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Sanjay S. Khandekar
Portland State University

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PURIFICATION AND CHARACTERIZATION OF

FUMARATE REDUCTASE FROM

METHANOBACTERIUM THERMOAUTOTROPHICUM

by

SANJAY S. KHANDEKAR

A dissertation submitted in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY
in
ENVIRONMENTAL SCIENCES AND RESOURCES - CHEMISTRY
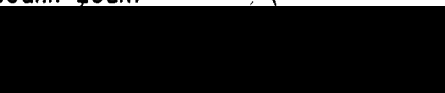
Portland State University
1986

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the dissertation of Sanjay S. Khandekar presented August 18, 1986.

Gordon Kilgour, Chairman

Dudley Eirich

Joann Loehr

Jack Myers

Carl Wamser

Pavel Smejtek

APPROVED:

David McClure, Head, Department of Chemistry

Bernard Ross, Dean of Graduate Studies and Research

AN ABSTRACT OF THE DISSERTATION OF Sanjay S. Khandekar for the Doctor of

Philosophy in Environmental Science and Resources - Chemistry presented

August 18, 1986.

Title: Purification and Characterization of Fumarate Reductase from

Methanobacterium thermoautotrophicum

APPROVED BY MEMBERS OF THE DISSERTATION COMMITTEE:

Gordon Kilgour, Chairman

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Anaerobic fermentation has been an established technology ever since
man started treating sewage. Recently this process has received increased
attention because of its inherent ability to produce methane gas, which

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apart from solar energy, is the cleanest, most non-polluting source of energy.

Methanobacterium thermoautotrophicum, a thermophilic bacterium, grows on CO_2 as a source of carbon as well as electron acceptor, using hydrogen as an electron donor. Labeling studies carried out with ^{14}C have shown a presence of partial reductive TCA cycle. In this work, the enzyme fumarate reductase, which belongs to this cycle, has been purified to homogeneity using various separation techniques.

In keeping with the thermophilic character of the organism, fumarate reductase is extremely heat resistant. Incubation at 75°C for 24 hours led to an increase in purification. In contrast, the enzyme was found to be very sensitive to oxygen. The crude extract, when exposed to air, lost half of its activity within 20 minutes. Reducing agents were helpful in protecting against loss of enzymatic activity provided that a strict anaerobic atmosphere was maintained. For this reason, the entire purification was performed inside a Freter-type anaerobic chamber using reducing agents.

The molecular weight of the native fumarate reductase, as determined by Sephacryl S-300 gel exclusion chromatography, was found to be approximately 80,000. SDS polyacrylamide gel electrophoresis data suggested that the enzyme is a tetramer. Treatment with sulfhydyl reagents as well as Cu^{++} caused loss in fumarate reductase activity, indicating that the enzyme contains at least one sulfhydryl group which is important to its activity.

The UV/Visible spectrum of fumarate reductase did not reveal the presence of a flavin moiety as a cofactor. Both UV/Visible and

fluorescence spectra of fumarate reductase from M. thermoautotrophicum instead, indicated the presence of an unusual cofactor, which could be similar to either tetrahydromethanopterin or F_{420}.

ACKNOWLEDGEMENTS

I am grateful to all the people who helped me in completing this dissertation project. I am thankful to Dr. Dudley Eirich, who introduced me to the novel type of methanogenic bacteria and taught me the anaerobic microbiological techniques with untiring patience.

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Finally, I must thank all my friends, both at school and away from school, who kept interest in me and my work, kept me going through my difficult period, and were always available whenever I needed them most.

Oh God,
    grant me the serenity
        to accept the things I cannot change
    and the courage
        to change that I can
    and the wisdom
        to know the difference.

No matter how discouraging the laboratory work turned out to be, I simply went back in and tried more approaches. ....As long as I could avoid asking myself the defeating question, "Should I really be in this?" I remained immune to the anxieties that accompany scientific research. For me it was always forward march, never halt, never retreat.

John Sheehan
Chemist

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INTRODUCTION

Methanogenic bacteria, commonly called "methanogens" are the group of microorganisms that are responsible for the production of methane, a colorless, tasteless and odorless gas first discovered by the Italian scientist, Volta in 1776. Since these bacteria are strict anaerobes, they are found in niches where the oxygen concentration is significantly low. These environments, include marine and fresh water sediments, anaerobic sludge digestors, the guts of insects, the large bowel of man and animals, and the rumen (1).

Although methane represents a relatively minor component of the carbon cycle as shown in Figure 1, its increasing concentration in the atmosphere has warranted considerable attention. Recent studies carried out by Rasmussen and Khalil (2,3) have indicated that the atmospheric concentration of methane is slowly but steadily increasing by about 2% every year. Methane, like CO_2, absorbs the outgoing infrared radiation from the earth's atmosphere; increased methane concentration may cause the warming of the earth's surface through the "green-house effect" by 0.2 - 0.4°K over the next 40 - 50 years (4). Although there are many possible sources leading to the increased methane levels in the atmosphere which may or may not be related to each other, basically the atmospheric methane concentration is due to the biogenic and the anthropogenic processes. The biogenic production of methane exceeds the anthropogenic as well as the

(CH₂O)ₙ

Organic compounds

Photosynthesis:
Algae
Green plants

Respiration:
Plants
Animals
Microorganisms

Methane-oxidizing
bacteria

CH₄

(CO₂)

Aerobic
Anaerobic

Methanogenic
bacteria

Sedimentation

Anaerobic respiration:
fermentation:
Anaerobic
microorganisms
including
photosynthetic
bacteria

Photo-
synthetic
bacteria

Organic compounds
(CH₂O)ₙ
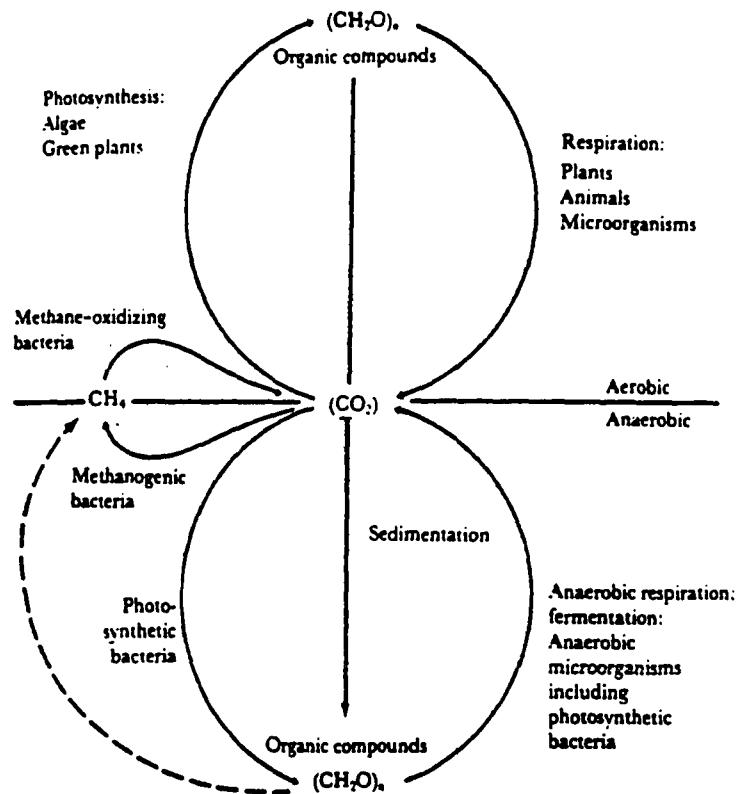
Figure 1.   Redox Cycle for Carbon.   (From Biology of
Microorganisms, Brock, T.D., Prentice-Hall,
Inc. N.J. (1979)).

fossil sources, as between 80 and 90% of the atmospheric methane is of biogenic origin (5); and methanogens are the sole biogenic source of methane.

Methane: A Potential Source of Energy

In recent years, due to the escalating prices of fuel and the increased awareness of the depletion of natural oil and gas, the idea of using biomass as a renewable source of energy has attracted a great deal of attention (6). Of all the available sources of fuels (apart from solar energy) currently in consideration, methane, a by-product of biomass degradation, is by far the cleanest and most non-polluting fuel. In the United States alone, about 25% of the energy is obtained by burning methane (7). There are a number of countries, especially in Asia and Africa which have many ongoing programs for utilizing the energy from biomass (8). It is assumed that the impact of full scale adoption of biogas plants by 2000 AD would provide about 90% of the rural energy requirements in India (9). The developed countries as well, in view of the potential scarcity of oil in the future, are considering methane as a potential candidate for motor fuel (10).

Significance of Methanogens in Anaerobic Environment

As mentioned earlier, methane generation takes place in an anaerobic

environment. Figure 2 depicts the fate of complex organic polymers such as proteins, carbohydrates and lipids in anaerobic environments (11). The fermentation of these polymers takes place in three consecutive steps. During the primary fermentation process, these complex polymers are broken down into their subunits, namely amino acids, methanol, sugars and the long chain fatty acids. The sugars are further broken into the mixed acids, while the long chain fatty acids are converted to the lower volatile fatty acids. These become the substrates for the secondary fermentation, which gives acetate as a major end-product along with formate, CO_2 and hydrogen. In the tertiary step hydrogen is rapidly oxidized, with concurrent reduction of CO_2 to methane by methanogenic bacteria. This terminal process of hydrogen removal is crucial for the overall anaerobic fermentation. If the hydrogen is not removed from anaerobic environments by methanogens, a mixture of products of the secondary fermentation would accumulate. This would then inhibit the whole anaerobic fermentation process. Thus, methanogens play a significant role in the process of terminal electron removal and thereby allow a more complete oxidation of the biopolymers to yield acetate, methane and CO_2. These coupled redox reactions between two or more anaerobic bacteria interacting with each other during the fermentation of complex organic substrates is commonly referred as "interspecies hydrogen transfer" (12). Labeling studies have indicated that as much as 67% of methane can be generated from acetate, while the remaining 33% can be produced from the reduction of CO_2 by hydrogen generated during the secondary fermentation process (13).
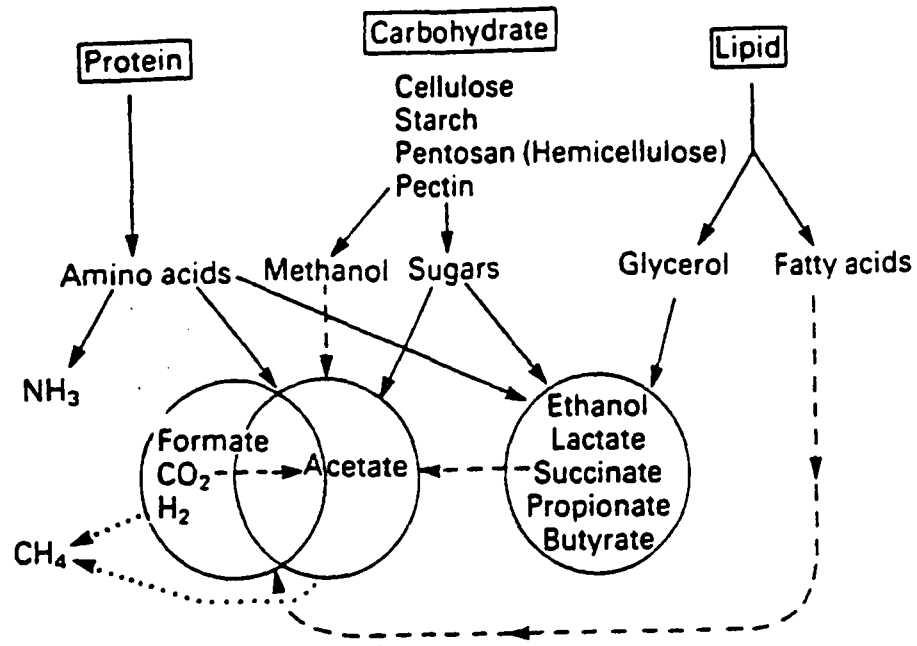
Figure 2. Anaerobic Breakdown of Biopolymers to Give Rise
to Methane. - Primary Fermentation Process, ---
Secondary Fermentation Process,···Tertiary
Fermentation Process. (From Large, P.J. (15)).

Taxonomy of Methanogens

All methanogens isolated so far have a common metabolic capacity to generate methane gas. However, as a group they vary considerably both morphologically and physiologically. Because of this, they are difficult to classify according to their metabolic, biochemical or physical properties. Fortunately, work done in the laboratories of Ralph Wolfe and Carl Woese, who used 16S ribosomal ribonucleic acid (RNA) sequence catalogs as their basis for the characterization of methane bacteria, has contributed greatly to the taxonomic classification of methanogens (14). During their studies, they digested the purified 16S ribosomal RNA from different methanogens with the enzyme ribonuclease T1. The oligonucleotide catalogs thus generated were then studied and compared using the standard taxonomic analysis.

A recent taxonomic scheme of methanogenic bacteria facilitated by the sequence analysis of 16S RNA is shown in Figure 3. When such studies were applied to other prokaryotes and eukaryotes and then compared with methanogens, the methanogens were found to be distinctly different (14). For this reason some taxonomists argue for placing them in a new kingdom called, archaebacteria. Table I shows the significant differences between methanogens and the other eubacteria, which justifies such classification (15).

ORDER | FAMILY | GENUS | SPECIES

Methanobacteriales
- Methanobacteriaceae
  - Methanobacterium
    - thermoautotrophicum
    - formicicum
    - bryantii
  - Methanobrevibacter
    - smithii
    - arboriphilus
    - ruminantium
- Methanothermaceae
  - Methanothermus
    - fervidus

Methanococcales
- Methanococcaceae
  - Methanococcus
    - vannielii
    - deltae
    - voltae
    - halophilus
    - thermolithotrophicus
    - jannaschii
    - maripaludis

Methanomicrobiales
- Methanomicrobiaceae
  - Methanomicrobium
    - mobile
    - paynteri
  - Methanogenium
    - cariaci
    - marisnegri
    - olentangyi
    - thermophilicum
    - tatii
    - wolfei
  - Methanospirillum
    - hungatei
- Methanoplanaceae
  - Methanoplanus
    - limnicola
- Methanosarcinaceae
  - Methanosarcina
    - barkeri
    - mazei
    - acetovorans
  - Methanothrix
    - sohngenii
  - Methanolobus
    - tindarius
  - Methanococcoides
    - methylutens

Figure 3.   A Taxonomic Scheme for Methanogenic Bacteria   (From Moore, T.B., M.S. thesis, PSU, 1985).

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TABLE I

DIFFERENCES BETWEEN EUBACTERIA
AND METHANOGENIC BACTERIA*

Eubacteria	Methanogens
1. Cell wall contains murein	1. Murein absent. Pseudomurein found in Methanobacteriales
2. Coenzyme M absent	2. Coenzyme M present in all species
3. Electron carriers are flavoproteins. quinones, cytochromes and ferredoxin	3. Contain unusual electron carriers — coenzyme F_{420}, an unusual flavin dervative — factor F_{430}, a nickel tetrapyrrole derivative Quinones and ferredoxin probably absent. Cytochromes and flavins rare
4. Membrane lipids are phosphatidyl derivatives and mono-, di- and tri-acylglycerols. all containing fatty acids.	4. Membrane lipids are C_{20} phytanyl and C_{40} biphytanyl glycerol *ethers* and isoprene hydrocarbons (mainly squalene)
5. No atractyloside-sensitive adenine nucleotide translocase has been found	5. Membranes contain an atractyloside-sensitive adenine nucleotide translocase
6. CO_2-fixation involves the Calvin cycle (except for the genus *Chlorobium*)	6. CO_2-fixation does not involve the Calvin cycle. nor the reductive tricarboxylic acid cylce of *Chlorobium*
7. RNA polymerase consists of a β' $\beta\alpha_2$ δ set of subunits	7. RNA polymerases do not have this structure
8. Transfer RNA contains ribothymine in the T ψC loop	8. T ψC sequence absent from tRNA
9. Peptide elongation factor EF-G not sensitive to diphtheria toxin	9. Peptide elongation factor is ADP-ribosylated by diphtheria toxin

* From Large, P.A. (15).

Bioenergetics and ATP Synthesis by Methanogens

Carbon dioxide and hydrogen are the substrates most commonly used by methanogens, CO_2 being the source of carbon and the electron acceptor and hydrogen being the electron donor. Methanogens are also known to grow on other substrates. These energy yielding reactions are shown in Table II with their respective Gibbs free energy of formation (16). ATP is the currency of energy in all living cells, and methanogens are no exception. Considering that in order to produce 1 mol of ATP about - 37 KJ/mol of energy is required, with the exception of acetate, about 2 to 3 ATP molecules could be generated per methane evolved from all the substrates given in Table II. Some work, although inadequate for solid conclusions, has been done in order to understand the mechanism of coupling of ATP synthesis to the methanogenesis (16-18). The current evidence, although indirect, suggests the absence of substrate level phosphorylation as well as the presence of a proton motive force that is coupled to the ATP synthesis during methanogenesis (19-20). It appears that the unusual cell wall associated with methanogens hampers the gentle preparation of membrane vesicles. Hence, the conclusive evidence of proton motive force in methanogens is yet to be presented.

Biochemistry of Methanogenesis

Figure 4 shows the sequential steps required for methane generation.

TABLE II

ENERGY-YIELDING REACTIONS USED
BY METHANOGENIC BACTERIA*

Reaction	ΔG^{o} per reaction (kJ)	$\Delta G^{o\prime}$ per CH_4 (kJ)
$4H_2 + CO_2 \rightarrow$ $CH_4 + 2H_2O$	-138.8	-138.8
$4HCOOH \rightarrow$ $3CO_2 + CH_4 + 2H_2O$	-119.5	-119.5
$4CH_3OH \rightarrow$ $3CH_4 + CO_2 + 2H_2O$	-310.5	-103.5
$4CH_3NH_3^+ + 2H_2O \rightarrow$ $3CH_4 + CO_2 + 4NH_4^+$	-225.7	-75.2
$4CO + 2H_2O \rightarrow$ $CH_4 + 3CO_2$	-185.6	-185.6
$CH_3COOH \rightarrow$ $CH_4 + CO_2$	-27.6	-27.6

* From Thauer et al (16).

$$CO_2 + X\text{-}H \longrightarrow X\text{-}COOH$$
$$\downarrow + 2H$$
$$X\text{-}CHO + H_2O$$
$$\downarrow + 2H$$
$$CH_3COOH + X\text{-}H \diagdown \quad -H_2 \qquad X\text{-}CH_2OH$$
$$-CO_2 \qquad \downarrow + 2H$$
$$CH_3OH + X\text{-}H \longrightarrow X\text{-}CH_3 + H_2O$$
$$-H_2O \qquad \downarrow + 2H$$
$$X\text{-}H + CH_4$$
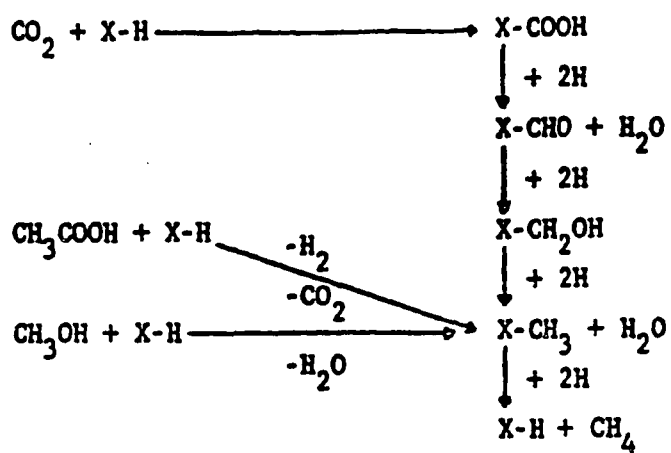
Figure 4. Steps Involved in the Reduction of Co_2 to CH_4. X is a carbon carrier which may or may not be the same in all reactions.

