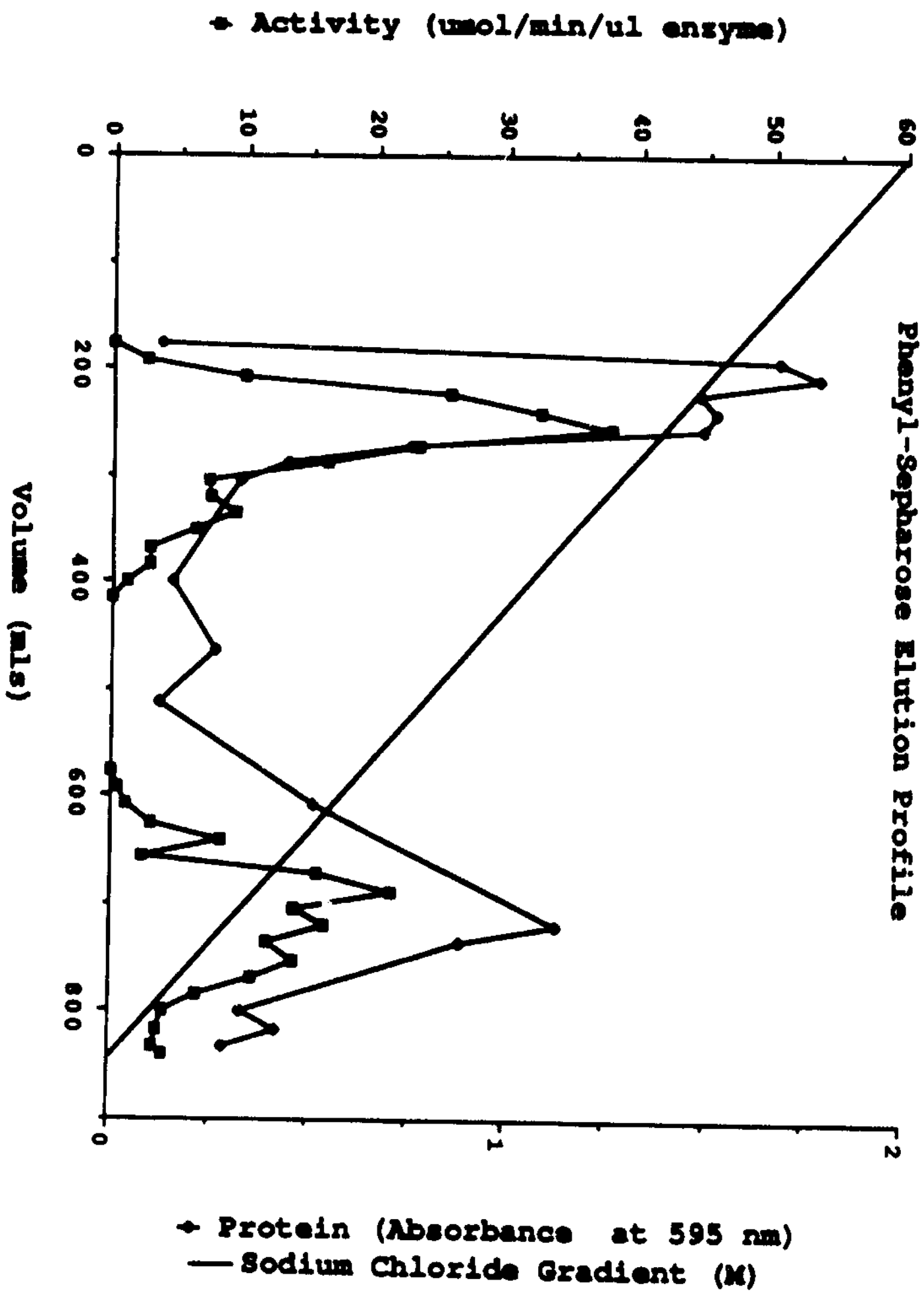
