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Portland State University

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AN ABSTRACT OF THE THESIS OF Scott D. Sheridan for the Master of Science in Biology presented July 26, 1985.

Title: Partial Purification and Characterization of F_{420} -dependent NADP Reductase from Methanobrevibacter smithii strain DE1

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

L. D. Eirich, Chairman

M. L. Taylor

P. L. O'Neill

D. Barnum, Graduate Office Representative

The F_{420} -dependent NADP reductase of Methanobrevibacter smithii has been partially purified employing a combination of affinity chromatography with Blue Sepharose (Cl-6B) and molecular sieve chromatography with Sephacryl S-200. The enzyme, which re-

quires reduced F_{420} as an electron donor, has been purified over 145 fold with a recovery of 6%. A molecular weight of 120,00 for the native enzyme was determined by Sephacryl S-200 chromatography. A subunit molecular weight of 28,200 was determined by SDS-PAGE, indicating that the native enzyme is a tetramer. The optimal temperature for enzymatic activity was found to be 45°C, with a pH optimum of 7.5. The NADP reductase had an apparent K_m of 42 μM for reduced F_{420} , and an apparent K_m of 41 μM for NADP. The enzyme was stable in 0.05 M sodium phosphate buffer (plus 10 mM cysteine) at pH 7.0, when gassed with nitrogen or hydrogen and stored at 4°C.

PARTIAL PURIFICATION AND CHARACTERIZATION
OF F_{420} -DEPENDENT NADP REDUCTASE
FROM METHANOBREVIBACTER SMITHII STRAIN DE1

by

SCOTT D. SHERIDAN

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

MASTER OF SCIENCE

in

BIOLOGY

Portland State University

1985

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

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PART I

INTRODUCTION

Man has known for some time that some form of "combustible air" is produced in areas where organic matter is being decomposed anaerobically, such as stream beds, bogs, swamps, and marshes (Volta, 1776). Although in 1867 Bechamp postulated that this methane production might be the result of microbial metabolism, it was not until twenty years later that Hoppe-Seyler demonstrated a firm relationship between a previously undiscovered group of organisms and this biogenesis [1]. It was left to Barker, who had the advantage of many years of microbiological advancements in culturing techniques, to correctly identify hydrogen and carbon dioxide as requirements for these bacteria, now called methanogens [2]. However, further advances in methanogen isolation and biochemistry had to await the advent of better techniques for anaerobically isolating and culturing methanogens [3].

The methanogens are very different from other bacteria; their cell wall structure, cell lipids, ribosomal and transfer RNAs, and metabolism are all markedly different from the eubacteria [4]. They are unique enough to have been assigned by some microbiologists to their own kingdom, the Archaeobacteria.

The methanogens are found occupying many ecological niches, and often are found at the bottom level in anaerobic food chains,

their ultimate carbon and energy source being the hydrogen and carbon dioxide or acetate produced by the metabolism of other bacteria. Methanogens also metabolize a few other substrates, namely formate, methanol, and methylamines. Though nutritional requirements may vary from species to species, all the methanogens utilize similar catabolic pathways, and all produce methane as a final end-product. Environments where methanogens are found include fresh water, estuarine, and marine sediments, flooded soils, animal digestive systems, and anaerobic sludge digestors [5]. It has been estimated that approximately 70% of the trace methane in the earth's atmosphere is from methanogenic metabolism [6].

This thesis will be a review of the currently known information about this unique group of organisms, and will include a description of a partial purification and characterization of an F_{420}^- dependent nicotinamide adenine dinucleotide phosphate (NADP) reductase from one of these organisms, Methanobrevibacter smithii strain DE1. It is hoped that the characterization of this enzyme may lead to an improved understanding of the phylogenetic relationships between the various species of methanogens.

ECOLOGY OF THE METHANOGENS

In normal aerobic systems, the problem of a final electron sink in bacterial metabolism is most often solved by using oxygen as the final electron acceptor. However, under anaerobic conditions (where oxygen is absent or sparse) quite a different system has evolved. Under these conditions, electrons are passed to protons,

nitrogen atoms, sulphur atoms, or carbon atoms, resulting in the products hydrogen gas, ammonia or nitrogen gas, hydrogen sulfide, or methane. In places where sulfate and light are limiting factors, organisms with methanogenic metabolisms are likely to flourish. In systems where sulphur reduction to hydrogen sulfide is an important pathway, methanogenesis is retarded, suggesting that the two reactions are competitive [7].