Although the steps may seem simple, a number of reactions are involved by which CO_2 is reduced to methane. Over the past decade the details of this cycle have been worked out in the laboratory of Wolfe demonstrating the presence of unusual cofactors, coenzymes, and enzymes participating in their own unique ways. The current model for methanogenesis is shown in Figure 5 (21).

In this unique model, the first reaction sequence involves the fixation of CO_2 to the formyl level of oxidation using a coenzyme called methanofuran (MFR), which was previously called CO_2 reduction factor(22). This formyl moiety is then transferred onto another carrier, tetrahydromethanopterin (H_4MPT) (23-24). Extensive work has been done on this oxygen sensitive cofactor (25-27), which is an activated form of methanopterin (MPT) (24). The H_4MPT carries the carbon of CO_2 to the methyl level and then transfers this group to Coenzyme M (2-mercaptoethane sulfonic acid).

The terminal step of this model is very complex. As shown in Figure 6, various protein components as well as cofactors are involved. The enzymatic function of component A-2 is not known. Component A-1, which is oxygen sensitive, contains hydrogenase along with other unknown proteins. The functions of these unknown proteins are not at all understood at this point. Component A-3 is another oxygen sensitive fraction of several unidentified proteins (21). Component C has been purified and has been shown to have a tightly bound cofactor F_{430} (28). The exact function of component B in this multi-enzyme system is still not clear (29). Interestingly, the system does not function to its optimum in the absence of both ATP and Mg^{++}. These seem to serve as effectors of the methyl
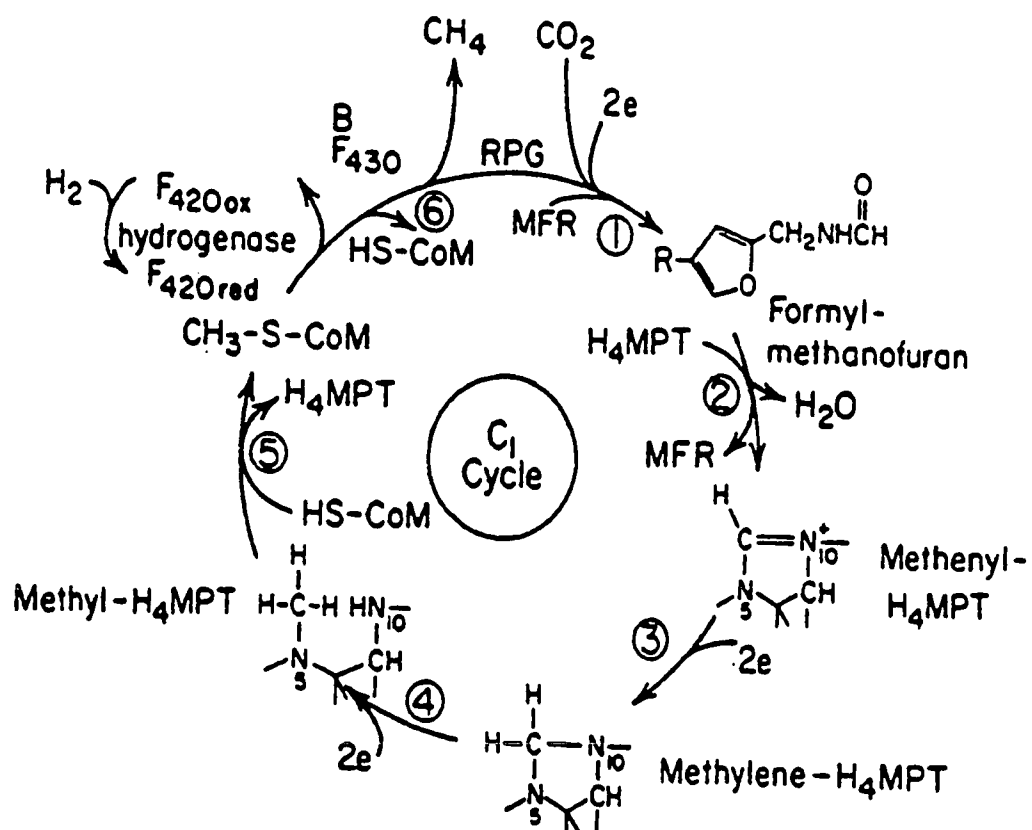
Figure 5. The C_1 Cycle for the Reduction of CO_2 to CH_4. (From Wolfe, R.S. (21)).

$$\begin{array}{c} \text{(Cofactors)} \\ \text{Component B} \\ F_{430} \\ F_{420} \\ \text{FAD} \\ \text{ATP} \\ Mg^{2+} \end{array}$$

$$CH_3 - S - CoM + H_2 \xrightarrow{\hspace{3cm}} CH_4 + HS - CoM$$

$$\begin{array}{c} \text{(Protein Components)} \\ A-1 \\ A-2 \\ A-3 \\ C \end{array}$$
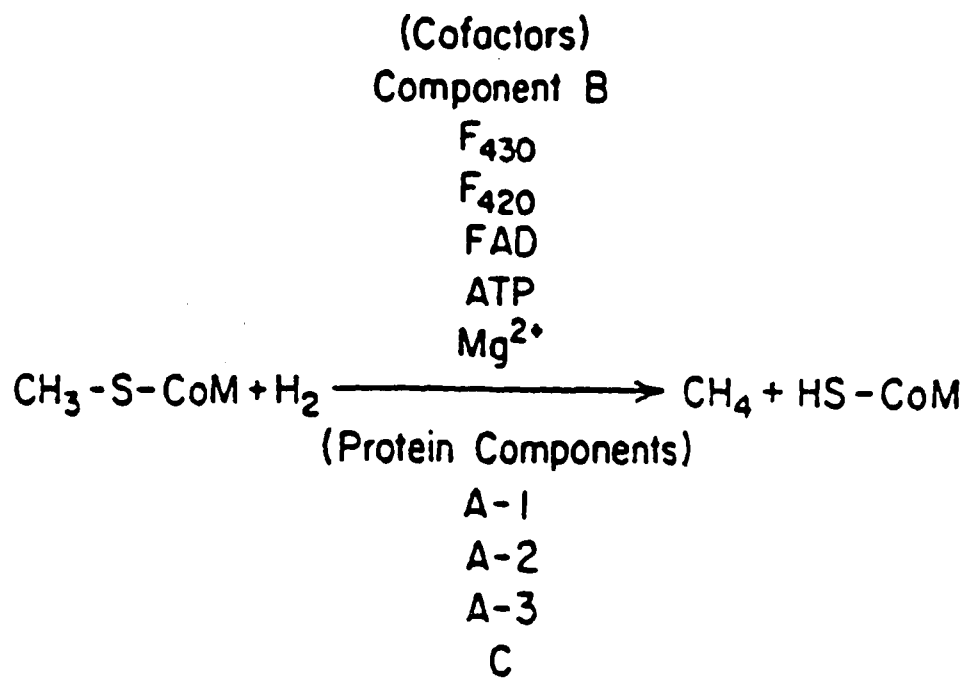
Figure 6.   The Methyl Reductase System (From Wolfe, R.S. (21)).

reductase. Due to the complexity of this reaction, precise details and tne roles of other cofactors involved are yet to be comprehended.

The structures of various novel cofactors/coenzymes that participate in this C_1 cycle are given in Figure 6a and 6b.

Carbon Fixation Pathway in Methanogens

In methanogens that grow on CO_2 and hydrogen, CO_2 not only serves as a source of electron acceptor but also as a sole source of carbon. Labeling studies carried out with radioactive $^{14}CO_2$ in Methanobacterium thermoautotrophicum indicated the absence of ribulose-1,5-bisphosphate carboxylase, the key enzyme in the pentose phosphate pathway, hydroxypyruvate reductase, an important enzyme in the serine pathway; and hexulose phosphate synthetase, a key enzyme in the hexulose phosphate pathway (30). Thus, it is assumed that these pathways do not play any role in the fixation of carbon in these autotrophic methanogens.

Interestingly, recent findings indicate the presence of a modified reversed citric acid cycle (31-32). This cycle is also called a "partial reductive TCA cycle". The detailed outline of this pathway is depicted in Figure 7. Since the activity of isocitrate dehydrogenase was virtually absent in the crude extract of M. thermoautotrophicum, the cycle is considered to terminate with the formation of α-ketoglutarate.

Unfortunately, little is known about the initial step of CO_2 fixation. It is thought that acetate is formed as a result of the condensation of two molecules of CO_2. Although this reaction resembles

HOOCCH$_2$CH$_2$CHCHCH$_2$CH$_2$CNHCHCH$_2$CH$_2$CNHCHCH$_2$CH$_2$CNHCH$_2$CH$_2$-⬡-OCH$_2$-furan-CH$_2$NH$_2$

with COOH groups and carbonyl O substituents

methanofuran

HS−CH$_2$−CH$_2$−SO$_3^-$

−Coenzyme-M

F$_{430}$

HSCH$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$CNHCHCHOPOH with CH$_3$, COOH, OH, and O substituents
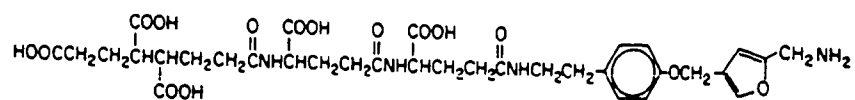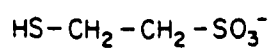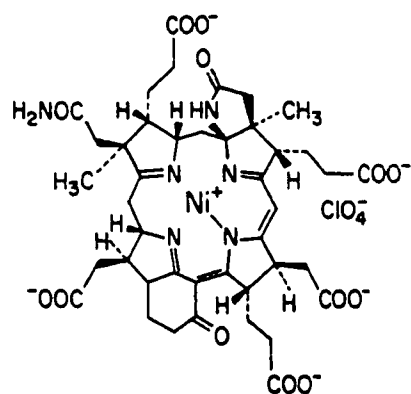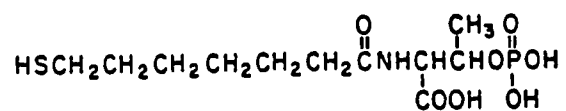
Component B

Figure 6a. Structures of Novel Cofactors/Coenzymes Present in Methanogens. (From Wolfe, R.S. (21)).

H₄ Methanopterin

Oxidized

Reduced

F₄₂₀
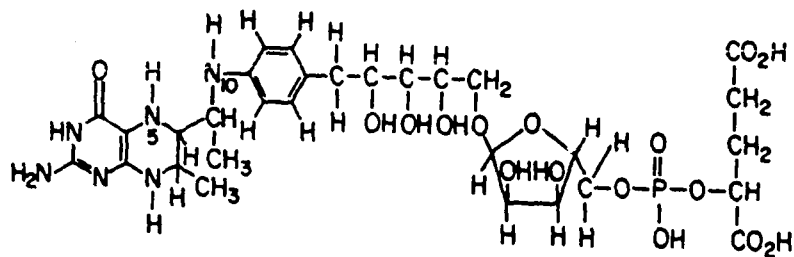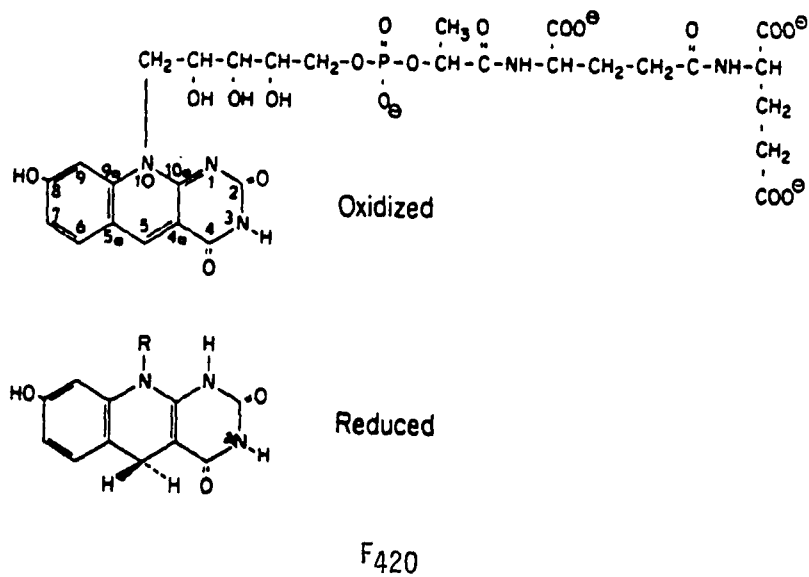
Figure 6b. Structures of Novel Coenzymes Present in Methanogens. (From Wolfe, R.S. (21)).
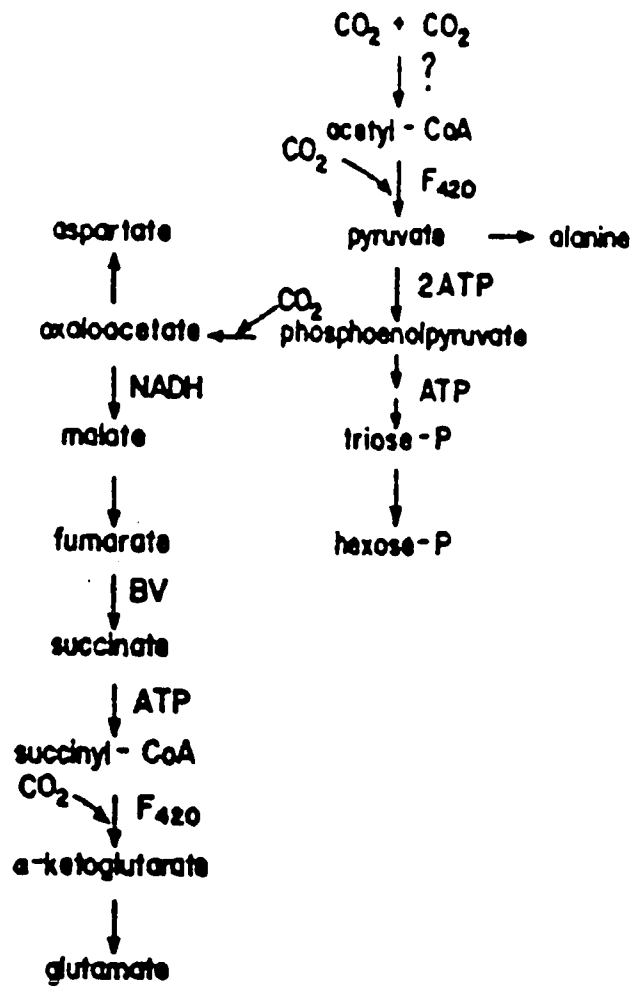
Figure 7. Pathway of Autotrophic CO_2 Assimilation in
M. thermoautotrophicum. (From Fuchs and
Stupperich (31)).

the one associated with the acetogenic bacteria (31), methanogens
conspicuously lack high levels of folate enzymes, which play a crucial role
in the folate pathway of these acetogenic bacteria (33). Nonetheless,
there has been definite evidence that activated acetic acid is the first
important intermediate in the overall fixation of CO_2 into cell carbon
(34). Acetyl CoA is then reductively carboxylated to pyruvate.
Phosphoenol pyruvate synthetase then converts pyruvate to the phosphoenol
pyruvate, a thermodynamically unfavorable reaction. Eyzaguirre et al (35)
have partially purified this enzyme, which requires at least two molecules
of ATP to carry out the direct synthesis of phosphoenol pyruvate. The
following step involves the carboxylation of phosphoenolpyruvate to
oxaloacetate. The enzyme, phosphoenolpyruvate carboxylase, has been
partially purified by Kenealy and Zeikus (36). The oxaloacetate generated
can then be used to synthesize aspartate and other related amino acids. It
can also be further reduced to malate by malate dehydrogenase. This enzyme
has been purified by Sprott et al (37) from Msp. hungatii. The enzyme has
been linked to the oxidation of reduced NAD. Fumarase catalyzes the
conversion of malate to fumarate simply by removing water. The next step
is the reduction of fumarate to succinate by fumarate reductase (38). The
electron donor for this reaction is yet to be identified. Succinate is
acetylated to succinyl CoA with the help of ATP. The formation of
α-ketoglutarate from succinyl CoA is the terminal step of this pathway,
which is catalyzed by α-ketoglutarate dehydrogenase (38). The enzyme uses
F-420 rather than NAD as a cofactor. The α-ketoglutarate is then converted
to the glutamate family of amino acids. Thus, it is clear that this field
of autotrophic carbon fixation is still in its early development.

Interestingly, studies performed with the extracts of M. barkeri

showed that α-ketoglutarate is synthesized via isocitrate (39). This

cycle, for comparison, is depicted in Figure 8.

Purpose of the Present Investigation

Figure 7 shows the proposed autotrophic CO_2 assimilation pathway in

M. thermoautotrophicum. Fuchs et al (40) and Zeikus et al (38) reported

activity of fumarate reductase in crude extract of M. thermoautotrophicum.

Zeikus et al (38) also reported that the activity of fumarate reductase was

found in the soluble cell fraction of these bacteria. This is surprising

because fumarate reductase purified from E. coli (41,42) as well as from

Vibrio succinogenes (43,44) was shown to be membrane bound. When E. coli

is grown in the presence of glycerol and fumarate (as electron acceptor),

fumarate reductase acts as the terminal enzyme in the electron transport

chain. This enzyme is expressed only during the anaerobic growth, and is

repressed aerobically (41). Both E. coli and V. succinogenes can generate

a sufficient membrane potential which can synthesize the ATP required for

the growth from the reduction of fumarate (42,44).

Fumarate reductase purified from both E. coli and V. succinogenes

showed the presence of covalently bound FAD attached to the histidyl moiety

of the larger subunit. The smaller subunit of both these enzymes contained

a binuclear iron-sulfur center. Although these two organisms have been

shown to have similar properties with respect to fumarate reductase,

surprisingly, DNA:DNA hybridization studies carried by Unden and Cole (45)
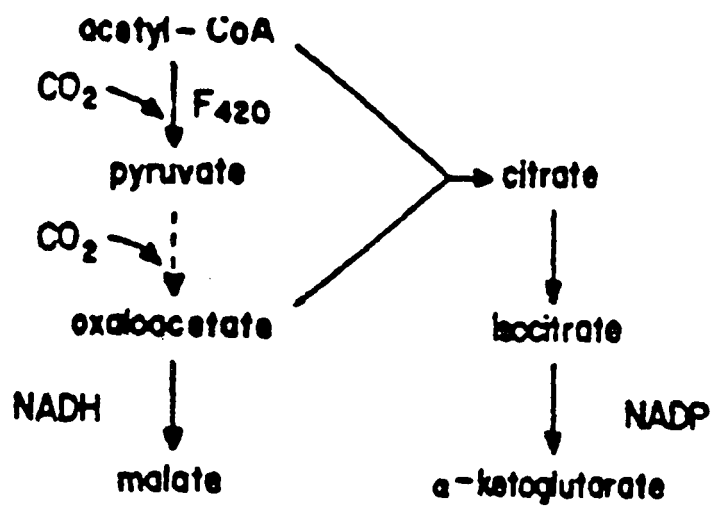
Figure 8. Incomplete TCA Cycle in Methanosercina
barkeri. (From Weimer and Zeikus (39)).

concluded that there are wide differences between the fumarate reductases of these two organisms.