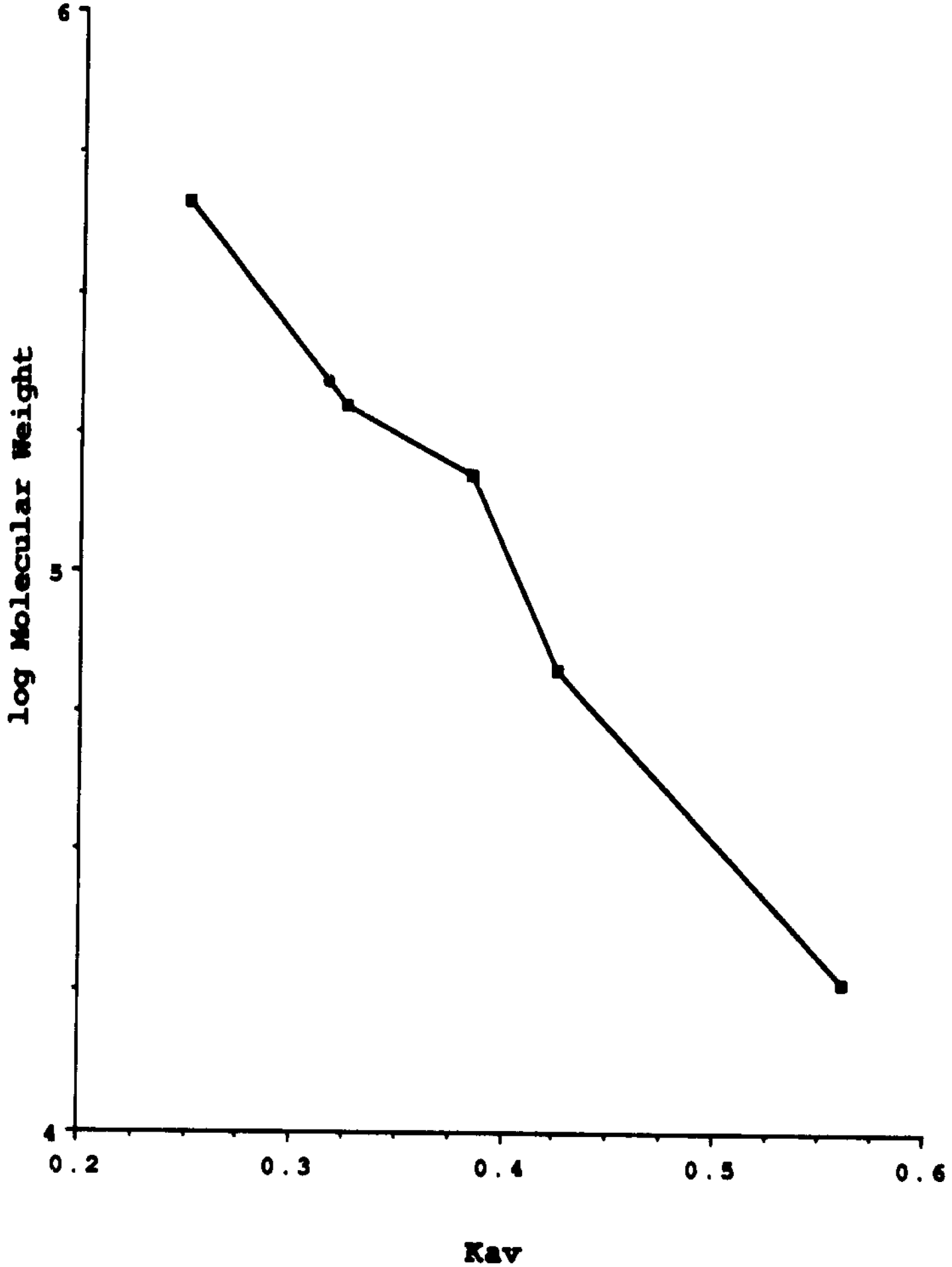