Methanogenic bacteria may be found in systems with widely varying conditions, cool to very hot, organically rich or poor, and high or low pressure. Some methanogens require only hydrogen and carbon dioxide, while others require additional nutrients. Those ecosystems containing mostly carbon dioxide of geochemical origin are known as the chemolithotrophic systems. They occur primarily in thermal springs and lakes where volcanic intrusion of some kind is present [7]. The methanogens present in these environments are often quite hardy, thermophilic, and obviously are efficient at obtaining energy and cell mass from compounds which are unmetabolizable to other organisms.

The other type of methanogenic ecosystem contains large to moderate amounts of organic monomers and polymers of biogenic and/or synthetic origin. This type of environment occurs in the digestive tracts of various animals, especially in the ruminants, and in places where material is being biodegraded anaerobically, such as swamps, bogs, sediments, water-saturated soils, sewage sludge, liquid waste digestors, biogas installations, garbage dumps, and landfills. It is in some of these environments that the presence

of methanogens is important, both as the final link in the anaerobic food chain, and as scavengers which help control the pH and partial pressure of hydrogen, factors which are extremely important for the complete dissimilation of organic material in anaerobic environments.

In these systems, which are almost always mixed cultures of several methanogenic and non-methanogenic species, large amounts of low molecular weight alcohols and fatty acids tend to be manufactured by non-methanogens. If these compounds are allowed to accumulate, the metabolic capacity of the system is damaged. Some non-methanogens will metabolize the higher molecular weight compounds (propionate, butyrate, ethanol), forming more oxidized products such as acetate, formate, methanol, and methylamines. NADH_2 , which is produced during the oxidation of these compounds to yield hydrogen, a thermodynamically unfavorable reaction unless the partial pressure of hydrogen is kept very low. This is exactly what the methanogens do, eventually converting the hydrogen and carbon dioxide passed to them into the relatively inert gas, methane. This process, known as interspecies hydrogen transfer, results in the stepwise degradation of the organic material coming into the system and prevents buildup of low molecular weight products which are unfermentable or which might inhibit metabolism at some point [5, 7]. In these systems, the methanogens have filled a unique ecological niche in which there is a mutualistic relationship between the methanogens and non-methanogens.

The rumen of various hoofed animals is a well known environ-

ment where methanogens are present. In this case, the methanogens are practically insignificant to the system as removers of propionate, butyrate, and acetate. The ruminants have a more elegant control over their internal conditions than non-animal systems; the lower molecular weight volatile fatty acids are absorbed by the rumen lining and utilized by the ruminant as an energy source. This helps to maintain a balanced pH between the rumen and the body fluid, and keeps the fermentation environment favorable for the mixed culture of organisms present [7]. Therefore, the rumen of animals is different from other organic degradation systems in that the ruminants are able to control the buildup of by-products by other than microbial means, while the non-animal systems require at least three metabolic types of bacteria. The methanogens are not benefitting the ruminants, since they produce methane from acetate, causing a loss of a potential nutrient for the animal.

Methanogens are also present in varying amounts in the digestive tracts of other animals, but their contribution or detriment to these animals is not clear. Recently, a link between increased amounts of methanogens in the human gut and colon cancer has been demonstrated [8]. However, it is not known whether the increased levels of methanogens are a cause of the disease or an effect.

The non-animal methanogenic ecosystems are more dependent on the presence of a mixed culture of organisms and on the process of interspecies hydrogen transfer. Many of these systems depend upon acetotrophs and hydrogenotrophic methanogens to keep the partial

pressure of hydrogen and acetate concentration down to an acceptable level, thus keeping the pH up [7]. It has been shown that most methanogens grow best when the pH is near neutrality or slightly alkaline.

In the organic sediments, such as lake bottoms, stream beds, and ocean and tributarial sediments, methane is usually bubbled out of the sediment; gas composed of 95% methane was collected from the bottom of Lake Erie by large funnels inverted above the sediment [9]. The formation of methane in these systems can be adversely affected by the presence of sulfate-reducing bacteria, presumably because the buildup of hydrogen sulfide is inhibitory to methanogenesis [10]. It is also possible that the sulfate-reducing bacteria outcompete the methanogens for available substrates and minerals [11].