Fumarate reductase isolated from Proteus mirabilis was also found to be repressed when oxygen or nitrate was available as an electron acceptor (46). This enzyme is also coupled to the synthesis of ATP.

The fumarate reductase of Clostrodium formicoaceticum is located on the outerspace of the cytoplasmic membrane (47). The activity was shown to be NADH dependent and reduced FMN rather than FAD was found to be an effective electron donor. Such studies indicate that the fumarate reductase of C. formicoaceticum is significantly different from those of V. succinogens and E. coli.

Contrary to all these membrane associated fumarate reductases, there is evidence for fumarate reductase activity in the soluble fraction of the cell extracts of baker's yeast (48) and brewers yeast (49). The only similarity between the soluble and the membrane bound fumarate reductase is that they are all flavoproteins. Muratsubaki and Katsume (48) purified fumarate reductase from the cytosol fractions of the cells of baker's yeast. The enzyme contains one molecule of non-covalently bound FAD . Their results are strikingly different from other organisms previously mentioned. This is not surprising since the role of fumarate reductase in yeast seems to be different. They proposed that fumarate reductase plays a regulatory role in the production of succinate when the cells are grown anaerobically. Other studies have indicated a higher amount of succinate accumulation in the medium during anaerobic cultivation than during aerobic cultivation (50). Thus, although not confirmed, it is suggested that the physiological function of fumarate reductase in yeast cells is to make

available high levels of succinate or succinyl CoA, which are important intermediates in the biosynthetic pathways (48).

Considering activities of fumarate reductase in the soluble fractions of the extracts of M. thermoautotrophicum, a role for this enzyme in the anabolic (biosynthetic) metabolism seems to be likely. Fuchs et al (40) performed preliminary studies on fumarate reductase from crude extract of M. thermoautotrophicum. Their studies showed the absence of catabolic oxidation of succinate to fumarate. On the basis of this evidence they excluded the operation of a fumarate-succinate cycle generating ATP. Hence, the preliminary studies do indicate the anabolic role of fumarate reductase, which is also supported by the very fact that its activity is essentially associated with the soluble fraction. It therefore appears, as Fuchs et al (40) proposed, that the sole purpose of fumarate reductase is to provide the cells with succinate, succinyl CoA and α-ketoglutarate, which are essential for the synthesis of various amino acids as well as other intermediates.

Such comparative studies will not lead to definitive conclusions for fumarate reductase of M. thermoautotrophicum since the results were primarily obtained with crude cell preparations; many times purified enzymes exhibit different properties than crude preparations of the same enzyme.

It is also impossible (in crude preparations) to determine the presence or absence of cofactors such as FAD or FMN, which are shown to be associated with the other purified fumarate reductases. Therefore, in order to understand the basic differences or similarities between the fumarate reductase of M. thermoautotrophicum and those of other organisms,

we undertook the purification of fumarate reductase in order to study its physical and chemical properties.

In conclusion, the studies involved in the present investigation will help in comparing the properties of purified fumarate reductase of M. thermoautotrophicum with those of other organisms. These studies would also contribute to an understanding of the unique biochemistry of methanogens.

EXPERIMENTAL

Culture Maintenance and Growth Conditions of
Methanobacterium tnermoautotrophicum

Maintenance of the Pure Culture

Methanobacterium thermoautotrophicum, strain ∆H was a generous gift

from Dr. R.S. Wolfe (University of Illinois, Urbana-Champaign). This

bacterium was originally isolated and characterized by Zeikus and Wolfe

(51) from sewage sludge. M. thermoautotrophicum was the choice for this

investigation since it is one of the most widely studied methanogens. The

bacterium is autotrophic, since carbon dioxide can serve as the sole source

of carbon; hydrogen gas supplies the reducing power. An additional

advantage in choosing M. thermoautotrophicum was that it is a thermophilic

methanogen. The optimum temperature for the growth of this organism is

between 65-70°C. This helps in minimizing the chance of contamination, as

well as improving the generation time. As a matter of fact, this bacterium

is one of the fastest growing methanogens known. The general properties of

M. thermoautotrophicum are given in Table III .

The pure culture was maintained in a minimal medium prepared

according to the method of Balch and Wolfe (52). The composition of this

medium is given in Table IV. The medium was first prepared by adding all

the reagents except the reducing agents, namely L-cysteine hydrochloride

and sodium sulfide. The medium (20 ml) was then transferred to 100 ml

serum bottles. The bottles were stoppered with thick walled rubber

TABLE III

GENERAL CHARACTERISTICS OF M. THERMOAUTOTROPHICUM*

Source   : Sewage Sludge Plant

Gram Reaction   : Gram + Ve

Shape   : Curved Rod, Forms Long Filaments

Motility   : Non-motile

Temperature
Optimum   : 65 - 70°C (Max. 75°C, Min. 40°C)

pH Optimum   : 7.2 - 7.6

G-C Content   :
of DNA   : 52%

Generation Time : 3 - 5 Hours in Optimum Conditions

Substrates   : CO_2 and H_2
                    $CO_2 + 4 H_2 ------> CH_4 + 2 H_2O$

                         $(\Delta G° = - 135.6$ KJ/mol$)$

* From Zeikus and Wolfe (51)

TABLE IV

COMPOSITION OF THE STANDARD MEDIA[1]

Components	Ingredients added to the distilled water to give a final volume of one liter
[2]Complete Salts	25 ml
NH_4Cl	1.25 gm
[3]Trace Minerals	10 ml
[4]Trace Vitamins	10 ml
Ferrous Ammonium Sulfate Heptahydrate	0.02 gm
[5]$NaHCO_3$	7.50 gm
[6]L-Cysteine-HCl	0.60 gm
[6]Na_2S, 9 H_2O	0.60 gm

1 From Balch et al (14).
2 See Table IVA
3 See Table IVB
4 See Table IVC
5 Na_2CO_3 was used instead of $NaHCO_3$ during mass culture.
6 Added last to maintain the reducing conditions.

TABLE IVa

COMPOSITION OF THE COMPLETE SALT SOLUTION

Components	Ingredients added to the distilled water to give a final volume of one liter
KCl	0.67 gm
$MgCl_2 \cdot 2\ H_2O$	5.5 gm
NH_4Cl	0.5 gm
$CaCl_2 \cdot 2\ H_2O$	0.28 gm
K_2HPO_4	0.28 gm

TABLE IVb

COMPOSITION OF THE MINERAL SOLUTION

Component	Dissolved 1.5 g NTA, adjusted pH to 6.5 with KOH and the following ingredients were added to the distilled water to give a final volume of one liter
$MgSO_4 \cdot 7\ H_2O$	3.0 gm
$MnSO_4 \cdot H_2O$	0.5 gm
NaCl	1.0 gm
$FeSO_4 \cdot 7\ H_2O$	0.1 gm
$CaCl_2 \cdot 2\ H_2O$	0.1 gm
$CoCl_2$	0.1 gm
$ZnSO_4$	0.1 gm
$CuSO_4 \cdot 5\ H_2O$	0.01 gm
$AlK\ (SO_4)_2$	0.01 gm
H_3BO_3	0.01 gm
$Na_2MoO_4 \cdot H_2O$	0.01 gm

TABLE IVc

COMPOSITION OF THE VITAMIN SOLUTION

Composition	Ingredients added to the distilled water to give a final volume of one liter
Biotin	2 mg
Folic Acid	2 mg
Pyridoxine HCl	10 mg
Thiamine HCl	5 mg
Riboflavin	5 mg
Nicotinic Acid	5 mg
DL Ca Pantothenate	5 mg
Vitamin B_{12}	0.1 mg
p-Aminobenzoic Acid	5 mg
Lipoic Acid	5 mg

stoppers (Belco Laboratories) and then sealed with aluminum crimp seals.
The bottles were evacuated for 5 to 10 minutes and then autoclaved at 121°C
for 20 minutes. The autoclaved bottles were then pressurized with CO_2 and
hydrogen (20:80) which supplied the source of carbon and electrons. The
gases were passed through a reduced copper column (oxygen scrubber) which
was constantly heated. The medium contained resazurin which acted as a
redox indicator. It is pink in the oxidized form and colorless in the
reduced form. Just prior to inoculation of the pure culture the medium was
reduced by adding the reducing agents via a sterile hypodermic syringe.
The syringe was flushed with H_2 and CO_2 gas mixture (80:20) for at least 10
times before the reducing agents or the pure culture were added to the
media. (Extreme care must be taken in order to maintain the anaerobic
atmosphere while transferring the reducing agents and the pure culture to
the pressurized bottles containing the medium). After inoculation the
serum bottles were pressurized with the mixture of H_2 and CO_2 to a total
pressure of 30 psi and incubated in a horizontal position at 65°C for three
days. This helped the diffusion of the gaseous substrates into the medium.

When the cultures were not in use, they were stored in the
refrigerator at 5°C. The bottles were repressurized with the mixture of
hydrogen and carbon dioxide (80:20) whenever it was necessary.

Mass Culture of M. thermoautotrophicum

M. thermoautotrophicum was mass cultured (53) using the techniques
described above with a few modifications. Two hundred ml of medium in a

one liter serum bottle was reduced and inoculated (5% inoculum) following the stringent anaerobic techniques described earlier. The pressurized (30psi, 20:80 CO_2: H_2) bottle was then incubated at 65°C.

A 14 liter fermentor (New Brunswick Microferm) was used to mass culture this organism. The assembly of the fermentor is illustrated in Figure 9. Hydrogen and CO_2 gases (80:20) were passed through a heated copper column (to remove O_2) before entering the fermentor jar. The fermentor jar containing 12 L of medium (Table IV) was steam sterilized by autoclaving for 50 minutes. The autoclaved fermentor jar was then attached to the fermentor unit. The medium was gassed with hydrogen and carbon dioxide employing a stirring rate of 400 rpm. The pH was adjusted to about 7.0-7.2 by addition of 100 ml of sterile 3.0 M HCl. The reducing agents were then added anaerobically, maintaining sterile conditions.

The reducing agents and the gaseous atmosphere help in attaining the proper anaerobic environment. This is indicated by the change in the color of resazurin which turns from blue to pink and then turns colorless. Once the appropriate anaerobic atmosphere was generated, the fermentor was inoculated with the starting culture (1.7% inoculum). The initial flow rate (200 ml/min) of the gas mixture was maintained for the first 24 hours. This was then increased periodically during the next two days. The steady growth of the cells was observed by the turbidity (light green in color). A more definitive way to measure the rate of growth was by determining the amount of methane produced, which was analyzed gas chromatographically using a Varian Gas Chromatograph equipped (Model 90-P) with Porapak Q column (5' x 0.25" size). The optimal growth was achieved within three days.
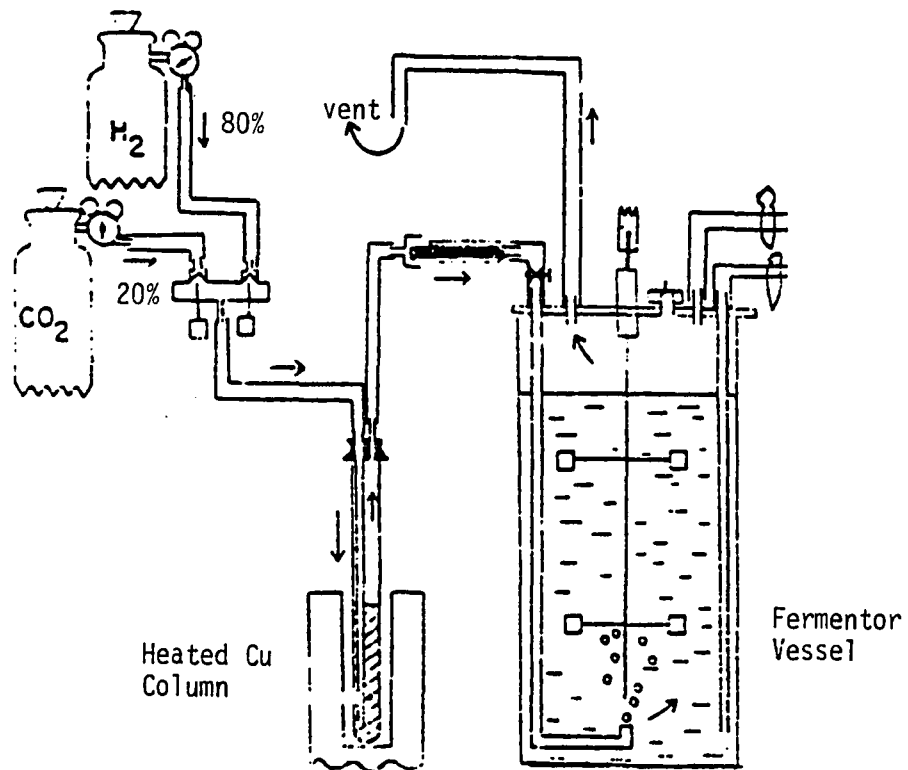
Figure 9. Digramatic View of the Fermentor used to Mass
Culture Methanogens. (From Byrant et al (53)).

The cells were harvested using a Cepa-Schnell continuous centrifuge. A typical yield of 50 - 100 gms/12 liter was routinely obtained. The cell paste was suspended in 0.1 M phosphate buffer, pH 7.5 (2 ml/g of wet cell paste) and stored at -20°C under a hydrogen atmosphere.

Preparation of the Crude Extract

The crude extract was prepared using a French pressure cell employing a pressure of 20,000 psi. The lysate thus formed was centrifuged at 20,000 rpm for a period of 45 minutes using sealed stainless steel centrifuge tubes under a nitrogen atmosphere. The collected supernatant was flushed with hydrogen for several minutes and then stored under a hydrogen atmosphere.

Anaerobic Freter-type Chamber

Early enzyme purification studies performed with extracts of M. thermoautotrophicum were not very successful due to the extreme oxygen sensitivity of certain enzymes and/or their components (14). In view of these problems, Gunsalus et al (54) described the use of a Freter-type anaerobic chamber with an air-lock system (55) which was employed by them during the fractionation of the oxygen-sensitive methyl reductase system of M.thermoautotrophicum. Their studies indicated that this type of chamber could be used for the fractionation and the purification of a variety of oxygen sensitive enzymes with minimal time and expense as compared with the

room size chamber described by Poston et al (56).

Since the proposed research involved the purification of an enzyme from an anaerobic bacterium, an anaerobic chamber (Coy Laboratory Prod., Inc., Ann Arbor, Michigan) was employed throughout the course of this investigation. The diagramatic view of this chamber is depicted in Figure 10. The chamber contained about 95% nitrogen and 5% hydrogen. The presence of hydrogen was checked periodically using a Varian Gas Chromatograph equipped with thermal conductivity detector. Hydrogen concentration should not exceed 5% since in the presence of oxygen it may act as an explosive. The anaerobic chamber, in addition, contained a catalyst diffusion box. The box contained an activated charcoal bed (2 cm deep) beneath the palladium catalyst. The catalyst helped in maintaining the anaerobic atmosphere by activating the reaction of hydrogen in the gas mixture with any oxygen diffused through the plastic walls of the chamber. The activated charcoal is used for the removal of the volatile sulfides which would otherwise inactivate the palladium catalyst. The palladium catalyst was reactivated every week by heating at 130°C for 2 hours in an oven. The activated charcoal was replaced every two months. The use of reactivated catalyst insured the anaerobic environment inside the chamber. The low level of oxygen was monitored using an oxygen analyzer (Chemical Sensor Development, Inc., California). The oxygen concentration in the chamber averaged less than 5 ppm once the equilibrium conditions were achieved. Glassware and plasticware as well as rubber stoppers were allowed to equilibrate with the chamber atmosphere for a period of 1 to 24 hours prior to their use. Neoprene gloves were used to handle the glassware inside the chamber.
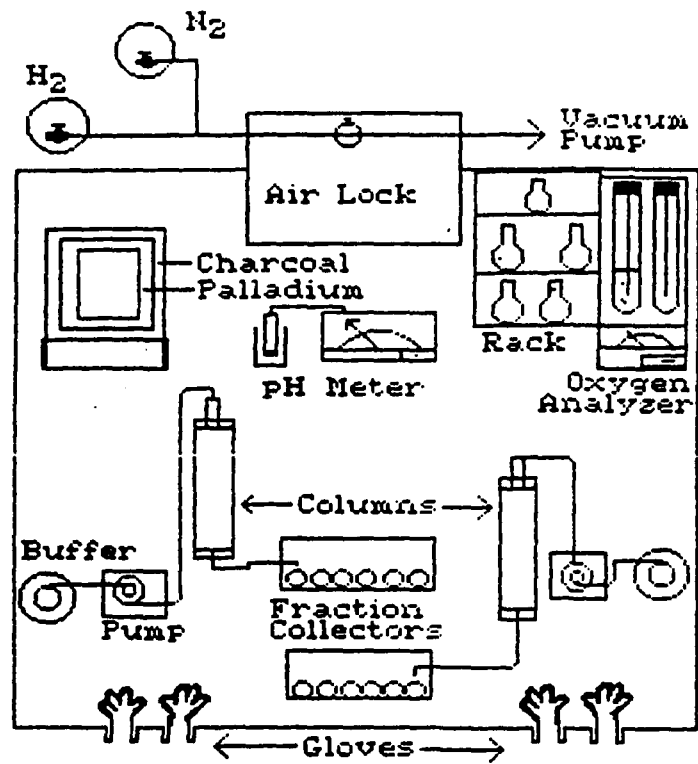
Figure 10. Digramatic View of an Anaerobic
Freter-type Chamber.

An electronic gas leak detector (Quantum Instruments, N.Y.) was frequently used to detect any gas leakage from the anaerobic chamber. Neoprene gloves were the major cause of gas leakage due to tearing or holes. However, they could be easily replaced while maintaining the anaerobic atmosphere inside the chamber. In general, careful use and precautions helped in minimizing the chance of damaging the glove box.

The anaerobic chamber contained a pH meter which was helpful in measuring the pH of the buffers, media, and extracts without exposing them to the outside atmosphere. The chamber also included a membrane filtratation apparatus, column chromatographic equipment, and other essential items required during the purification processes. These will be discussed in detail in the Results section.

Assay Procedure for the Fumarate Reductase

The enzyme fumarate reductase catalyzes the reduction of fumarate to succinate. Since the natural electron donor in these bacteria is not known, an artificial electron donor, benzyl viologen (E_0 -350 mV at pH 7.0) was incorporated during the course of this investigation. Benzyl viologen is colorless when oxidized and blue when reduced. Thus the rate of oxidation of reduced benzyl viologen by fumarate could be monitored spectrophotometrically. The assay conditions and the reactions involved are given in Figure 11 and 11a respectively. The assay procedure, explained below, was performed according to the method of Zeikus et al (38), with modifications.

```
BENZYL VIOLOGEN (red.) ╲    ╱ FUMARATE
(Colored)               ╲  ╱
                         ╲╱
                       F.R.
                         ╱╲
BENZYL VIOLOGEN (Oxi.)◄─╯  ╲─► SUCCINATE
(Colorless)
```

(Monitor the Oxidation of Reduced B.V. at 578 nm)

Typical Assay Mixture

A. 3.0 ml total volume
   ----0.1 M Sodium Phosphate buffer (pH 7.0) with
   ----17.5 M 2-mercaptoethanol, 1mM EDTA and 1mM DTT
   ----0.31 mM Benzyl Viologen
   ----1.66 mM Sodium Fumarate
   ----Enzyme (20 - 50 µl)

B. Combine buffer and enzyme under nitrogen atmosphere
   outside the chamber. Add benzyl viologen and reduce a
   small amount of it with sodium dithionite until A_{578}
   of approximately 1.0 is achieved. Begin the reaction
   with the addition of sodium fumarate.