Activity (umol/min/ul enzyme)
Phenyl-Sepharose Elution Profile
Volume (mLs)
0
200
400
600
800
0
10
20
30
40
50
60
0
1
2
Protein (Absorbance at 595 nm)
Sodium Chloride Gradient (M)

Figure 4. Molecular weight determination of dehydrogenase by Superose 12 gel filtration chromatography. The Kav value for the dehydrogenase is represented by O.

Gel Filtration Standard Curve



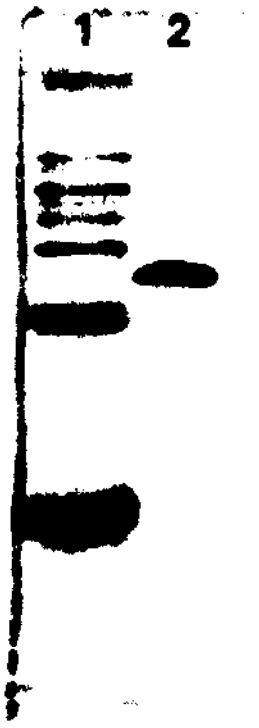
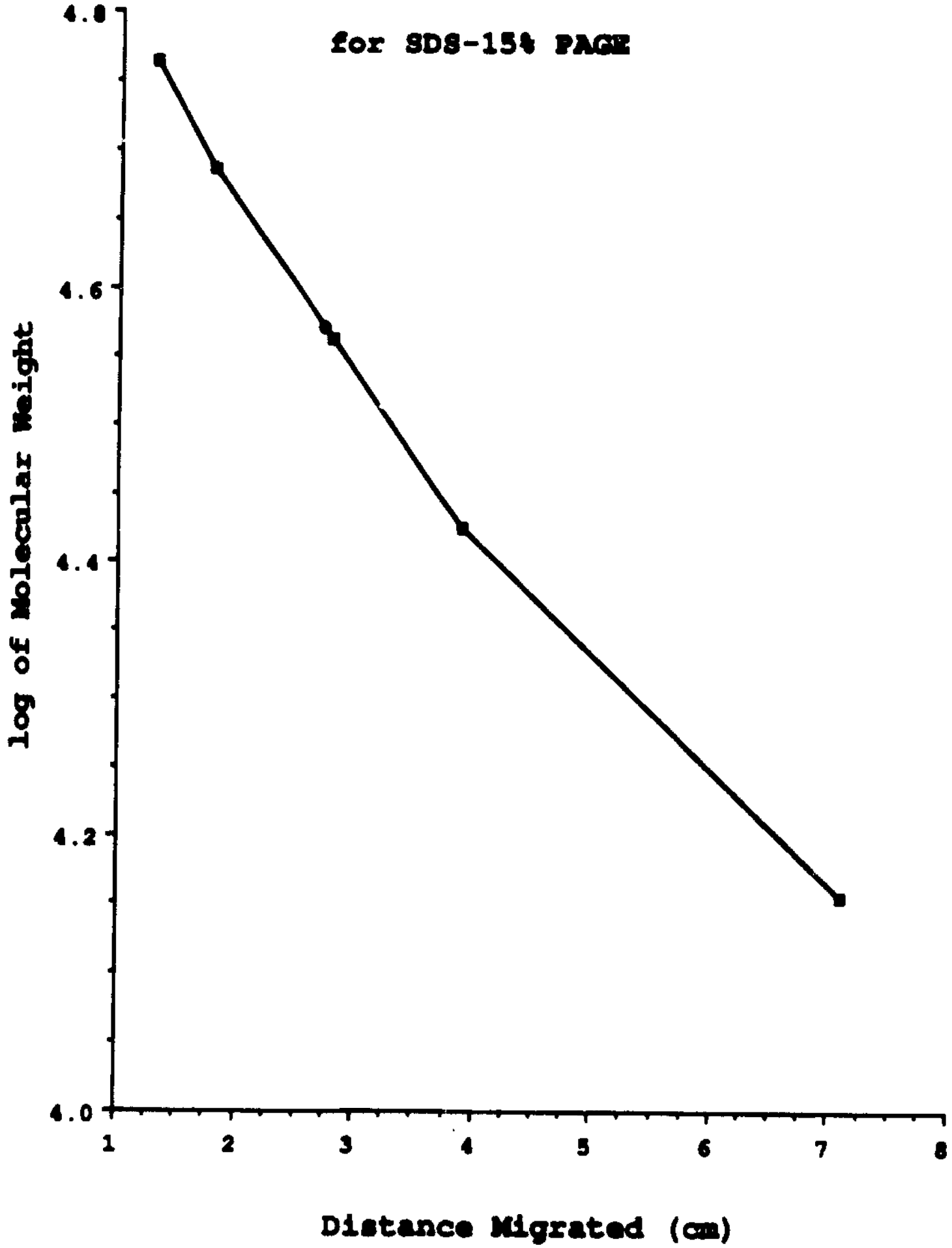