With the discovery that microbes could convert what we humans would normally consider garbage (i.e., animal wastes, industrial effluent, and other by-products of our society) into a clean, easily used energy source (methane), the technology and art of growing and sustaining cultures of methanogens has taken on new importance. In addition, these organisms help to remove toxic wastes from sewage that might otherwise be dumped directly into the nearest body of water [12]. It has just been within the last few years that commercial and private production of methane for use or sale has become refined enough to be profitable, and since garbage is a part of human existence, methanogenic energy production and biodegradation research seems destined to intensify. Research focused upon the physiology and genetics of the methanogens will undoubtedly have some useful application in this growing field.

PHYLOGENETICS OF THE METHANOGENS

Early efforts in research on methanogens were often hampered by crude culturing techniques; methanogens are strict anaerobes, and until the advent of efficient anaerobic culturing techniques, pure cultures of many methanogens were difficult to obtain. Before the late 1970's, an adequate explanation of the phylogenetic relationships between the various methanogenic genera, families, and orders did not exist. To further complicate the problem, the methanogens are morphologically very diverse and have varying growth characteristics. Early classification schemes distributed them among the prokaryotes in relation to their morphology. Thus, the methanogens were spread out all over the taxonomic map.

Upon examination of the unique physiology of the methanogens, Barker [2] placed all of them in one family, the Methanobacteriaceae. Unfortunately, this did little to distinguish between the various species; a single family contained rods and cocci, Gram negative and positive, and guanine + cytosine molar percentages ranging from 25-51% [4]. Since that time research has shown that the methanogens are unique among bacteria. They have either modified cell wall components which are not found in other bacteria, or lack components which other bacteria typically possess. In addition, cell lipids of methanogens are structurally different from eubacterial lipids, as are their tRNA and rRNA. They are physiologically unique in their ability to metabolize hydrogen and carbon dioxide to methane, and they possess unique coenzymes and cofactors not present in other bacterial types [4].

The most sensible classification scheme yet suggested places the methanogens in a separate division from the Eubacteria and Cyanobacteria. This division, the Archaeobacteriae, possibly represents the result of a divergence from the eubacterial cell line which took place long ago [3]. In addition to the methanogens, the extreme halophiles and some of the acidothermophiles have been placed in this division [12]. A genetic taxonomic technique involving analysis of oligonucleotide fragments from ribonuclease T_1 digested 16S rRNA supports the separation between the Archaeobacteria and the Eubacteria, and has also been used to elucidate the phylogenetic relationships within the methanogens [4,13].

This method involves the digestion of 16S rRNA from two organisms with ribonuclease T_1 , and analysis of the fragments (only those in hexamers or larger) to determine their nucleotide sequences. These data are collected, catalogued, and used to calculate an association coefficient, the S_{AB} value. The equation is:

$$S_{AB} = \frac{2N_{AB}}{(N_A + N_B)}$$

where N_A and N_B are total numbers of nucleotides found in the catalogues and N_{AB} represents the number of nucleotides in oligomers common to the two catalogues. Balch [4] took the known data on 13 methanogens, which included S_{AB} values, morphology, metabolism, and guanosine + cytosine molar percentages, and developed the system shown in Figure 1.

This scheme distributed the known methanogens among three orders, encompassing four families, seven genera, and 13 species.

Order I, the Methanobacteriales, are all Gram positive rods, have similar cell wall composition, and high 16S rRNA homology. The Methanococcales (order II in Figure 1) are made up of only one family, and are irregular cocci with cell walls made of protein. The third order, the Methanomicrobiales, is quite varied and has cells with highly variable metabolism and morphology. Since Balch first outlined the previous taxonomic system, more than 17 new species have been isolated, and five new genera have been designated. Though there are still only the three main orders, two new families have been added to the system. Figure 2 shows the current standing of the taxonomy of methanogens in light of the known data. However, current data indicates that the family Methanosarcinaceae, which possesses unique physiological characteristics, might better be placed in a separate order [14, 15].