C. Record the oxidation of benzyl viologen by observing
   the loss of A_{578} over time at 75°C.

Figure 11. Spectrophotometric Assay for Fumarate Reductase.

$$\text{(benzyl viologen structure)} \equiv BV^{+2}$$

(1) $BV^{+2} \xrightarrow{S_2O_4^{-2}} BV^{\cdot +}$

(2) $2BV^{\cdot +} + HOOC\text{-}CH\text{=}CH\text{-}COOH + 2H^+ \xrightarrow{F.R.} 2\,BV^{+2} +$

        (Fumarate) $HOOC\text{-}CH_2\text{-}CH_2\text{-}COOH$
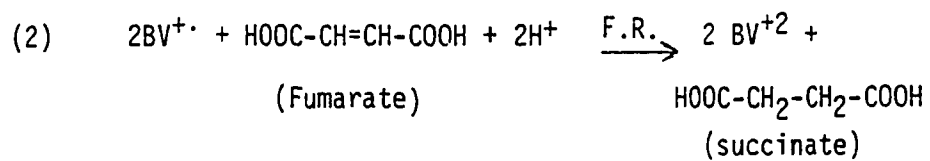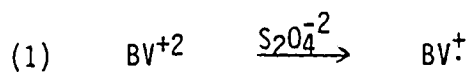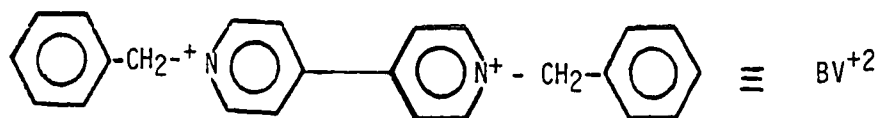
                                                   (succinate)

Figure 11a. Reactions Involved in the Assay for Fumarate Reductase. BV^{+2} is Benzyl Viologen, $BV^{\cdot +}$ is Benzyl Viologen Radical, $S_2O_4^{-2}$ is Dithionite.

Bausch and Lomb spectrophotometric tubes were routinely used for the assay. The cleaned tubes were transferred to the anaerobic chamber and were equilibrated with chamber atmosphere for at least an hour. To these tubes was added 3.0 ml of anaerobic sodium phosphate buffer, 0.1 M, pH 7.0, containing 15 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM dithiothreitol. The tubes were tightly sealed with rubber stoppers (No. 00 in size) and were then transferred outside. Nitrogen gas, passed through a heated copper column to remove residual oxygen, was bubbled through these stoppered tubes for a few minutes. The apparatus employed for this is shown in Figure 12. The tubes were then incubated at 75°C for 15 minutes. The temperature of the cuvette holder was regulated with a temperature controlled water bath that was attached to the Turner spectrophotometer (Model 330). For the routine assays, the reaction mixture contained benzyl viologen (0.3 mM), sodium fumarate (1.7 mM), and 10-50 µl of enzyme. Some of the benzyl viologen was initially reduced by adding sodium dithionite. The dithionite was added until the absorbance at 578 nm reached approximately 1.0. The reaction was started with the addition of fumarate. The rate of oxidation of reduced benzyl viologen was followed spectrophotometrically at 578 nm using a recorder (Sergent Welch, Model SRG-2) attached to the Turner Spectrophotometer.

Determination of Protein

Elution profiles of the proteins separated by column chromatography were followed at 280 nm using a Beckman D.U. spectrophotometer. The
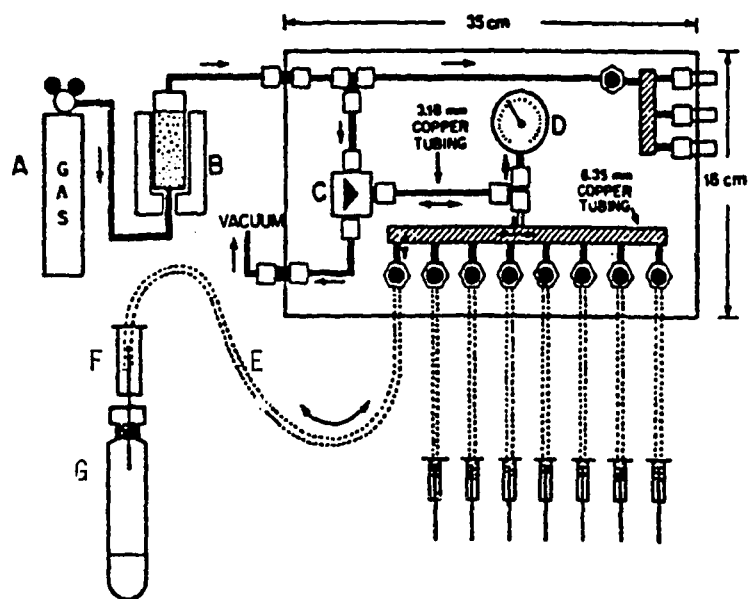
Figure 12. Gassing Manifold with Apparatus for Supply
O_2 free Nitrogen Gas. A, N_2 Cylinder;
B, Heated Cu column; C, valve; D, Pressure
Gauge; E, Thick-Walled Plastic Tubing
F, Vaccutainer Needle; G, Assay Tube.
(From Balch, et al (14)).

protein concentrations for each step of purification were determined by the method of Bradford (57). Bovine serum albumin (1mg/ml) was employed as a protein standard.

Acrylamide Gel Electrophoresis

The discontinuous polyacrylamide gel electrophoresis (PAGE) was performed at various stages of the purification by following the general procedure of Davis (58). The components of both the upper and the lower gels were prepared according to the method of Hames (59). The gels were stained overnight with Coommassie Blue R and destained with a aqueous mixture of 30% methanol and 7% acetic acid (59).

Subunit Determination

The subunits of fumarate reductase were determined by employing PAGE in the presence of sodium dodecyl sulfate. The samples were boiled in the presence of 5% SDS and 5 % 2-mercaptoethanol for 10 minutes. The compositions of both the lower and the upper gels containing 10% SDS were as described by Hames (59).

RESULTS

Stability of Fumarate Reductase in Crude Extract

Various enzymes characterized from Methanobacterium
thermoautotrophicum have been shown to be either oxygen sensitive or oxygen
resistant (60). It was essential to carry out similar studies with the
crude preparations of fumarate reductase from M. thermoautotrophicum since
this would determine whether an anaerobic Freter-type chamber (55) would be
required for its purification.

In order to study the oxygen sensitivity of fumarate reductase, the
crude extract of M. thermoautotrophicum was exposed to air for a period of
5, 10, 30, 60 and 120 minutes. The assay was performed as described
earlier in the Experimental section. The results are shown in Figure 13,
and demonstrate that the fumarate reductase is extremely sensitive to the
oxygen in air. About 50% of the activity is lost upon 5 minutes exposure,
while 80% is lost after the enzyme is exposed to air for 2 hours. Reducing
agents, either of biological or chemical origin, are generally helpful in
mimicking the reducing atmosphere of an anaerobic bacterial cell (61).
These reducing agents protect the active site of the enzyme by reacting
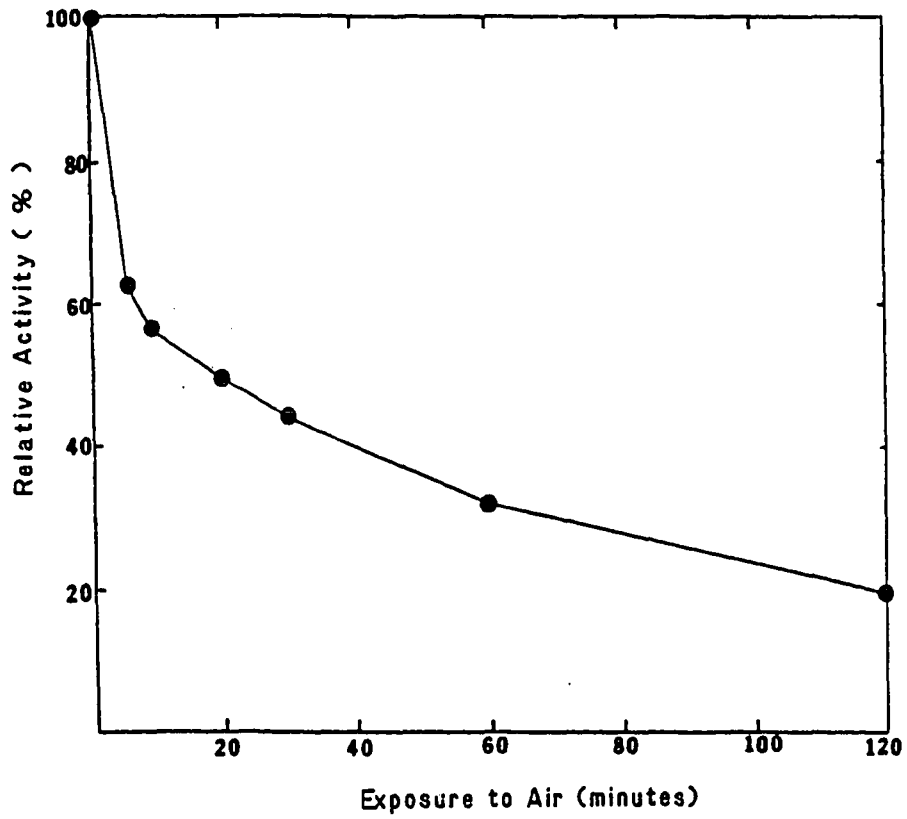with molecular oxygen.

Figure 13.   Effect of Air on the Activity of Fumarate
Reductase in Crude Extract.

In order to study whether such reducing agents are helpful in
preventing the loss in activity caused by oxygen, a variety of reducing
agents, dithiothreitol (DTT), dithioerythritol (DTE), 2-mercaptoethanol,
cysteine HCl and glutathione were transferred anaerobically to the
anaerobic chamber. The crude extract was incubated separately with each of
these reducing agents for 3 days. The final concentrations of these
reducing agents were 17.5 mM for 2-mercaptoethanol and 10 mM for the rest.
A control was prepared under identical conditions, except that buffer of
the same amount was added instead of the reducing agent. All the samples
were kept in stoppered and sealed serum vials. At the end of three days
the activities of fumarate reductase in all the samples were determined
following the routine assay procedure. The results shown in Figure 14
indicate that there is no adverse effect on the fumarate reductase activity
on incubation with any of the above mentioned reducing agents. In order to
compare the effect of reducing agents on exposure to air, the bottles with
the samples were then exposed to air for a period of 2.5 and 4 hours. The
results are also depicted in Figure 14.

When the crude extract was not incubated with reducing agents, about
80% of the activity was lost within 2.5 hours exposure to air. Although
none of the reducing agents was able to maintain 100% of the fumarate
reductase activity upon exposure to air, the loss in activity was greatly
reduced. All reducing agents showed a similar effect. At the end of 4
hours of air exposure, only 60% of the activity was lost. On the contrary,
the control lost most of the activity on 4 hours exposure to air. The
results confirmed the usefulness of the reducing agents in protecting the
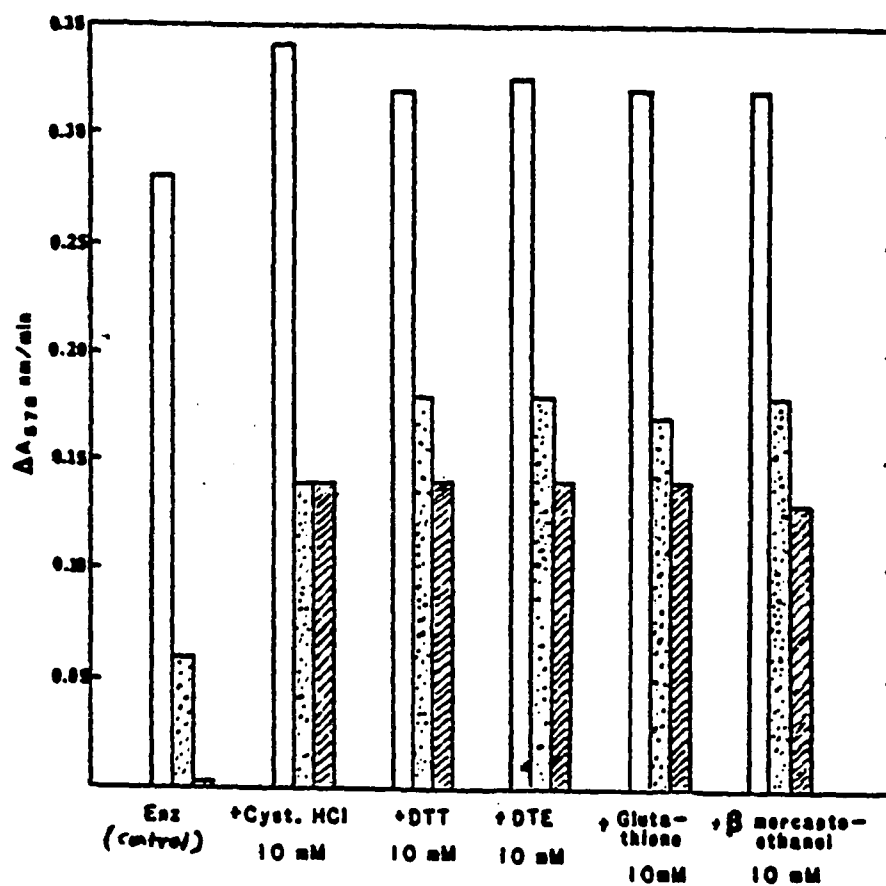fumarate reductase activity.

Figure 14. Effect of Various Reducing Agents on the Activity
of Fumarate Reductase. The bar graphs show
original activity after 3 days of anaerobic
incubation, followed by activity after 2.5 and
4.0 hours of exposure to air.

The above results were further confirmed by exposing the crude extract that was previously incubated with 5 mM cysteine HCl and 1 mM DTT to air for different time intervals. The activity remaining was then determined following the assay procedure as described earlier. The results are described in Figure 15.

Further studies were carried out to find out whether the loss in the enzyme activity could be recovered upon the addition of the reducing agents. Crude extract that did not have any added reducing agents was exposed to air for 2 hours. The bottle was then tightly stoppered and evacuated for 15 minutes. It was then pressurized with oxygen free nitrogen and incubated with 1 mM DTT and 5 mM cysteine HCl. The fumarate reductase activity was determined after 30 minutes, 1 hour and 2 hours of incubation. The results are outlined in Figure 16. It is unfortunate that the loss in fumarate reductase activity could not be recovered upon the addition of reducing agents. This indicates that the loss in activity is irreversible. However, as seen earlier, the loss in the activity slows down with the addition of reducing agents.

Taking into account the above results, all the buffers used during the purification of fumarate reductase contained 5 mM cysteine HCl and 1 mM DTT. The buffers were evacuated for 30 minutes and gassed with oxygen-free nitrogen for a few minutes before they were transferred to the anaerobic chamber.

Metal ions that are essential for the enzyme activity, if not tightly bound, may dissociate during the purification process. To investigate this, crude extract was passed through a Chelex 100 column that was previously degassed and flushed with oxygen-free nitrogen before its
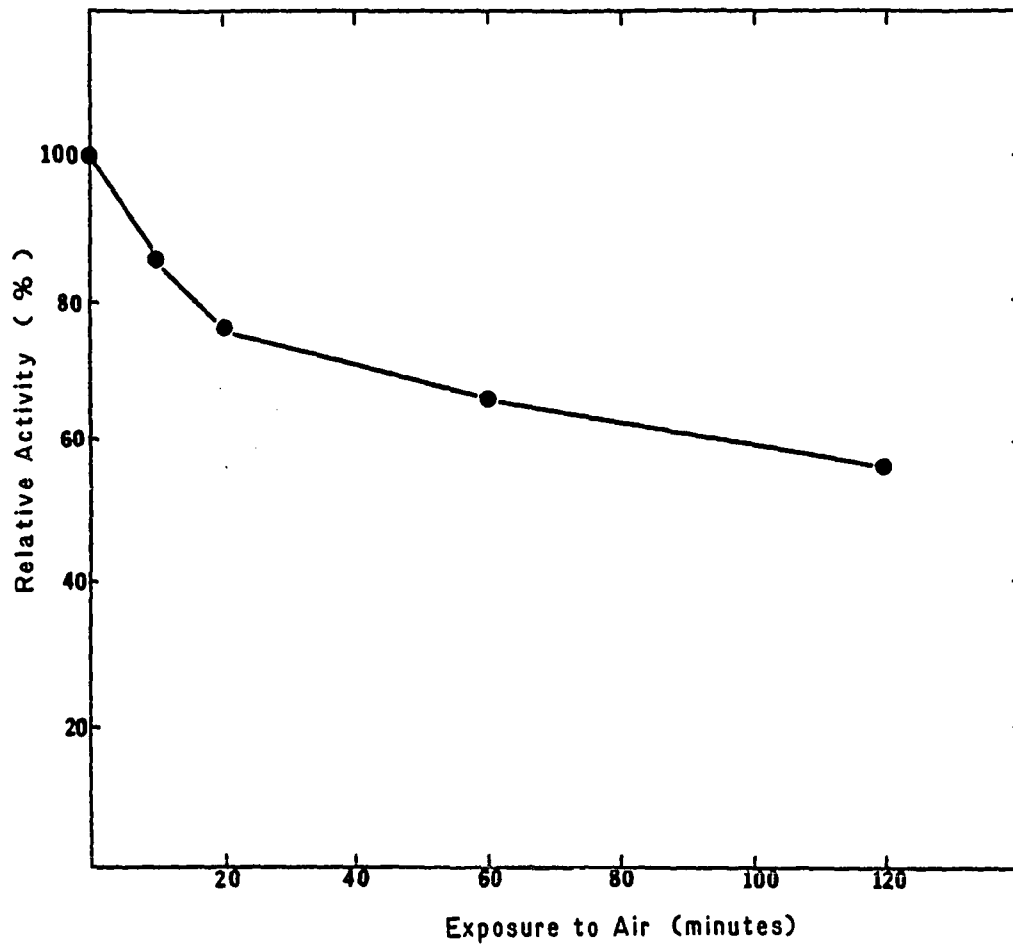
48

Figure 15. Effect of Air on the Activity of Fumarate Reductase in the Presence of DTT (1 mM) and Cysteine HCl (5 mM).
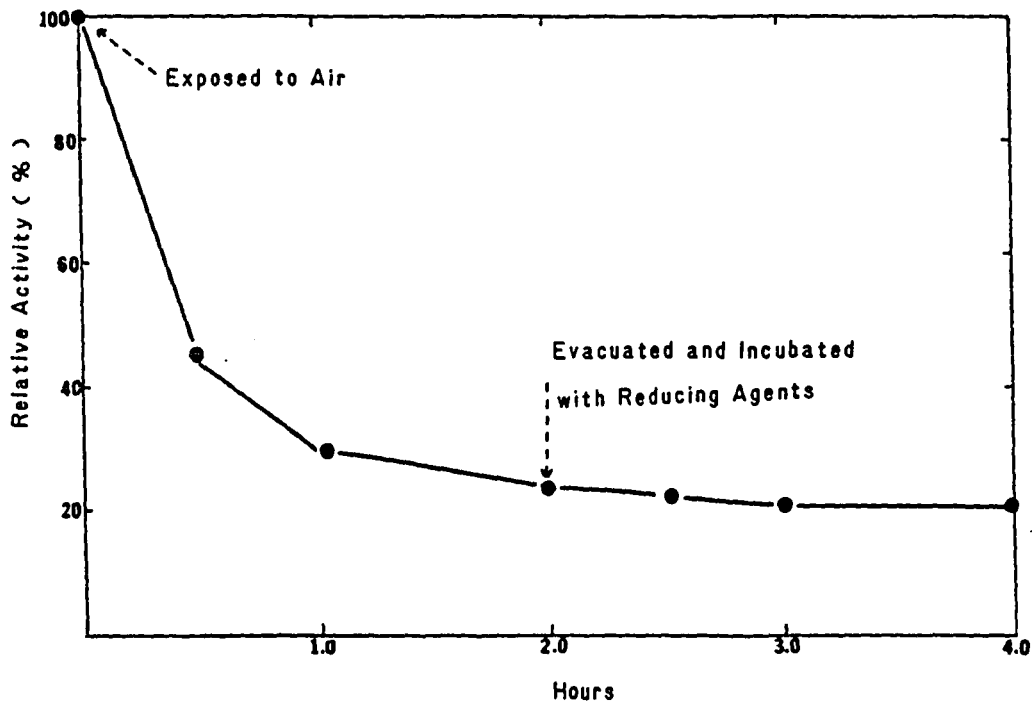
Figure 16. Irreversible Loss in the Fumarate Reductase Activity
on Exposure to Air.

transfer to the anaerobic chamber. The sample was eluted with 0.1 M sodium phosphate buffer, pH 7.0, with appropriate reducing agents. The sample eluted off the column was collected in the serum bottle. It was then stoppered, sealed, pressurized and analyzed for the fumarate reductase activity. Results indicated that all of the activity was maintained after passing the enzyme through the Chelex column. The Chelex 100 chelating ion exchange resin is extremely selective in chelating copper, iron, nickel, zinc, cobalt and other heavy metal ions (61). Since all of the fumarate reductase activity is maintained, it appears that if any of these metal ions is essential for activity, it must be tightly bound to the enzyme's active site.