Figure 5. SDS-PAGE (15% acrylamide) of purified dehydrogenase. Lane 1, 20µl of molecular weight markers as described in Materials and Methods. Lane 2, 5µg of purified dehydrogenase.

Figure 6. Subunit molecular weight determination of the dehydrogenase by relative mobility on SDS-PAGE (15% acrylamide). Distance migrated by dehydrogenase represented as O.


Molecular Weight Standard Curve
for SDS-15% PAGE
Log of Molecular Weight
4.8
4.6
4.4
4.2
4.0
1
2
3
4
5
6
7
8
Distance Migrated (cm)

B. Amino Acid Sequence Data.

Two samples were submitted for amino acid sequencing, one from each purification. 1.175 nmols of dehydrogenase from the first purification was submitted. The R_f value of 0.75 obtained on a 10% PAGE matched the R_f value previously observed(9). However the sequence showed two amino acids at each position in the ratio of two tc one (figure 7). This suggested that the enzyme was not pure. Furthermore, enzyme sliced from a 10% native PAGE as described in the materials and methods did not show activity.

Approximately 600pmols of dehydrogenase from the second purification was submitted for amino acid sequencing. The sequence (figure 7) clearly showed single amino acids at 18 of the first 20 residues and 12 out of the first 20 are hydrophobic residues. If this type of stoichiometry were to continue throughout the molecule, over 50% would be hydrophobic. The R_f value was 0.178, quite different from previously observed, but the enzyme sliced from 10% PAGE demonstrated an activity of .30 nmols/min/µl (there was not enough protein for an accurate determination of specific activity). A piece of acrylamide incubated with buffer A overnight served as the control and showed no activity.

Figure 7
Protein sequence results of the 5,10-methylene$_4$MPT:coenzyme F_{420} dehydrogenase.

First Purification			Modified Purification		
Cycle	Amino Acid	Amount (pmol)	Cycle	Amino Acid	Amount (pmol)
1	T/S/G/E/A/K		1	V(G/M)	443(1443)
2	TYR/PRO	169/77	2	VAL	404
3	LEU/PRO	416/120	3	LYS	606
4	ALA/PRO	497/166	4	ILE	428
5	ILE/GLU	333/201	5	GLY	283
6	LEU/THR	350/66	6	ILE	405
7	GLY/VAL	249/145	7	ILE	474
8	ALA/ASN	351/54	8	ARG	109
9	GLY/VAL	255/135	9	CYS/TYR	?/19
10	THR/ILE	46/76	10	GLY	248
11	TYR/ILE	207/113	11	ASN	168
12	ARG/PRO	89/59	12	ILE	294
13	THR/MET	87/48	13	GLY	222
14	HIS/VAL	40/96	14	THR	193
15	ALA/VAL	213/116	15	SER	81
16	ALA/ILE	253/86	16	PRO	107
17	SER/VAL	35/95	17	VAL	131
18	GLY/LYS	114/75	18	LEU	133
19	ILE/LEU	134/84	19	ASP	62
20	THR/LYS	53/96	20	LEU	170
21	ASN	66	21	LEU?	226
22	PHE	123	22	LEU?	249
23	SER	22	23	???	
24	ARG	26	24	D/E/R	96/77/27
25	ALA	128	25	ARG	22
26	???		26	ALA	172
27	GLU	69	27	ASP	65
28	VAL	106	28	???	
29	ALA	120	29	???	
30	GLU/TYR	61/36	30	ASN	37
31	MET	72	31	ILE/ASN	103/52
32	VAL	110	32	ASP/ILE	66/108
33	???		33	VAL/ALA	98/137
34	LYS	83	34	???	
35	PRO	52.3	35	VAL	117
36	GLU	47			
37	ILE	80			
38	ALA	88			