Another technique for assigning phylogenetic relationships, i.e., examination of the characteristics of enzymes which perform similar functions, can be applied to the methanogens. Eirich has recently shown that significant differences exist between the structures of NADP reductases among the methanogens [16]. Purification and characterization of more NADP reductases from methanogens is likely to result in increased understanding of the taxonomy of these bacteria.

CHARACTERISTICS OF THE METHANOGENS

The methanogens possess so many unusual characteristics that it is difficult to know where to begin describing them. In order to avoid confusing the reader, this section will cover, primarily, morphological, biochemical, and genetic variations that make the methanogens different from the eubacteria, and will leave description of their unusual metabolism to the next section.

Cell Wall Composition

In normal bacteria, the cell wall is composed of varying amounts of the substance known as peptidoglycan, a compound of alternating n-acetylglucosamine (NAG) and n-acetylmuramic acid (NAM) moieties cross-linked together by a polypeptide bridge composed of alternating D-and L-amino acids. It is the amount and structural arrangement of this peptidoglycan that give a bacterial cell its Gram staining characteristics, as well as serving various metabolic and protective functions. However, the peptidoglycan material of certain methanogens is significantly different from eubacterial peptidoglycan.

The peptidoglycan of methanogens, termed pseudopeptidoglycan, is found in the Gram positive species that make up the order Methanobacteriaceae. It is similar to peptidoglycan in that it contains NAG (and in some cases n-acetylgalactosamine), but does not contain NAM. Instead, another amino sugar, n-acetyltalosaminuronic acid is

found linked to the NAG or n-acetylgalactosamine [17]. In addition, though the peptide linkage in pseudopeptidoglycan is variable within the various genera of the Methanobacteriaceae, no methanogen cell walls contain any D-amino acids, further distinguishing them from the eubacteria. The structures of peptidoglycan and pseudopeptidoglycan are shown in Figure 3. The organization of the cell wall differs among genera. The genus Methanobacterium has a single layer of pseudopeptidoglycan 15-20 nanometers thick, while Methanobrevibacter possesses three distinct layers, which together are a total of 30-40 nanometers in thickness [18].

In contrast to the Methanobacteriales, most of the Methanococcales and Methanomicrobiales have cell walls composed of protein and show up negative in the Gram stain. The Methanococcales are irregular cocci, usually motile, and possess a single cell wall layer about 18 nanometers thick. The genera Methanomicrobium and Methanogenium are similar to Methanococcales in their proteinaceous cell wall, but show a greater diversity of morphology and motility [4]. Members of the genus Methanospirillum are long, curved rods, due to a rigid, proteinaceous sheath which covers several cells. This has the effect of causing them to form long, regular spirals, hence their name. The only Gram positive representatives within the Methanomicrobiales, the Methanosarcinaceae, have a cell wall composed of an acid heteropolysaccharide containing galactosamine, neutral sugars, and uronic acids, but no muramic or glutamic acids or glucosamine [19].

Cell Lipids

Research has shown that methanogen cell lipids are different from eubacterial lipids in several important respects. Eubacterial lipids are linked to the polar "head" groups by ester linkages, while the methanogens utilize more stable ether linkages. In addition, contrary to the straight chain, unbranched fatty acids seen in the eubacteria, the fatty acids of methanogens are predominantly polyisobranched [20]. Presumably both these features would tend to increase the stability of the membranes in which they are included, an asset which might become especially important at high temperatures.

Of the lipids commonly found in the methanogens, approximately 20-30% are non-polar lipids, while the remaining 70-80% are polar. The polar components have been demonstrated to be mostly C_{20} and C_{40} phytanyl and biphytanyl glycerol ethers; exceptions to this rule occur in the Methanococcaceae and Methanosarcinaceae, which contain only the C_{20} phytanyl ethers [21]. The non-polar components of methanogen lipids were found to exist in a large range of sizes (C_{14} to C_{30}), but the most common components were the C_{30} , C_{25} , and C_{20} isoprenoids, each present in a number of hydroisoprenoid derivatives. The C_{30} (squalene) component was found in all the families of methanogens except the Methanosarcinaceae [22]. Viewing this evidence, it is tempting to hypothesize a microbial origin for many of the multibranched, isoprenoid components commonly found in sediments and petroleum deposits [21].