EDTA is also a fairly strong chelating agent selective towards heavy metal ions. To check the stability of fumarate reductase in the presence of EDTA, the crude extract was incubated anaerobically with 1 mM EDTA for 4 days. A control was prepared under identical conditions which did not contain EDTA. Activity of fumarate reductase was tested in both the control and the EDTA sample. Results obtained indicated that fumarate reductase was stable in the presence of 1 mM EDTA. Consequently, 1 mM EDTA was routinely added to the assay buffer, as well as to the purification buffers, along with the other reducing agents mentioned earlier in order to protect the enzyme from reacting with trace quantities of various metal ions which would otherwise affect the enzyme activity.

Purification of Fumarate Reductase

Since fumarate reductase was most stable in an anaerobic environment, all purification steps were performed in an anaerobic atmosphere.

Heat Step

Seventy five ml of crude extract of M. thermoautotrophicum was transferred to a serum bottle inside the anaerobic chamber. The bottle was then stoppered, sealed, and pressurized (30 psi) with oxygen free nitrogen. This bottle was heated in an oven at 75°C for 24 hours. The crude extract was brought back to the anaerobic chamber and was transferred to stainless steel centrifuge tubes which were then tightly sealed. The tubes were centrifuged at 20,000 rpm for 45 minutes at 4°C. The centrifuged extract was opened inside the chamber. The supernatant solution and the pellet (dissolved in a small amount of assay buffer) were both tested for fumarate reductase activity. All of the activity was associated with the supernatant solution. Typically this represented 60-70% of the activity in the crude extract. A small amount of this supernatant was saved for protein determination, and the rest was subjected to DEAE-Sephacel chromatography.

DEAE-Sephacel Chromatography

The DEAE-Sephacel column (2.6 x 34 cm) was packed inside the anaerobic chamber. The column was equilibrated with 0.05 M sodium

phosphate buffer, pH 7.0, containing 1 mM DTT and 5 mM cysteine HCl. The
column was washed with at least two column volumes of the same buffer in
order to establish the proper anaerobic atmosphere. The flow rate was 46.0
ml/hr. The supernatant solution (62 ml) obtained following the heat step
was applied to this preequilibrated column using a peristaltic pump. A
salt gradient was used to elute the proteins. Initially a linear salt
gradient (500 ml total volume, 0.0 to 0.2 M NaCl in 0.05 M sodium phosphate
buffer) was applied. Fractions containing 6.0 ml/tube were collected. The
tubes were tightly stoppered and were immediately tested for fumarate
reductase activity. None of the fractions showed the presence of fumarate
reductase activity.

In order to elute the tightly bound fumarate reductase from the
column, a linear gradient using 0.2 and 0.4 M NaCl in 0.05 M phosphate
buffer (250 ml of each) was applied, which was followed by 0.4 M NaCl in
0.05 M phosphate buffer. Again the fractions were collected as explained
earlier. The activity was tested and was found to be present in fraction
numbers 164 through 175, i.e. at the end of the gradient. The absorbance
of the tubes was measured at 280 nm to obtain an elution profile of the
proteins. In order to protect the fumarate reductase from exposure to air,
0.1 ml of the sample was withdrawn using a syringe and was then diluted
with the column buffer before measuring the absorbance. Care was taken not
to expose these tubes to air. The tubes with fumarate reductase activity
were then pooled inside the chamber. Activity of the pooled sample was
determined, saving a small amount for protein determination. The elution
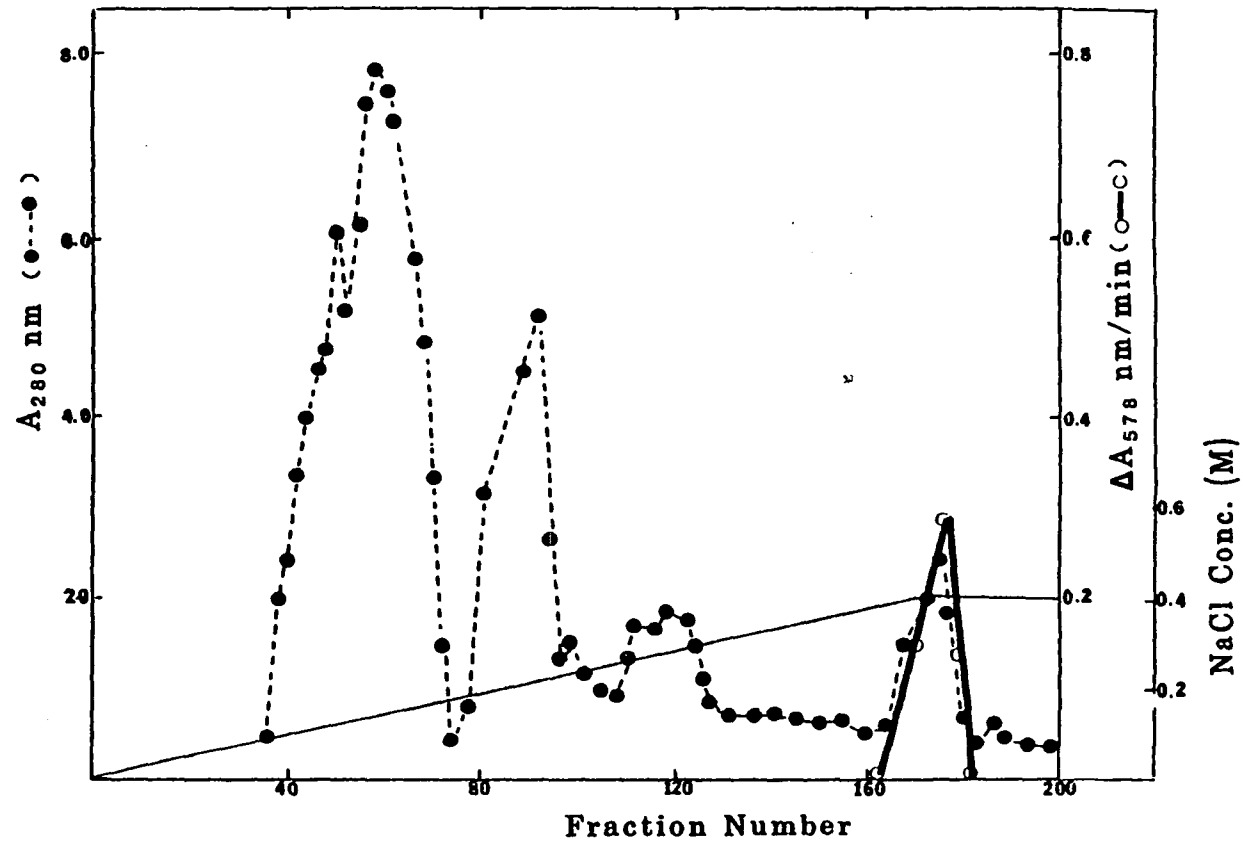profile for fumarate reductase is shown in Figure 17.

Figure 17. Elution Profile of Fumarate Reductase on DEAE-Sephacel Column
Following Heat Treatment.

Sephacryl S-200 (I) Chromatography

The pooled fractions (65 ml) from DEAE-Sephacel were transferred to an Amicon membrane filtration device (utilizing a PM-10 membrane) inside the anaerobic chamber. The enzyme was concentrated to 4.0 ml.

Two separate Sephacryl S-200 columns (1.6 x 26 cm each) were packed inside the anaerobic chamber using a peristaltic pump with a flow rate of 45 ml/hr. The columns were joined together using thick walled plastic tubing. The columns were equilibrated with 0.02 M sodium phosphate buffer, pH 7.0 containing 1 mM DTT and 5 mM cysteine HCl. The columns were washed with at least two column volumes of this buffer and then the concentrated fumarate reductase (4.0 ml) was applied to the column using the peristaltic pump. The proteins were eluted with the above mentioned buffer. The flow rate was 42 ml/hr; the fractions containing 2.5 ml/tube were collected. The tubes were stoppered and tested for fumarate reductase activity. The activity was found in fraction 20 through 31. The fractions were also tested for absorbance at 280 nm. The elution profile for fumarate reductase is shown in Figure 18. The active fractions (21 through 29) were pooled together and a small portion of the pooled solution was saved for the protein as well as the activity determination.

Hydroxyapatite Column Chromatography

The pooled fraction (25 ml) obtained following Sephacryl S-200 chromatography was concentrated to 4.2 ml.

A hydroxyapatite column (1.6 x 26 cm) was packed inside the anaerobic chamber and was equilibrated with 0.02 M sodium phosphate buffer, pH 7.0, containing 2 mM DTT, 5 mM cysteine HCl and 5 mM sodium fumarate. The column
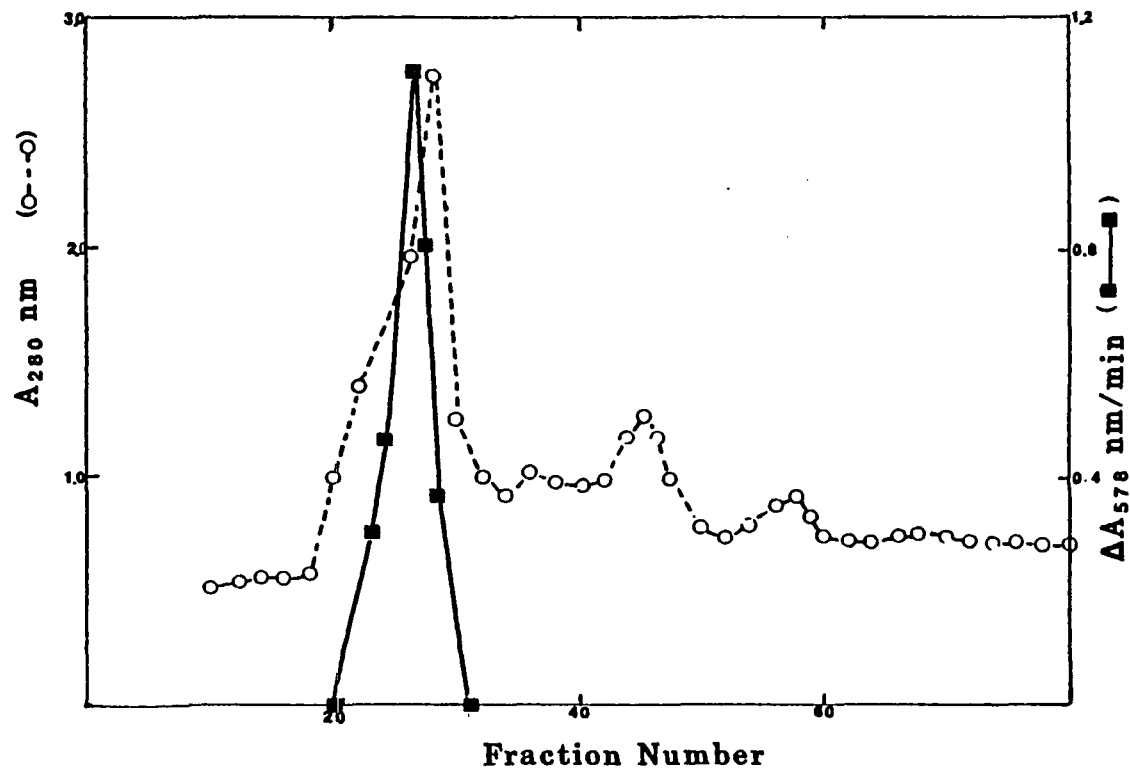
Figure 18. Elution Profile of Fumarate Reductase on Sephacryl S-200
Column Following DEAE-Sephacel Chromatography.

was washed with at least 2 column volumes using this buffer. The concentrated enzyme preparation (4.2 ml) was then applied to this column using the peristaltic pump and was then eluted with a linear phosphate gradient using 0.02 and 0.2 M sodium phosphate buffer, pH 7.0, containing 2 mM DTT, 5 mM cysteine HCl and 5 mM sodium fumarate. The flow rate was 46 ml/hr. Fractions of 2.7 ml/tube were collected using a fraction collector. The stoppered tubes were analyzed for fumarate reductase activity. The activity was detected in fraction numbers 72 - 86. The fractions were also analyzed for absorbance at 280 nm. The elution profile of fumarate reductase is depicted in Figure 19. The active fractions (73-85) were pooled together. A small amount of this solution was used for protein and activity determinations.

Sephacryl S-200 (II) Chromatography

The pooled sample of fumarate reductase from hydroxyapatite chromatography (32 ml) was once again concentrated to 4.2 ml and was then applied to a Sephacryl S-200 column (2 columns each of 1.6 x 26 cm) which had been previously equilibrated with 0.05 M sodium phosphate buffer, pH 7.0, containing 2 mM DTT and 5 mM cysteine HCl. The flow rate was 30.0 ml/hr. Fractions of 2.0 ml/tube were collected. The tubes were tightly stoppered and assayed for fumarate reductase activity and for absorbance at 280 nm. Enzyme activity was observed in fraction numbers 30 through 39. The elution profile is shown in Figure 20. Tubes 32 - 38 were pooled together and the protein concentration and the activity were determined.
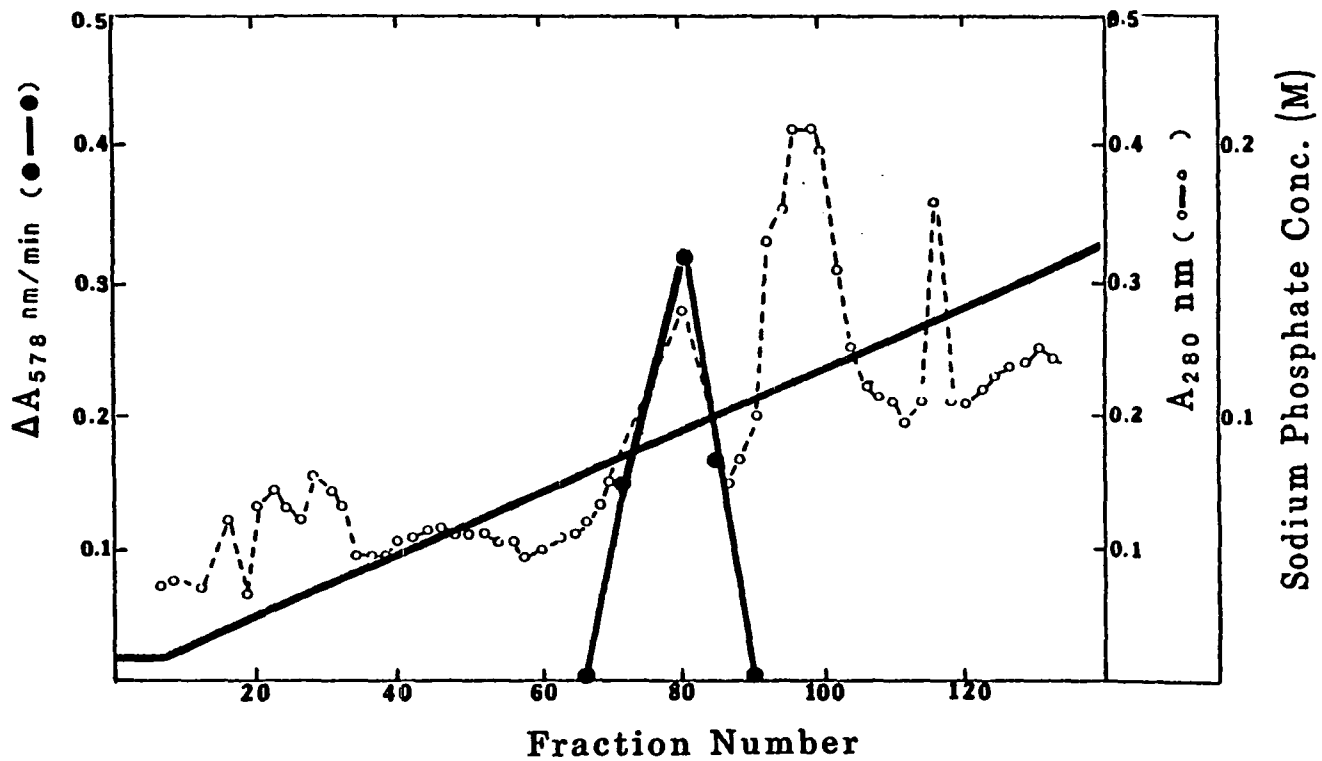
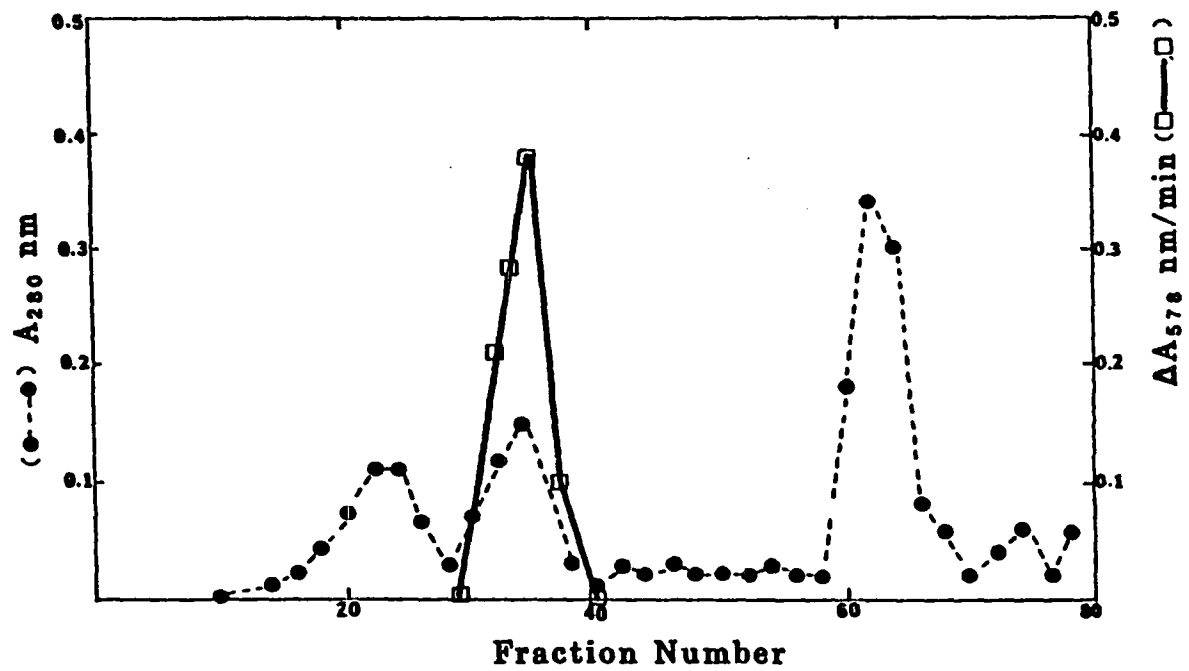Figure 19. Elution Profile of Fumarate Reductase on Hydroxyapatite Column.