C. Immunological Techniques.

Injections were made using dehydrogenase from the first purification method. WESTERN blots were used to check the specificity of the antibodies and the enzyme reacted solely with the band of R_f =0.75 (figure 8). Antibody incubated with the enzyme inhibited activity 3.1 fold as compared to incubation with the same amount of normal rabbit serum. However a titer was not performed because it was apparent from the amino acid sequence that the antibody had been raised to possibly two enzymes in the cell, and could not be used for accurate *in situ* labeling.

Figure 8. WESTERN blot of 70% ammonium sulfate as described in Materials and Methods.

IV. Discussion.

A. Problems Encountered During Purification.

The methylene-H_4MPT:coenzyme F_{420} dehydrogenase was purified from CFE of *M. thermoautotrophicum* and the purified enzyme had a specific activity of 15,983 nmol/min/mg(table 1). Chromatography on phenyl-sepharose showed two peaks of activity(figure 3), one eluting at 1.56M NaCl and one eluting at 0.45 M NaCl. The active peak at 1.56 M contained a high percentage of methylcoenzyme M component C as determined by native PAGE. Hartzell proposed that component C was associated to the cell membrane through the more hydrophobic dehydrogenase(9). The high percentage of component C in the first dehydrogenase peak suggests an association between the dehydrogenase and component C, while the second peak of activity may correspond to dehydrogenase which has been resolved from the methylreductase during chromatography. The elution molarity of the second dehydrogenase activity peak from the phenyl-sepharose column indicates the enzyme is hydrophobic compared with the very acidic component C. The results of the modified purification amino acid sequence tend to support that the dehydrogenase is hydrophobic; however the sample number is 20, which may be too low for accurate speculation on the nature of the rest of the enzyme. Therefore it could play a role in attaching component C to the cell membrane or the methanoreductosome, since it is doubtful that a direct association of component C to the hydrophobic cell membrane exists.

Though the reaction rates of the Mono Q and Superose fractions are extremely low, the activity assay is very sensitive due to the high

extinction coefficient of 21,600 M^{-1} cm^{-1} at 335 nm for the methenyl-H_4MPT double bond. A major problem throughout the purification was a decrease in specific activity which made an unambiguous characterization impossible. The dehydrogenase lost activity through the methotrexate chromatography step, but increased activity was observed at the Mono Q and Superose steps. Designating methotrexate activity as 1, Mono Q showed a 28.8 fold increase in activity, and Superose showed a 48.8 fold increase. It was found that the dehydrogenase failed to bind F_{420} when resolved from component C(9). If the dehydrogenase is associated to component C *in vivo*, the loss in activity may be from the disassociation of the enzymes and a loss of biologically active structure. This enzyme needs to be studied further to understand why specific activity is lost during purification.

The native M_r of the dehydrogenase from the modified purification determined by gel filtration was 217,000 and the M_r of the single subunit determined by SDS-PAGE was 32,800. This data suggests seven subunits. However, the decreased R_f value of .178 more likely indicates an association or aggregation of dehydrogenase.