DNA

Examination of the DNA of methanogens has revealed a wide range of guanosine + cytosine molar percentages, from 27.5% at the low end to 61%. However, within the various genera the G + C molar percentages are fairly restricted. Species in the genus Methanobrevibacter contain G + C molar percentages of 27.5% to 32%, while the genus Methanobacterium ranges from 32.7% to 40.7% for the mesophiles, and 49.7% for the thermophile M. thermoautotrophicum [4]. The Methanococcales show G + C contents of around 30%, which is in sharp contrast to the 51.6% and 61.2% found in two marine cocci within the genus Methanogenium. The other genera in the order Methanomicrobiales contain a range of G + C molar percentages.

RNA

Both the rRNA and the tRNA of methanogens are substantially different from typical eubacterial RNAs. Examination of S_{AB} values for 16S rRNA of methanogens, eubacteria, and eukaryotes reveals no greater similarity between the methanogens and the eubacteria than between the eubacteria and eukaryotes [4]. Subsequently this was confirmed by discovery of the lack of similarity between methanogen and other 5S rRNA [23]. In addition, post-transcriptional modification patterns in 16S and 23S rRNA differ from those of typical bacteria. While the eubacterial tRNAs contain a universal arm sequence GTYCG (Y being pseudouracil), the methanogens contain one of two analogs, either GYYCG or GUYCG [4].

PHYSIOLOGY OF THE METHANOGENS

Work on the biochemical systems present in the methanogens is difficult due to the sensitivity to oxygen of many of the enzymes and coenzymes involved. Breakthroughs in this area had to wait for the development of better anaerobic purification and assay techniques. Fortunately, the last decade has yielded much new information about the process which results in the biogenesis of methane.

Although several substrates are used by methanogens in general (formate, methanol, acetate, methylamines), the most commonly metabolized substrates are hydrogen and carbon dioxide. The equation for the reduction of carbon dioxide and oxidation of hydrogen to methane is as follows:

The redox potentials for this process are shown in Figure 4. The first steps of the process are quite unfavorable, while the others are very favorable. By some as yet undetermined mechanism, the methanogens are able to use some sort of coupling mechanism which allows the energetically favorable final steps to promote the earlier unfavorable ones.

Different C_1 of varying oxidation states along the pathway from carbon dioxide to methane, such as formate, formaldehyde, and methanol, do not appear to be involved in the actual reductive process although they may be present in the organism. Instead, the methanogens make use of several novel compounds, which function as carbon

carriers at various levels. Examination of these compounds, which are found only in the methanogens, is necessary for understanding of the methanogenic process.

CDR Factor

CDR, or carbon dioxide reduction factor, is present in the low molecular weight fraction of cell extracts, and appears to be responsible for the reduction of carbon dioxide or carboxyl groups to the level of formaldehyde. Recently, the structure of CDR factor was elucidated, and its structure is shown in Figure 5 [24]. The molecule has been identified as 4[N-(4,5,7-tricarboxyheptanoyl- γ -L-glutamyl- γ -L-glutamyl)-p-(β -aminoethyl)phoxymethyl-2-(amino-methyl) furan, or methanofuran for short [25].

Methanopterin and FAF

Methanopterin, or N-[1'-(2"-amino-4"-hydroxy-7"-methyl-6"-pteridiny)ethyl]-4-[2',3',4',5',-tetrahydroxypent-1'-yl(5'-1")-0- α -ribofuranosyl-5"-phosphoric acid] aniline, is present in all known methanogens, and it and many of its derivatives are important in methanogenesis [26]. Methanopterin itself, may be a species which occurs as an artifact of purification, and a direct role for it in methanogenesis has not been established. However, one of its derivatives, tetrahydromethanopterin, also called formaldehyde activation factor (FAF), appears to be a carbon carrier at the formyl, methenyl, and methyl levels of oxidation [27,28,29,30]. This is not surprising, since methanopterin bears a structural resemblance

to folic acid, which participates in other well known metabolic processes as tetrahydrofolate and other derivatives. Other compounds found with or associated with methanopterin and FAF may be artifacts caused by the breakdown of the unstable and easily oxidized FAF [24,26,31]. The structure of methanopterin is shown in Figure 5.