Figure 20.   Elution Profile of Fumarate Reductase on Sephacryl S-200
Column (II) Following Hydroxyapatite Column.

Summary of Purification

The results of the purification are outlined in Table V. The heat step achieved about 2-fold purification, with a loss of about 35% activity. Similar results were obtained when crude extract was heated for a lesser time, but a 24 hour period was found to be satisfactory during purification.

DEAE Sephacel chromatography gave a much better purification, 24-fold, for a total purification of about 36-fold. The yield was also relatively high. The elution profile showed that fumarate reductase was tightly bound to the anion exchanger and only 0.4 M NaCl could elute it from the column. The pooled fractions were pale yellow in color at this stage of purification.

The Sephacryl S-200 (I) column principally served as a desalting column. For this purpose, the column was run with a relatively high flow rate (43 ml/hr.). The elution profile shows that proteins with similar molecular weights were eluted along with fumarate reductase, although some small molecular weight proteins were separated from fumarate reductase.

The hydroxyapatite column gave a very significant purification of 13-fold. The overall yield at this stage of purification was about 10%. The elution profile shows that fumarate reductase was eluted with 0.1 M sodium phosphate buffer. The enzyme at this stage was essentially colorless.

The final step of purification was Sephacryl S-200 (II)

TABLE V

PURIFICATION OF FUMARATE REDUCTASE

Purification Step	Total Protein (MG)	Total Activity (Units)	Specific Activity (Units/MG)	Purification Fold	Yield (%)
Crude extract	2625.0	2917.5	1.1	1.0	100
Heat (75°C for 24 hours, pH 7.0)	1240.0	2000.0	1.6	1.5	66
DEAE-Sephacel	42.3	1718.6	40.7	36.6	59
Sephacryl S-200 (I)	29.3	982.5	30.5	27.5	31
Hydroxy-apatite	1.0	396.8	389.0	350.5	10
Sephacryl	0.3	192.0	711.1	640.6	7

1 Protein was determined following the Bradford method using Bovine Serum Albumin as a standard protein.

2 One unit of activity is defined as the amount of enzyme necessary to cause a change in absorbance at 578 nm of 1.0 per minute at 75°C and pH 7.0 under the assay conditions.

chromatography which was used to remove the remaining contaminating proteins present and a 2-fold purification was achieved. The overall purification from original crude extract was 640 fold and the yield was 7%. It can be estimated that the fumarate reductase composed 0.16% Of the total protein in crude extract.

Polyacrylamide Gel Electrophoresis

Ten μg of purified enzyme following the second Sephacryl S-200 step was subjected to nondenaturing polyacrylamide gel electrophoresis (7% running, 4% stacking gel concentration). The results shown in Figure 21 indicate that the fumarate reductase was purified to homogeneity following the second Sephacryl S-200 column chromatography. For comparison polyacrlamide gel electrophoresis (10% running, 4% stacking) was also performed at various stages of purification. The results are shown in Figure 22. Since the samples were prepared under different conditions, they did not have a similar protein concentration.

Properties of Fumarate Reductase

Molecular Weight

In order to achieve a better resolution, 3 identical columns (each of 1.6 x 26 cm) joined in series were packed inside the anaerobic chamber with Sephacryl S-300 using the peristaltic pump. Each column was equilibrated with 0.1 M phosphate buffer, pH 7.0 containing 2 mM DTT, 5 mM cysteine HCl
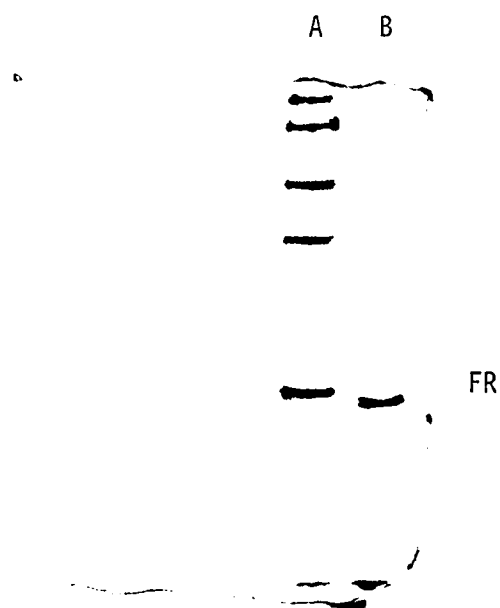
Figure 21. Non-Denaturing PAGE of the Purified Fumarate
Reductase. Lane A: Standard Mixtures of
Proteins Containing, Thyroglobulin (669 kd),
Ferritin (440 kd), Catalase (232 kd),
LDH (140 kd), and Albumin (67 kd).
Lane B: Purified Fumarate Reductase
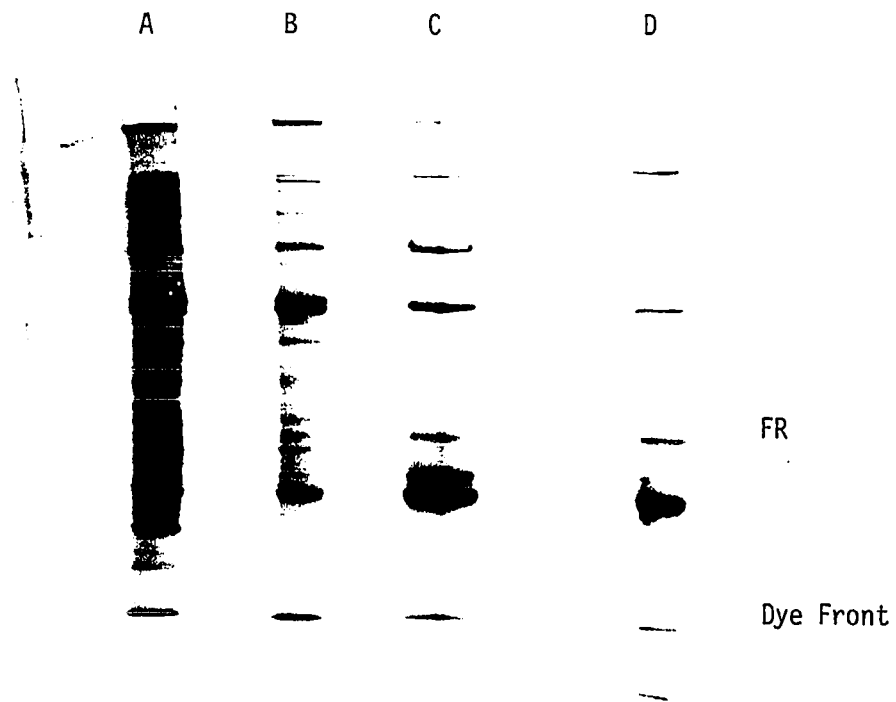Following Sephacryl S-200 (II) Column.

Figure 22: Non-Denaturing PAGE at Different Stages of
Protein Purification. Lane A: Crude Extract;
Lane B: Heat Treatment; Lane C: DEAE
Sephacel; Lane D: Hydroxyapatite.

and 0.4 M NaCl for at least 24 hours. The flow rate was 11 ml/hr. The void volume of the column was determined using 2.0 ml of blue dextran (conc. 5 mg/ml). Under the identical conditions, 2.0 ml of standard protein mixture containing 6 mg ovalbumin, 10 mg γ globulin, and 10 mg carbonic anhydrase was applied to the column and was eluted with the above mentioned buffer. The collected fractions were read at 280 nm to obtain an elution profile. Upon resolution of the above proteins, another set of protein standards, containing 10 mg aldolase and 10 mg myoglobin was eluted under the identical conditions. Finally 2.0 ml of partially purified fumarate reductase was applied to this standardized column maintaining the identical conditions. The fumarate reductase activity was tested in the eluted fractions. The ratio of Ve/Vo vs log molecular weight was plotted. The results are summarized in Figure 23. From the graph it is evident that the molecular weight of fumarate reductase, as determined by gel exclusion chromatography, is approximately 78,000.

Subunit Molecular Weight

Purified fumarate reductase (3 ml) was concentrated to 0.2 ml using a Centricon filtration device. Ten µg of concentrated fumarate reductase was boiled in the presence of 5% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol for 10 minutes. The sample was then subjected to SDS polyacrylamide gel electrophoresis employing the method of Hames (59). Figure 24 shows the presence of 4 bands each of almost equal intensity. A plot of relative mobility of the molecular weight markers and enzyme to bromphenol blue is shown in Figure 25. Four bands each of 22 kd, 40 kd, 58 kd and 78 kd are seen, with the major one at 22 kd. Since the native
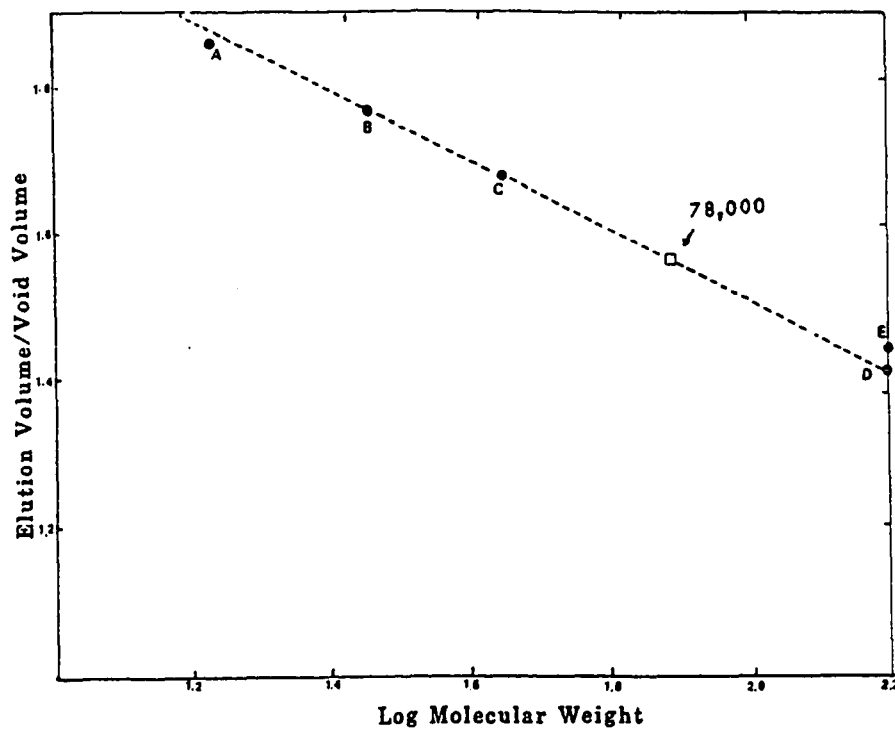
Figure 23.   Molecular Weight Determination of Native Fumarate
             Reductase Using Sephacryl S-300 Column. A
             standard mixture of proteins contained; A, Myoglobin
             (17 kd), B, Carbonic Anhydrase (29 kd); C, Ovalbumin
             (45 kd); D, γ Globulin (158 kd); E, Aldolase (158 kd).
             □ , Fumarate Reductase.
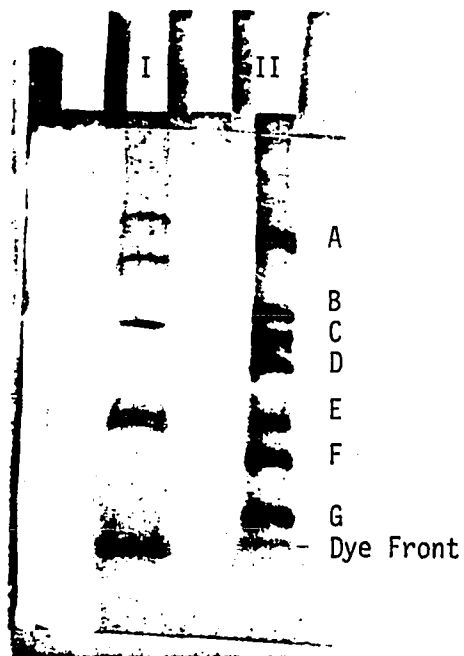
Figure 24. SDS-PAGE of the Purified Fumarate
Reductase. Lane I: Fumarate Reductase.
Lane II: Standard Protein Mixture
containing, A, BSA (68 kd); B, Ovalbumin
(43 kd); C, Glyceraldehyde 3-Phosphate
Dehydrogenase (36 kd); D, Carbonic
Anhydrase (29 kd); E, Trypsinogen (25.7
kd); F, Trypsin Inhibitor (20 kd) and
G, α-Lactalbumin (14 kd).

Figure 25. Calibration Curve Established Using Low
Molecular Weight Standard Proteins on
SDS-PAGE. Standards A through G are the
same as explained in Figure 24.

molecular weight of fumarate reductase is around 80,000, the results

obtained following the SDS gel electrophoresis indicate that fumarate

reductase is composed of four subunits of 20 kd each; the higher molecular

weight components being due to incomplete dissociation.

Optimum Temperature

The assay buffer was added to the Bausch and Lomb Spectrophotometric

tubes inside the anaerobic chamber. The tubes were tightly stopppered and
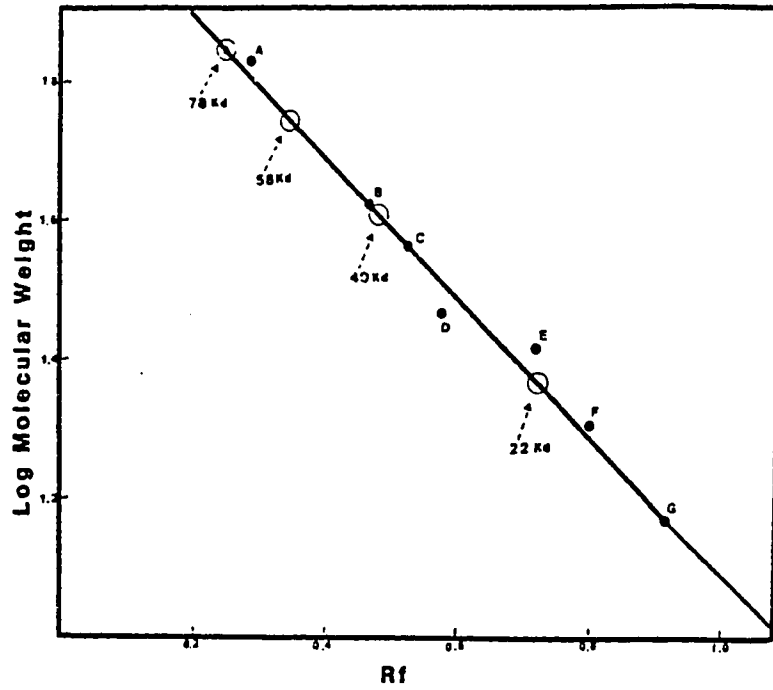
were removed from the chamber. The tubes were then incubated in the

presence of oxygen-free nitrogen at various temperatures ranging from

40°C-90°C for a period of 15 minutes. The assays were performed as

indicated in the Experimental Section at the respective incubation

temperature. The results are outlined graphically in Figure 26. The

activity increased with temperature, as expected, to a maximum of

approximately 75°C. The activity decreased rapidly on increasing the assay

temperature above 70°C presumably due to the heat-denaturation. The enzyme

was totally inactive at 90°C.

Optimum pH

In order to check the optimum pH for fumarate reductase, various

buffers, namely: sodium citrate (0.05 M) pH 4.5-6.5; sodium phosphate (0.1

M) pH 6.5-8.0; MOPS (0.05 M), pH 7.0-8.0; and Tris HCl (0.1 M) pH 7.5-9.0,

all containing 5 mM cysteine HCl and 2 mM DTT, were evacuated for 30

minutes and then transferred to the anaerobic chamber. The assay tubes

containing buffers at various pH were incubated at 75°C for 15 minutes in

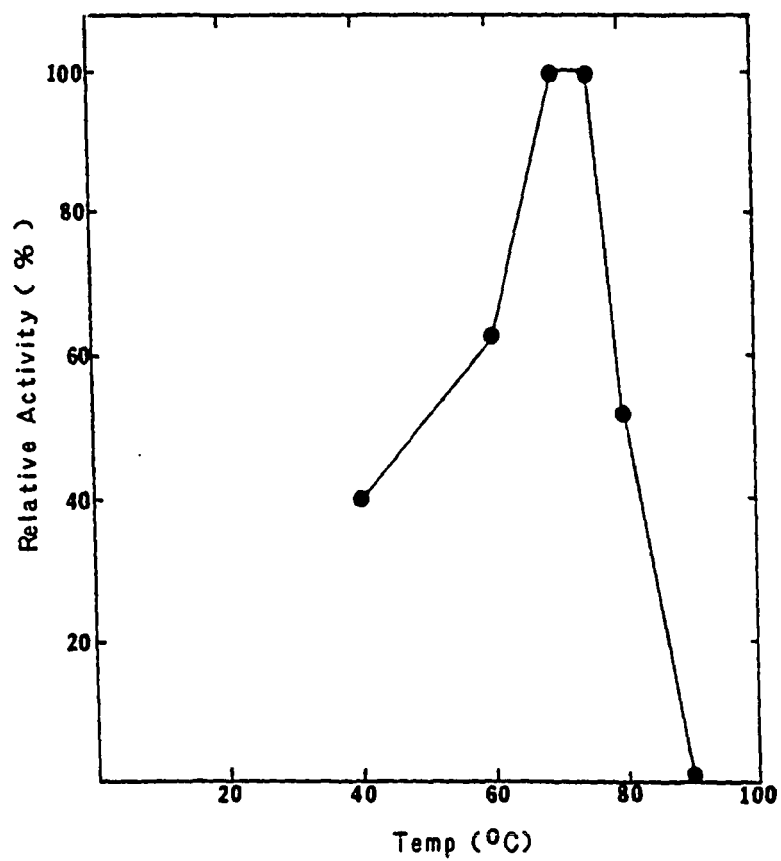the presence of oxygen-free nitrogen. The activity of fumarate reductase

Figure 26. Effect of Temperature on the Activity of
Fumarate Reductase.

was studied following the routine assay explained in the Experimental Section. The results are depicted in Figure 27. It is apparent that the optimum pH for fumarate reductase under the assay conditions is close to 7.0.