B. F_{420} Affinity Column.

F_{420} is one of the unique coenzymes of methanogenesis which has a brilliant yellow color and a green-blue fluorescence(17). Hartzell found the dehydrogenase exhibited sigmoidal cooperativity with an estimated Hill coefficent of 3.8 when titrated with F_{420}(9). An F_{420} affinity column was synthesized as a possible step in the purification procedure of the dehydrogenase. The F_{420} molecule, which contains three carboxyl groups (figure 9)(6), was linked to amino-Sepharose 4B affinity resin. EDC promoted the condensation between the free amino groups attached to the resin and the F_{420} carboxyl groups to form a peptide bond by acid catalyzed removal of H_2O (figure 10). The resin changed colors from white to brilliant yellow and exhibited fluorescence under UV light after synthesis.

The non-specific elution profile (data not shown) showed enzyme bound to the resin and eluted at .7 M NaCl with another peak of activity eluting at 1 M NaCl, with specific activities of 2490 and 1860 nmol/min/mg enzyme respectivly. The presence of two activity peaks is again not understood, and may be due to differential aggregation. Specific elution with 4mM F_{420} eluted enzyme with a specific activity of 1640 nmol/min/mg enzyme. F_{420} affinity chromatography was not pursued further because it was not an efficient purification step. However, this type of chromatography could be used to identify other enzymes that require F_{420}.

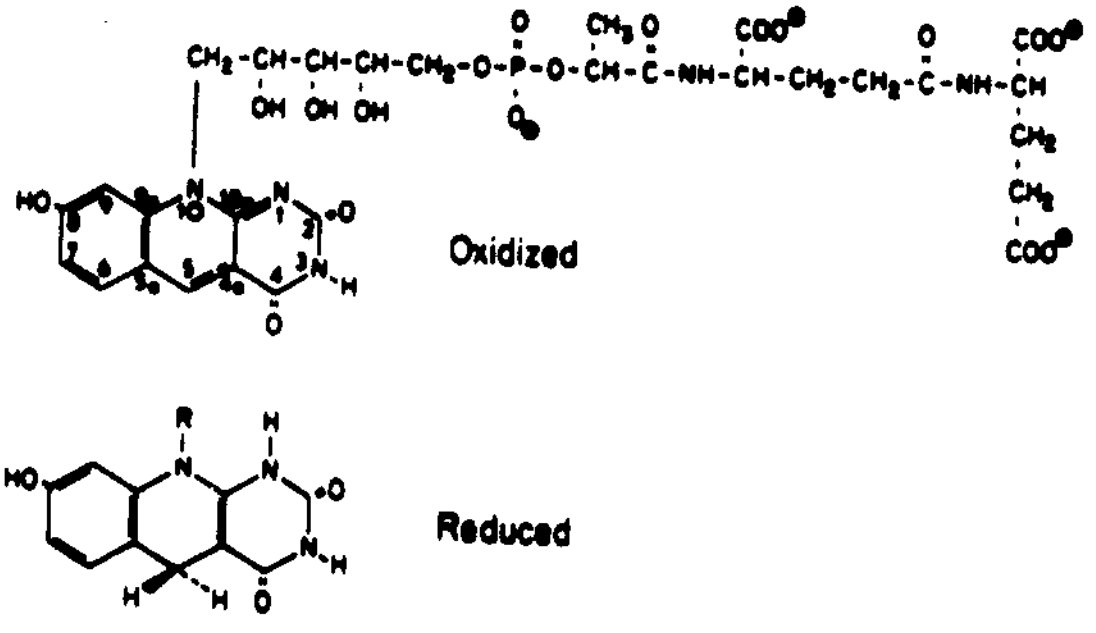


Figure 9. The F_{420} molecule(6).