Coenzyme M, F_{430} , and the Methyl Reductase Complex

Coenzyme M (CoM) is yet another compound unique to the methanogens. It has been identified as 2-mercaptoethanesulfonic acid, and has long been identified as being involved with the binding of a methyl group with a subsequent demethylation yielding methane [5]. Earlier studies postulated that CoM might be a carbon carrier at the formyl as well as the methyl level, but recent studies show that it operates exclusively at the methyl level [30,32]. Other investigations have revealed that CoM is part of a larger methyl reductase complex, and associated with several other components [24].

The protein part of the methyl reductase complex can be separated into three parts. Component A was found to be a protein with a molecular weight of approximately 500,000 which possesses hydrogenase activity and contains FAD; component B, a small molecular weight fragment, has an as yet unclear role in methanogenesis. The third component in the system, component C, is the part of the complex that possesses the methyl reductase activity. It was found to be closely associated with F_{430} , a nickel-containing tetrapyrrole unique to methanogenic species [33]. There is evidence that F_{430}

may be connected to both the CoM moiety and the component C of the methyl reductase complex [34,35]. In addition, the methyl reductase complex requires ATP, Mg^{2+} , FAD, and possibly vitamin B_{12} and another unique flavin derivative, F_{420} .

F_{420} and NADP

Although F_{420} , a unique flavin analog, is not specifically identified with the process of methanogenesis, its role in biosynthesis seems certain. F_{420} seems to act primarily as an intermediary electron carrier between hydrogen or formate and NADP. F_{420} also appears to be involved in the formate dehydrogenase complex, which converts formate to hydrogen and carbon dioxide, with the assistance of NADP. In addition, it may play an as yet unknown role in the methyl reductase complex. A more comprehensive explanation of F_{420} and NADP's possible cellular functions will be presented in the experimental section of this thesis.

The most current model of the methanogenic process was developed by Escalante-Semerena et al [24], and involves a tricyclic pathway. The model is shown in Figure 6. Initially, carbon dioxide or a carbonyl group from an organic acid is passed to the CDR factor, which, with electrons generated from an available hydrogen molecule, is reduced from the acid to the aldehyde oxidation level. The next step involves the passing of the aldehyde moiety from CDR to FAF to form methenyl-FAF, which is then reduced to methylene-FAF. At this point, methylene-FAF may release formaldehyde, which can be utilized

in other biosynthetic processes, or may be reduced further to methyl-FAF. Presumably, methylamines, methanol, and acetate can enter the cycle at this point. The third part of the cyclic model consists of the CoM-F₄₃₀ methyl reductase complex, which serves to pick up the methyl group from methyl-FAF and transfer it to CoM. Then, with the final addition of electrons from hydrogen, methane is released, and CoM immediately picks up another methyl moiety.

One interesting phenomenon, the RPG effect (after R.P. Gunsalus) is an elegant system for driving the methanogenic process. It appears that the demethylation of CoM in the final step of methanogenesis somehow increases the rate of initial carbon dioxide reduction. This phenomenon may be due specifically to the change in CoM, or to the change in FAF which occurs prior to methane release [24]. In any case, it is interesting that the methanogens have found a way to get around the energetically unfavorable first steps in the methane-producing process.

Although the biochemical pathway from hydrogen to carbon dioxide is better known than it was five years ago, it is by no means totally clear. Several problems remain to be worked out, including the specific roles of F₄₃₀ and F₄₂₀ in the metabolic pathway, exact nature of the interactions between CDR factor, FAF, and the CoM-methyl reductase complex, and precise elucidation of the RPG effect. In addition, much more needs to be discovered about the processes of carbon assimilation, biosynthesis, and ATP production in the methanogens.

The exact mechanism by which the methanogens produce ATP is not clear. However, recent research points to the speculation that methane production and ATP synthesis are both membrane associated processes. It has been shown that when preparations from disrupted Methanobacterium thermoautotrophicum cells were centrifuged at 226,600 x g, the resulting supernatant lacked intact membrane vesicles. Moreover, that fraction's ability to catalyze the production of methane from carbon dioxide and hydrogen was no longer present [36]. Other studies have demonstrated that a proton gradient exists across these vesicle membranes [37,38,39]. In light of what is known about chemiosmotic coupling in other organisms, it is reasonable to suspect that methane production and ATP synthesis are coupled. The proton gradient may also be partly or wholly responsible for the RPG effect.