Determination of K_m for Fumarate

Assays were performed as explained earlier, except that the concentration of sodium fumarate in the assay mixture was varied. The Figure 28 gives the values for K_m for fumarate which is the true substrate for fumarate reductase. The K_m was found to be 0.2 mM. Higher concentrations of fumarate did not inhibit fumarate reductase. The turnover number was calculated to be 916 mn^{-1}.

Effect of Metal Ions

Enzyme preparation obtained following the DEAE Sephacel chromatography was anaerobically incubated for 30 minutes in the presence of various metal ions. The metal ions used were, $Fe(NH_4)_2SO_4$ 6 H_2O; $CoCl_2$ 6 H_2O; $NiCl_2$ 6 H_2O; $FeSO_4$ 7 H_2O; $MgCl_2$ 6 H_2O; $MnSO_4$ H_2O; $CaCl_2$ 2 H_2O; Na_2SeO_4, and $CuCl_2$ 2 H_2O. The final concentration of each these compounds in the incubation mixture was 5 mM. The assay was performed as explained in the Experimental Section. The results are shown in Figure 29 . None of the metal ions except Cu(II) affected the enzyme activity. Incubation with copper caused 80% loss in the fumarate reductase activity.

Effect of Sulfhydryl Inhibiting Reagents

Partially purified fumarate reductase obtained following step II of
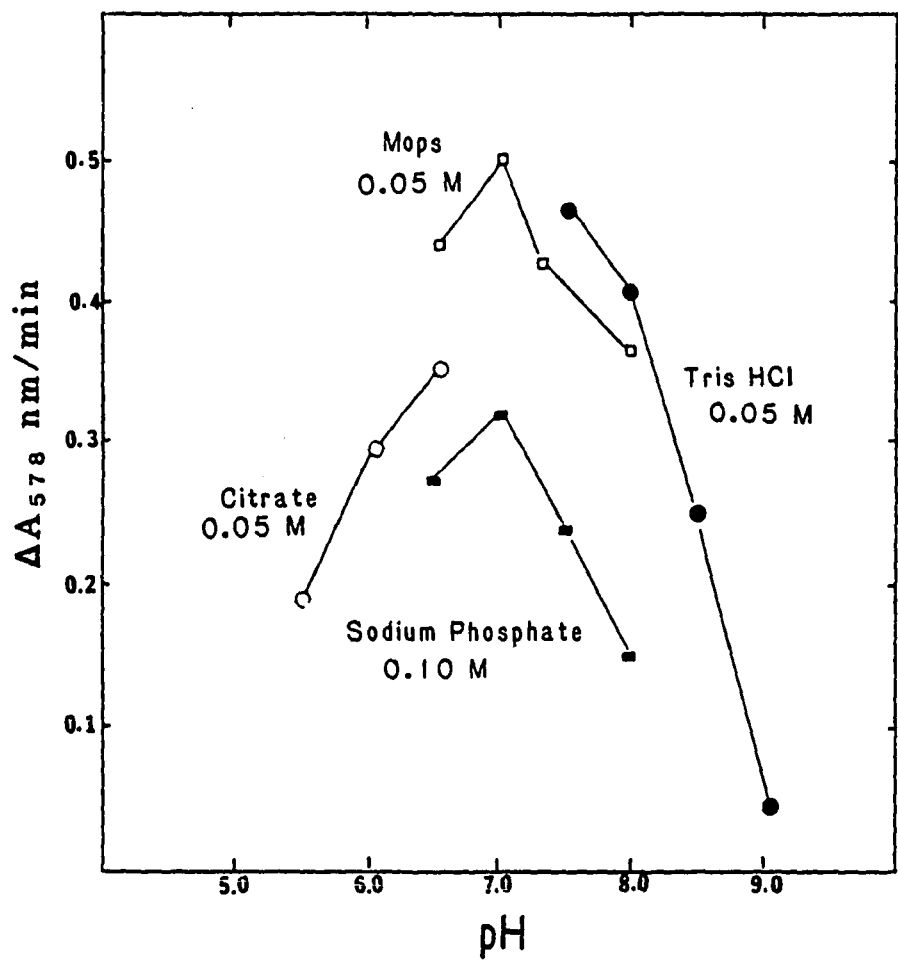
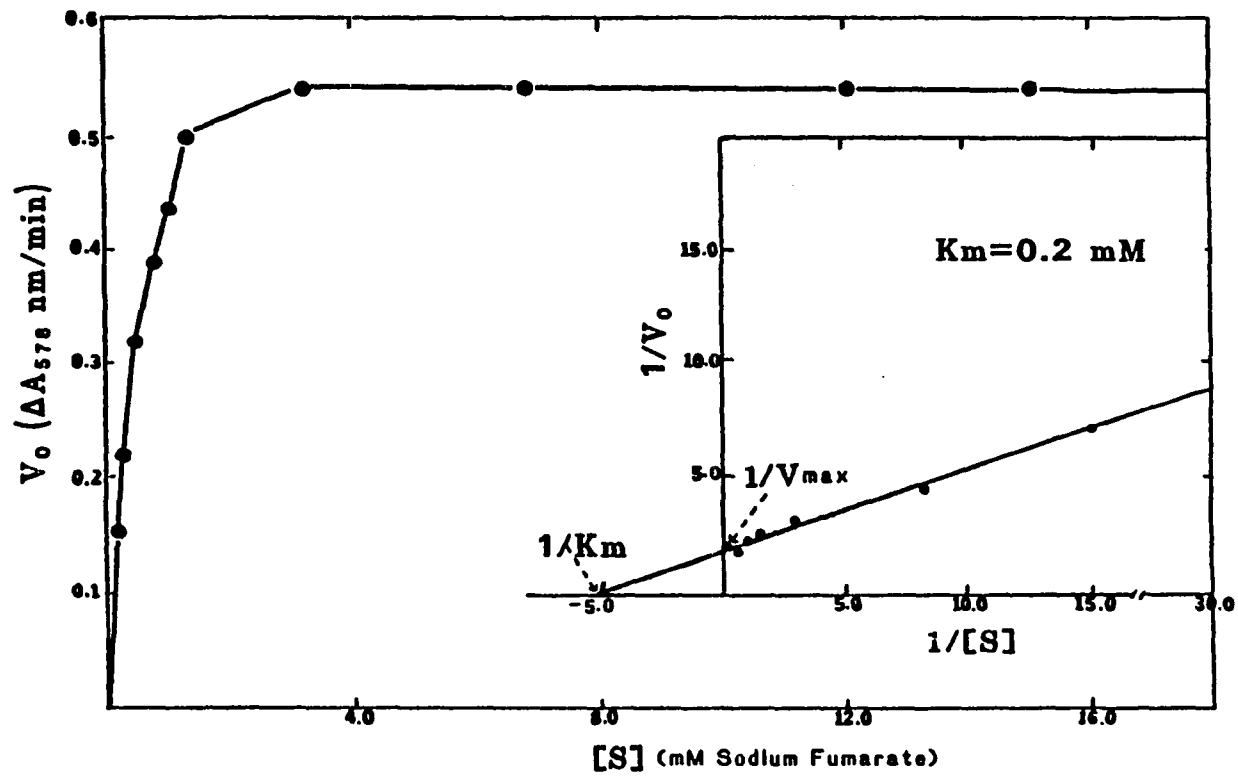Figure 27. Effect of pH on the Activity of Fumarate Reductase.

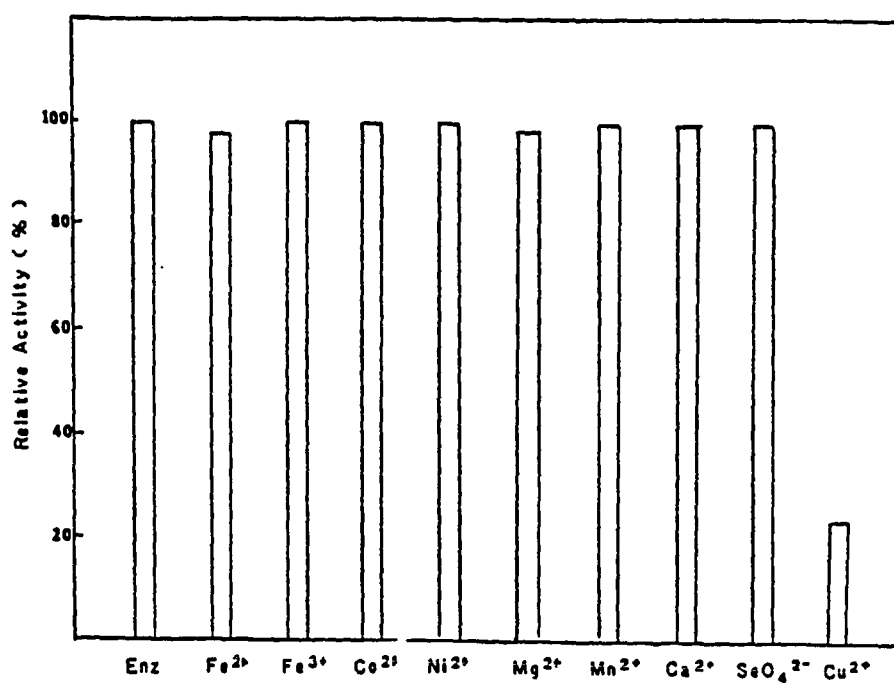Figure 28. Kinetic Parameters of Fumarate Reductase.

Figure 29. Effect of Various Metal Ions on the Activity of
Fumarate Reductase Obtained Following
DEAE-Sephacel Chromatography.

purification was incubated anaerobically in the presence of 5 mM

iodoacetamide or 5 mM para hydroxy mercury benzoate (pHMB). The activities

remaining after 2 hours of incubation were compared with the control which

did not contain any of these reagents. The results are shown in Figure

30. Both reagents inhibited about 60% of the enzyme activity indicating

the involvement of sulfhydryl groups at or near the active site of the

enzyme. When the enzyme was incubated in the presence of both

iodoacetamide (5 mM) and sodium fumarate (5 mM), inhibition was not noticed

(Figure 30). This implies that binding of fumarate protects enzymatic

groups which are inactivated by iodoacetamide.

These results were further confirmed with the purified preparation of

fumarate reductase. Purified fumarate reductase was incubated with 1 mM

iodoacetamide in the assay mixture at 75°C for a period of 5, 10 and 20

minutes respectively. The reaction at the end of each incubation period

was started with the addition of sodium fumarate. The results are depicted

in Figure 31. The loss in enzyme activity on incubation with iodoacetamide

was time dependent. About 80% of the activity was lost on 20 minutes

incubation with 1 mM iodoacetamide.

Attempts to study the effect of pHMB on purified fumarate reductase

in the assay mixture failed since Hg precipitated as HgS due to the

presence of reducing agents in the assay mixture.

Effect of Exposure to Air

Purified fumarate reductase was exposed to air for a period of 5, 10,

30, and 60 minutes. The activity remaining was studied following the

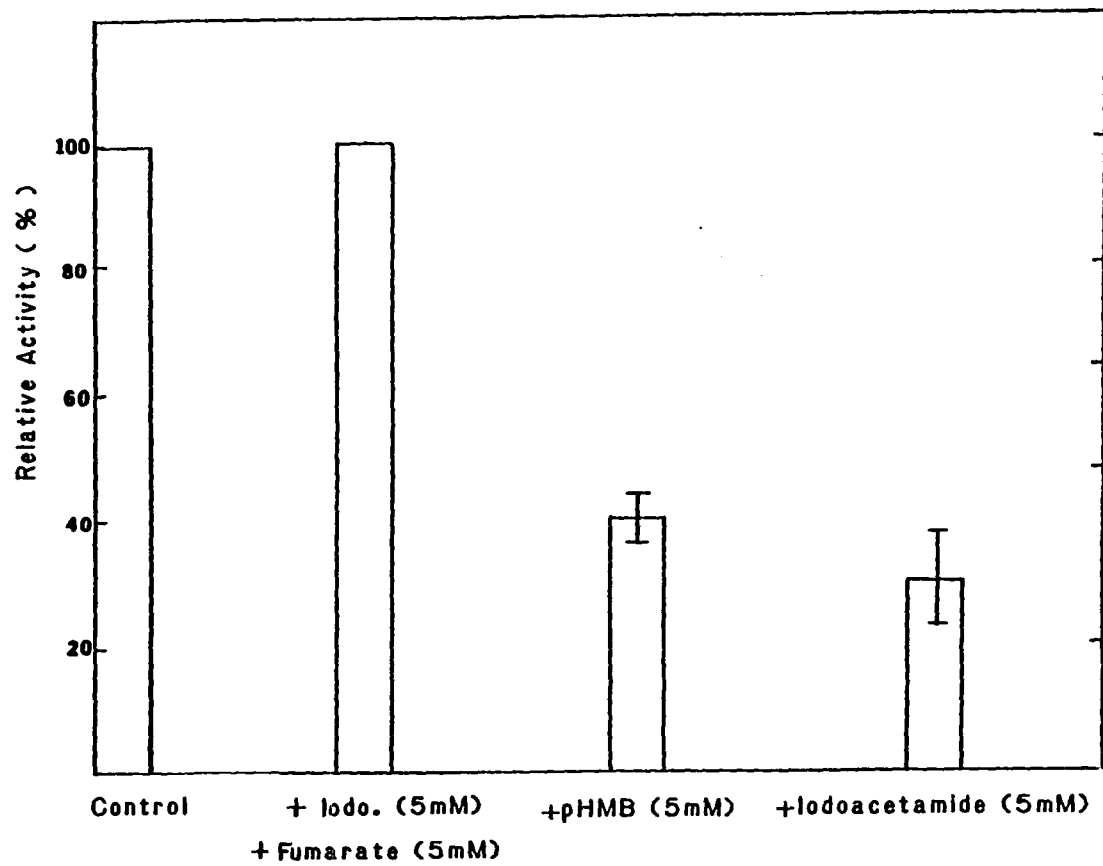routine assay procedure. The results are shown in Figure 32. Results

Figure 30. Effect of Sulfhydryl Inhibiting Reagents on the Activity of Fumarate Reductase Obtained Following Heat Treatment.
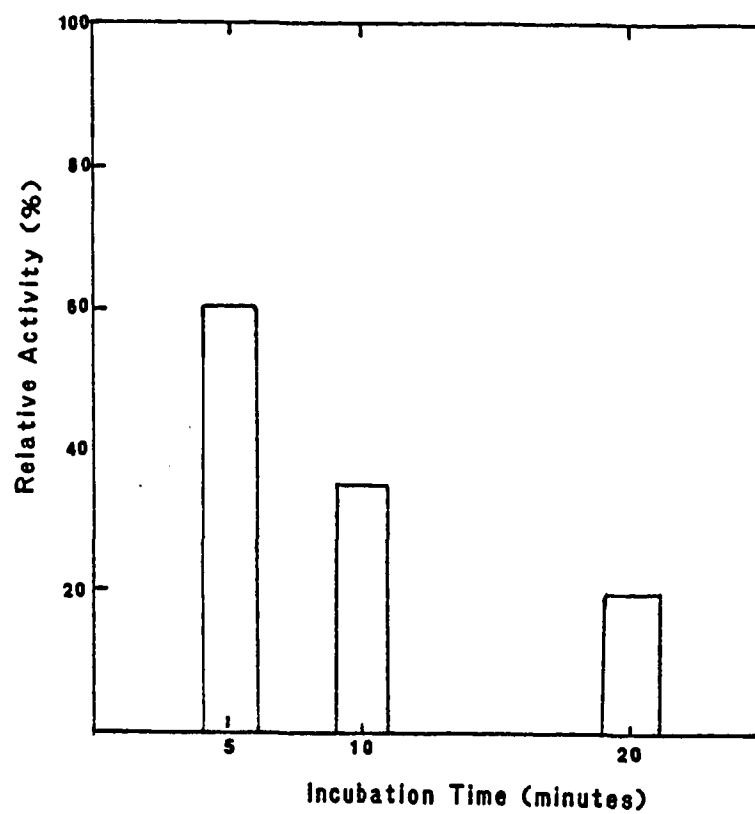
Figure 31. Effect of Iodoacetamide (1 mM) on the Activity of Purified Fumarate Reductase.
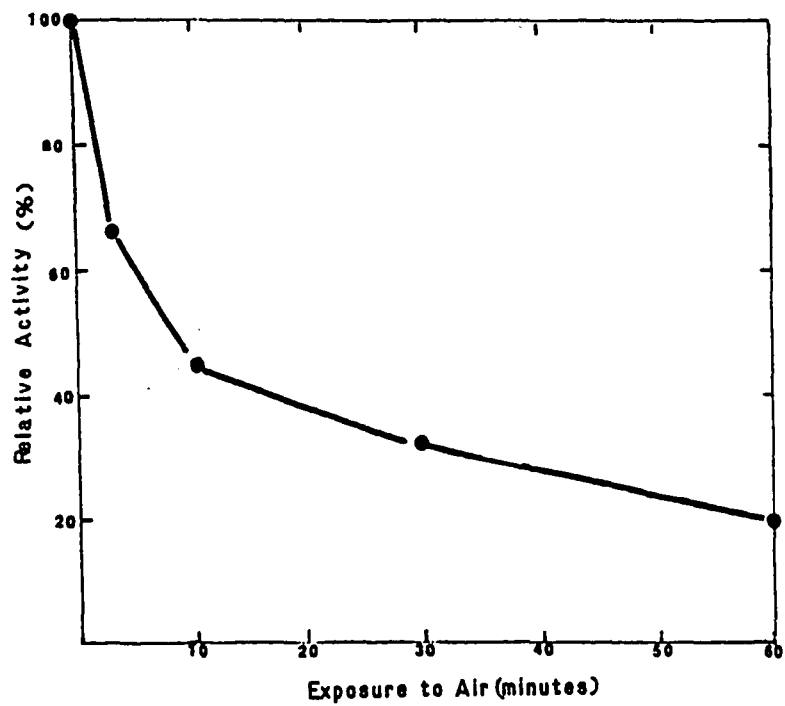
Figure 32. Effect of Air on the Activity of Purified Fumarate Reductase.

indicate a similar pattern in the loss of fumarate reductase activity that was noticed with the crude extract, except that, upon purification fumarate reductase became more susceptible to oxygen inactivation.

Absence of Succinate Dehydrogenase (SDH) Activity

Both partially purified (following step (II) of purification) and purified fumarate reductases were tested for SDH activity using potassium ferricyanide as an electron acceptor (61a). Results indicated tnat under the assay conditions employed, succinate is not oxidized to fumarate. Of course, this only means that ferricinide is not an appropriate electron acceptor for this reaction.

Fumarate as an Electron Acceptor for Growth of M. thermoautotrophicum

The redox potential of fumarate-succinate couple is high enough (30 mV) to accept electrons from hydrogen (62). However, even in the presence of 50 mM sodium fumarate and 30 psi hydrogen, cells of M. thermoautotrophicum refused to grow. The growth was monitored for a period of seven days. This clearly indicates that, like E. coli and V. succinogenes, M. thermoautotrophicum cannot use fumarate as an electron acceptor, suggesting the role of fumarate reductase to be biosynthetic.