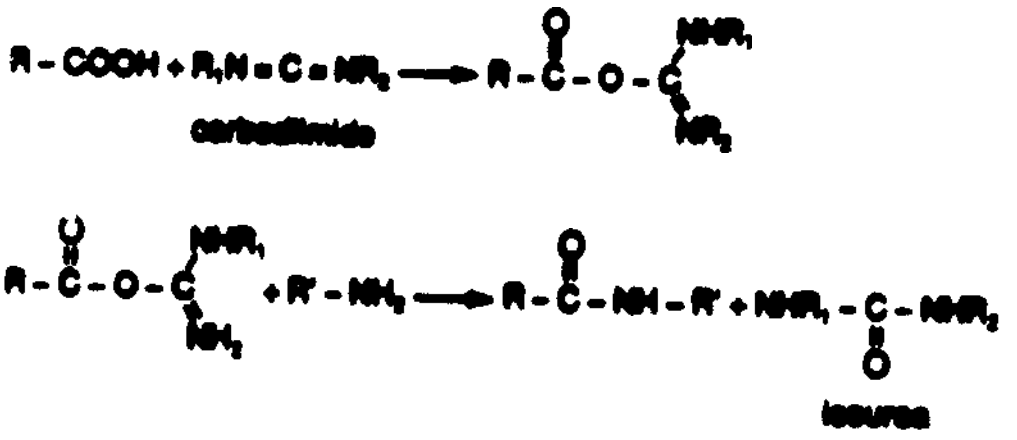


Figure 10. Carbodiimide coupling reaction as explained in text. R-COOH, any of the three carboxyl groups on the F_{420} molecule; R'-NH_2, amino groups attached to the sepharose resin bead.

C. Amino acid sequence and anti-dehydrogenase antibodies.

The amino acid sequence from the first preparation of enzyme demonstrated that it was possibly contaminanted or the wrong enzyme, since it showed no similarity to the second sequence (figure 7) and was not active in assays when cut from a PAGE. The second preparation showed single amino acids for 18 of the first 20 positions and demonstrated activity in assays when cut from a PAGE. If cloning experiments are attempted, a DNA probe derived from the second amino acid sequence should be used.

The inhibition assays demonstrated anti-dehydrogenase antibody were raised, but since the protein samples injected into the rabbit appear to be contaminated, they cannot be used for *in situ* labeling experiments. Also, most enzyme purifications demonstrate an increase in specific activity which indicates the enzyme is being selectively purified from other proteins. However the dehydrogenase loses specific activity throughout the purification procedures and one cannot be certain a single protein band or native or SDS PAGE corresponds to the dehydrogenase enzyme. The dehydrogenase must be purified unambiguously before reliable *in situ* labeling studies can be pursued.

V. Summary

The methylene-H_4MPT: coenzyme F_{420} dehydrogenase was purified to electrophoretic homogeneity in four chromatographic steps using phenyl-Sepharose, immobilized methotrexate, Mono Q, and Superose. The dehydrogenase is beleived to associated with Component C of the methylreductase and may have a secondary function of attaching the acidic component C enzyme to the hydrophobic cell membrane for ATP production via a transmembrane proton motive force. The amino terminal amino acid sequence and chromatography on phenyl-Sepharose indicate the dehydrogenase is hydrophobic and could participate in this type of interaction. Furthermore, the decrease in specific activity throughout the purification may be caused by a loss of biologically active structure when the dehydrogenase is resolved from component C.

The native M_r of the dehydrogenase was 217,000. The subunit molecular weight was 32,800; we suspect that the enzyme has formed an aggregate.

The antibodies raised against the dehydrogenase can not be used for *in situ* labeling experiments because the dehydrogenase preparation used for injections was probably contaminated. However the amino-terminal amino acid sequence of the dehydrogenase derived from the modified purification procedure showed single amino acid residues for 18 of the first 20 positions and could be used to generate genetic probes for cloning experiments

The purification of the 5,10 methylene-H_4MPT: coenzyme F_{420} is complicated by the loss in specific activity; further experimentation aimed at an unambiguous purification procedure is needed. More research is also needed to establish the relationship between component C, the dehydrogenase, and their function in the production of a proton motive force.

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