UV/Visible Spectrum of Fumarate Reductase

The UV/visible spectrum (Figure 33) of fumarate reductase was recorded in a stoppered cuvette under a nitrogen atmosphere using a Cary 14 spectrophotometer. No absorbance was observed in the visible region. The UV spectrum showed a presence of absorption maxima at 310 nm and at 280 nm
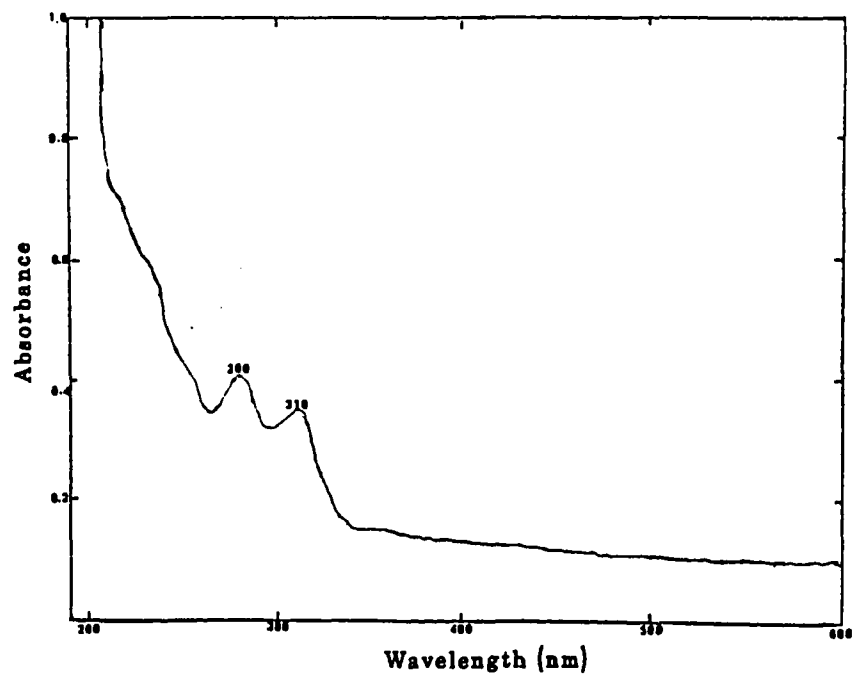
Figure 33.   UV/Visible Spectrum of Fumarate Reductase.

with shoulders at 260 nm and 230 nm. In order to check the effect of oxygen, the cuvettes were exposed to air. Due to the presence of reducing agents, the changes in the spectrum were visible only after prolonged exposure (more than 24 hours) to air. The ratio of 310/280 decreased upon exposure to air. No absorbance was observed in the visible region even after 4 days of exposure to air.

Fluorescence Spectrum of Fumarate Reductase

The fluorescence spectrum of fumarate reductase was determined using an Aminco-Bowman Spectrophotofluorometer (model No. 4-8202). Due to the unavailability of anaerobic cuvettes, the fluorescence spectrum was recorded on fumarate reductase that was exposed to air for about five minutes. The fluorescence spectrum is shown in Figure 34. The excitation spectrum (for 410 nm emission) showed 235 nm and 295 nm peaks while the emission spectrum (for excitation at 295 nm) showed a 410 nm peak.
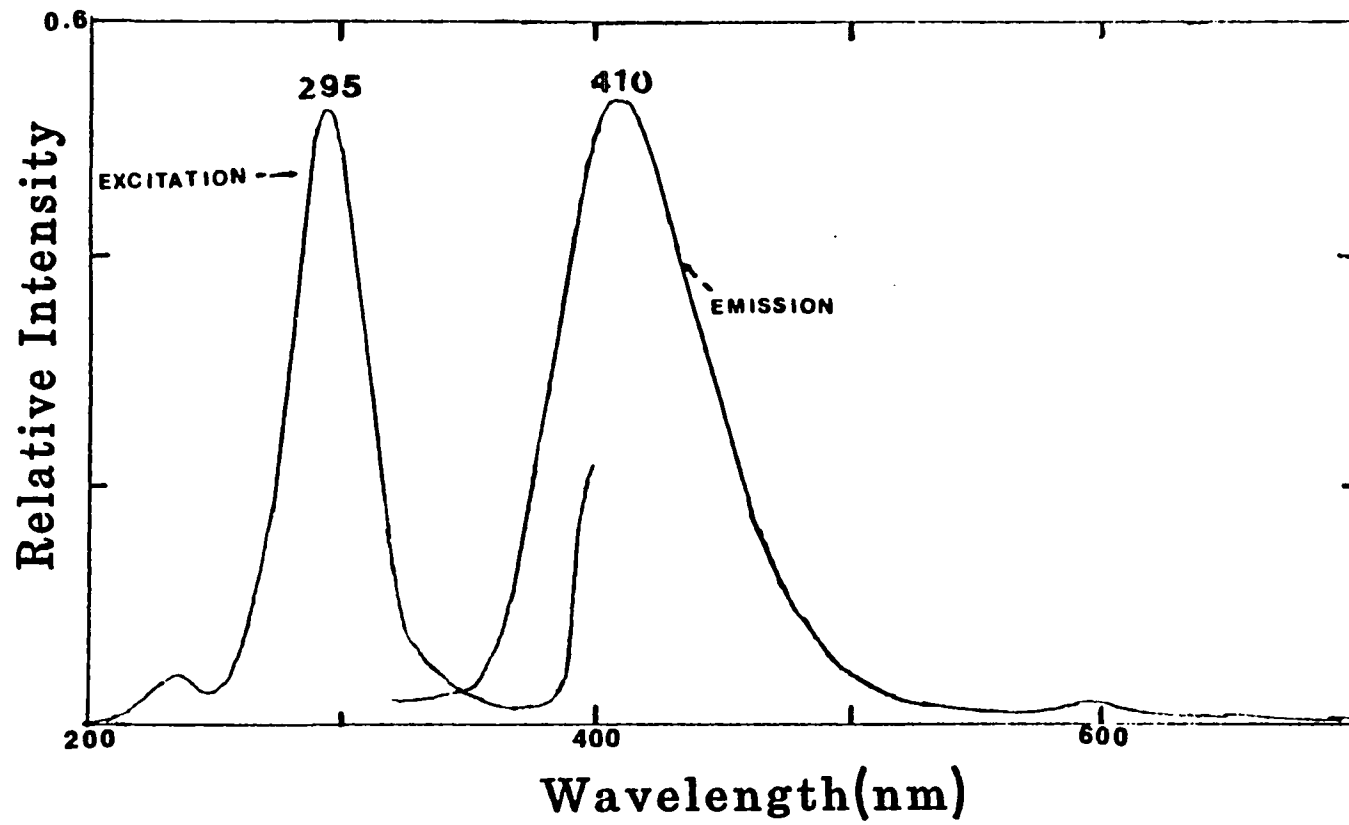
Figure 34. Fluorescence Spectrum of Fumarate Reductase.

DISCUSSION

This is the first detailed study on fumarate reductase from the
methanogenic bacteria. We have been able to purify fumarate reductase to
homogenity and have determined the properties of fumarate reductase that
are listed in Table VI.

Comparison of Fumarate Reductases from Various Sources

It has been established that when E. coli is grown anaerobically on
fumarate and glycerol-3-phosphate, fumarate serves as a terminal electron
acceptor, whereas glycerol-3-phosphate serves as a source of carbon (62).
Under these circumstances, fumarate reductase is induced. This
membrane-bound fumarate reductase has been purified and characterized by
Dickie and Weiner (63). Its properties are summarized in Table VII. Unden
et al (64) as well as He et al (personal communication) have reported the
isolation and purification of similar membrane-bound fumarate reductase
from Vibrio succinogenes and Desulfovibrio multispirans respectively. The
properties of this enzyme are also given in Table VII. Preliminary studies
were also carried out on the membrane-bound fumarate reductases from
Clostrodium formicoaceticum (47) and Proteus mirabilis (46).

On the contrary, fumarate reductases isolated from brewers yeast (65)
and bakers yeast (49) were found to be in the cytosol fraction of the

TABLE VI

A SUMMARY OF PROPERTIES OF FUMARATE REDUCTASE PURIFIED
FROM METHANOBACTERIUM THERMOAUTOTROPHICUM

1) Molecular Weight   approx. 78,000 dalton

2) Subunit Mole. Wt.   approx. 20,000 dalton

3) No. of Subunits   four

4) Temperatutre Optimum   approx. 75°C

5) pH Optimum   approx. 7.0

6) Km for Fumarate   0.2 mM

7) Presence of at least one -SH group in the vicinity of the active site of enzyme

8) Flavins are absent

9) Possible involvement of compound similar to that of tetrahydromethanopterin, or F_{420} as cofactor of fumarate reductase. Possibility of a new cofactor can also not be ruled out

10) Fumarate reductase seems to be involved in the biosynthetic reactions

TABLE VII

PROPERTIES OF FUMARATE REDUCTASES ISOLATED FROM VARIOUS SOURCES

Source	Location	Mol.Wt.	Total No. of subunits	Subunit mol.wt	Cofactor	K_m for Fumarate	Function	Reference
Vibrio succino-genes	membrane	121 kd	2	80 kd 30 kd	cov. FAD	0.35 mM	catabolic	64
E. coli	membrane	100 kd	2	69 kd 24 kd	cov. FAD	0.42 mM	catabolic	63
Desulfo-vibro Multispirans	membrane	118 kd	4	45 kd 32 kd 30 kd 27 kd	noncov. FAD	2.50 mM	catabolic	personal communication
Brewers yeast	cytosol	62 kd	1	62 kd	noncov. FAD	not detected	biosynthetic	65
Bakers yeast	cytosol	58 kd	1	58 kd	noncov. FAD	0.20 mM	biosynthetic	49
M. thermo-autotrophicum	cytosol	80 kd	4	20 kd	unusual, could be sim to F_{420} or H_4MPT	0.20 mM	biosynthetic	

cells. The properties of these enzymes are also summarized in Table VII. These enzymes, with few exceptions, exhibit many different properties from the membrane bound fumarate reductases mentioned earlier, but they have in common the use of FAD as a cofactor. The enzyme from M. thermoautotrophicum is clearly a different protein as judged by its cofactor requirement and also by its molecular weight.

Purification of Fumarate Reductase from M. thermoautotrophicum

Like the many other enzymes isolated (60) from methanogens, fumarate reductase of M. thermoautotrophicum is very oxygen-sensitive. This often caused difficulty in obtaining a suitable yield of the purified enzyme. Reducing agents helped in protecting against loss in the enzyme activity, provided that a strict anaerobic environment was maintained. Furthermore, unlike the results Nozaki et al (66) observed, the inactivated enzyme could not be reactivated by incubation with reducing agents under anaerobic conditions. For this reason, as a routine, the entire purification was performed inside the anaerobic chamber using reducing agents. This was somewhat cumbersome, especially when packing the columns inside the chamber. It was also necessary to plan the entire purification ahead of time. Since time was a critical factor, all the purifications, once started, were completed within a period of 4 days.

Since the enzyme was to be isolated from a thermophilic bacterium, we took advantage of a heat step for purifying fumarate reductase. Jones and Stadtman (67) have also reported the use of a heat step while purifying formate dehydrogenase from M. vannielii. The reason for heating fumarate

for 24 hours was that it gave enough time for getting ready for the purification. DEAE Sephacel gave good purification and the yield was also fairly high. During earlier purification we used DEAE cellulose, however, DEAE Sephacel gave a far better purification. The enzyme was eluted with 0.4 M NaCl, indicating the tight binding of fumarate reductase to the anionic exchanger. This could have been due to the presence of excess acidic amino acids which gave the protein a net negative charge at pH 7.0. The PAGE results shown in Figure 21 also support this finding. The Sephacryl S 200 (I) column chromatography, perhaps because of a high flow rate, did not give the expected resolution. It also resulted in considerable loss in specific activity. We have ruled out the possibility of removal of an essential cofactor based upon our results with Sephadex G-25 chromatography, dialysis, and membrane filtration.

Hydroxyapatite (HA) chromatography is known to separate complex mixtures when all other separation methods fail (61). In this purification hydroxyapatite proved immensely valuable, as it gave an excellent overall purification. The HA column was equilibrated and eluted with the buffer containing 5 mM sodium fumarate. This seems to have stabilized the enzyme (64). The final Sephacryl S-200 step improved the purification and resulted in a homogenous preparation of the enzyme. The overall yield after this step was 7%. Attempts to obtain a better yield were hampered on other occasions for various reasons. Other methods of purification were also attempted, including ammonium sulfate precipitation, alcohol precipitation, cellulose phosphate ion exchange chromatography, phenyl sepharose chromatography, and Sephadex G-200 chromatography were also attempted. These methods did not provide better results either due to a

loss in yield, specific activity, or both.

Properties of Fumarate Reductase in M. thermoautotrophicum

The molecular weight of fumarate reductase, as obtained using Sephacryl S-300 chromatography, is approximately 80,000. This is small compared to those of V. succinogenes , E. coli, and Desulfovibrio multispirans (Table VII). The SDS gel electrophoresis showed four bands with approximate molecular weights of 20,000, 40,000, 60,000 and 80,000. We attribute this to the aggregation of subunits, and thus we propose that fumarate reductase is a tetramer joined together with four identical polypeptides.

The temperature optimum for fumarate reductase is around 75°C. This property made it easier to perform all purification steps at room temperature. The high temperature stability of fumarate reductase is not very surprising considering that the enzyme isolated was from a thermophilic organism, although very few bacterial enzymes have such a high temperature optimum. Alber et al (68) have used phage T4 lysozyme as their model to study the protein thermal stability. Their studies based upon site directed mutagenesis revealed that various factors such as hydrogen bonding, Van der Waal's interaction, ion pair interaction, dipole moment, covalent cross linkages, binding of ligands, and disulfide bonds are potentially important for the thermal stability of proteins. We feel that the presence of disulfide bonds, along with other (unknown) factors are responsible for the thermal stability of fumarate reductase.

The K_m for fumarate is 0.2 mM. Zeikus et al (38), based on their preliminary studies with a crude extract of fumarate reductase from M. thermoautotrophicum, determined the K_m for fumarate to be 0.9 mM. Their assay conditions, however, were substantially different from ours. Dickie and Weiner (63) reported an apparent K_m near 0.42 mM for the enzyme from E. coli, whereas Unden et al (64) reported an apparent K_m of 0.35 mM for the enzyme from V. succinogenes. Interestingly enough, the fumarate reductase from Baker's yeast exhibits a K_m of 0.2 mM for fumarate. Thus, it is interesting to note that fumarate reductases, purified from different organisms have K_m values in a similar range.

The inhibition of fumarate reductase due to Cu(II) suggests oxidation at the active site of fumarate reductase. Cu(II) is known to interact with thiol or imidazole group(s) (61,69); inhibition due to copper indicates the possible involvement of such group(s) at or near the active site. Air exposure studies also indicate that at least one thiol group is important for maintaining the activity, since on exposure to air there is a rapid loss of activity. The thiol group of cysteine is known to react with alkyl halides as well as mercuric reagents (61,69). Incubation with iodoacetamide or para hydroxy mercury benzoate caused significant inhibition of the fumarate reductase. Similar results were obtained by Yamazaki and Tsai (70), Robinson and Weiner (71) and Muratsubaki and Katsume (65) with fumarate reductases of other organisms. We were unable to obtain a 100% inhibition on incubation with these reagents because of the presence of reducing reagents in the enzyme preparations as well as the assay buffer. Nonetheless, these results do confirm the involvement of cysteine residues in close proximity to the active site of fumarate

reductase. When the partially purified fumarate reductase was incubated in the presence of fumarate and iodoacetamide, inhibition was not noticed. This suggests that the binding of substrate protects enzymatic group which otherwise is inactivated by iodoacetamide.

Cofactor of Fumarate Reductase

The purified fumarate reductase preparation was colorless. Fumarate reductases purified from other organisms are bright yellow in color. They all have either covalently or non-covalently bound FAD which has absorption maxima at 450 and 380 nm. Fumarate reductase of M. thermoautotrophicum does not have any measurable absorbance in the visible region. This is the first fumarate reductase which does not have a flavin moiety as a cofactor. Instead, fumarate reductase has a rather atypical UV absorbance and fluorescence spectra. The possible cofactors or components that have similar UV absorbances and fluorescences are summarized in Table VIII. The physical properties as well as the absorbance and fluorescence characteristics of H_4MPT or reduced F_{420} do resemble those of fumarate reductase, although they are clearly not identical. Since pterins are known as carbon carriers in the C_1 cycle of methanogenesis (Figure 5), it is difficult to justify pterins as cofactors; although H_4MPT does participate as a redox intermediate. The coenzyme F_{420} participates in the redox reaction (72,73) and therefore seems also to be a likely candidate. However, the spectrophotometric characteristics of oxidized fumarate reductase do not match with those of oxidized F_{420}. Thus, although the

TABLE VIII

SPECTRAL PROPERTIES OF COFACTORS KNOWN TO BE PRESENT IN M. THERMOAUTOTROPHICUM THAT RESEMBLE FUMARATE REDUCTASE

Cofactor/ Coenzyme	λmax (Uv/Vis)	Fluorescence maxima	Properties (physical)	Assigned Role (Reference)
Methano-pterin (MPT)	342,280,232	Yellow fluorescence	Oxygen and Heat stable	Carbon Carrier, CO_2 red. to methyl CoM(23,24)
H_4MPT	302,247,220 (Anaerobic) 325,280,230 (Aerobic, upon 17 hours exposure)	302 (Exci.) 420 (Emiss.)	Oxygen labile, Heat and Acid stable	Carbon Carrier; Terminal CO_2 Red. (21)
F_{420}	320,258 (Anaerobic) 420,295,267 (Aerobic)	320 (Exci.) 390 (Emiss.)	Acid labile, Ionic, Heat stable,	Electron Carrier (72,73)

spectral results need to be investigated extensively, they suggest the possibility of the presence of an unusual cofactor. Furthermore, the cofactor seems to be very tightly bound to the enzyme, since there was no apparent dissociation during purification. The presence of an unusual cofactor with fumarate reductase is not very surprising since methanogens are shown to have unusual coenzymes and cofactors (22,33,74). The absence of flavins also indicates that fumarate reductase of M. thermoautotrophicum is strikingly different from other fumarate reductases.

In the present investigation, reduced benzyl viologen was used as an artificial electron donor. We, as well as Zeikus et al (38) have tried reduced F_{420}, reduced NAD(P), and reduced flavins as electron donors, but were unsuccessful. M. thermoautotrophicum does not have quinones and cytochromes (33,74), and hence their involvement as electron donors must be ruled out. Although it will be premature to comment on the nature of the electron donor without any experimental evidence, we think that iron sulfur proteins might be involved. However, further studies are necessary before arriving at any conclusion.

Function of Fumarate Reductase in M. thermoautotrophicum

Fumarate reductase of M. thermoautotrophicum, unlike that of E. coli or V. succinogenes, is purified from the soluble fraction of the cells. The membrane bound fumarate reductase in E. coli and V. succinogenes is used to generate ATP via oxidative phosphorylation (41,43). Fuchs et al (40) on the basis of their labeling studies have ruled out the possibility

of direct participation of fumarate reductase in oxidative phosphorylation.

We were also unable to grow M. thermoautotrophicum in the presence of fumarate and hydrogen. Fumarate acts as an electron acceptor when many facultative organisms are grown anaerobically in the absence of oxygen (62). Therefore, the function of fumarate reductase in M. thermoautotrophicum seems to be different. Muratsubaki and Katsume (48) have proposed a biosynthetic role for fumarate reductase in the cells of bakers yeast. Both succinate and succinyl CoA are important intermediates in the partial reductive TCA cycle of M. thermoautotrophicum. Succinate is essential for the synthesis of tetrapyrrole (75,76). α-ketoglutarate is the precursor of various amino acids, including glutamate, arginine, and proline. Thus we propose a biosynthetic role of fumarate reductase in the cells of Methanobacterium thermoautotrophicum.

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