Exactly how the methanogens assimilate carbon has long been a mystery, but recent studies point to an incomplete reductive carboxylic acid cycle (IRC) [40]. Several key TCA cycle enzymes are not found in the methanogens, most critically, citrate lyase and isocitrate hydrogenase, preventing synthesis of α -ketoglutarate. Therefore, the oxidative TCA cycle cannot be utilized to obtain α -ketoglutarate, a necessary precursor for several important cellular compounds.

The IRC cycle starts when carbon dioxide is converted to acetate, and then to acetyl-CoA, a central intermediate. Acetyl-CoA is then converted to pyruvate, and subsequently phosphoenolpyruvate. From there, the reaction proceeds in a backward fashion around the

TCA cycle, ending with α -ketoglutarate.

The process by which the methanogens convert carbon dioxide to acetate has just recently been identified. Methanobacterium thermoautotrophicum, an organism which does not normally come into contact with acetate in its environment, has been shown to synthesize activated acetic acid from two carbon dioxide molecules; one of these is passed partway through the methanogenic cycle to the level of methyl-FAF, and another is converted to carbon monoxide through the action of a carbon monoxide dehydrogenase [41]. Presumably, this carbon monoxide is then added to the methyl group from methyl-FAF, creating a highly activated acetate which is rapidly converted to acetyl-CoA in the presence of CoA. A representation of the IRC cycle is shown in Figure 7 [41,42].

Acetyl-CoA is a useful intermediate which can be converted into several important products, including carbohydrate. This pathway to carbohydrate is shown in Figure 8 [43]. According to this scheme, ATP, F_{420} , and NADPH are all quite important [44]. Methanogens are known to assimilate various fatty acids if they are provided in the medium, most notably propionate and acetate [45,46], which may then be built into other cellular compounds. When propionate is provided, methanogens will often convert it to isoleucine, rather than synthesizing it from α -ketobutyrate [47]. Lysine biosynthesis has been postulated to take place via the diaminopimelic acid pathway, a route common to other bacteria [48].

The amount that has been learned about the methanogens and their metabolic, catabolic, and anabolic pathways, particularly within the

last eight years, has been enormous. Yet, there is much more that must be discovered. As these organisms are better known, their use as a way to convert refuse to clean energy in the form of methane will become cheaper and more feasible for larger segments of the world's population. In view of the eventual depletion of coal, oil, and natural gas deposits worldwide, this research takes on added importance.

PART II

INTRODUCTION

Presently, the best accepted classification scheme for the methanogens is based on homology of ribonuclease T₁ digested 16S rRNA fragments, and to a lesser extent, morphological and biochemical parameters. Another useful technique for the fine discrimination between closely related species involves examination of the properties of common enzymes. One of these enzymes, an F₄₂₀-dependent NADP reductase, has been studied previously in two other organisms, Methanobacterium thermoautotrophicum [16], and Methanococcus vanniellii [49]. In addition, the NADP reductase is found in all methanogens, and its activity is easily assayed [50]. The purpose of this investigator's work has been to isolate the NADP reductase from Methanobrevibacter smithii strain DE1, and to compare the properties of this enzyme to those of the two previously purified enzymes. This information may help indicate whether specific differences in NADP reductases are of a phylogenetic origin, or if some other factor is responsible.

Methanobrevibacter smithii strain DE1

Methanobrevibacter smithii is a Gram positive, lancet shaped coccus or short rod, which may form pairs, chains, or small aggregates, depending on growth conditions. Mbv. smithii can be distinguished from other members of its genus by several distinctions.

Mbv. arboriphilus does not utilize formate as an energy source, while Mbv. ruminantium and Mbv. smithii do; in addition, both Mbv. ruminantium and Mbv. smithii require acetate as a carbon source. Mbv. smithii can be distinguished from Mbv. ruminantium by its ability to synthesize coenzyme M. Mbv. smithii strain DEL is a mesophile, growing optimally at 40°C. The culture used in this study was isolated from sewage sludge.

F₄₂₀ and NADP

One of the unique substances found universally in methanogens, F₄₂₀ has been found to be N-[N-[0-[5-(8-hydroxy-5-deazoisalloxazin-10-yl)-2,3,4-trihydroxy-4-pentoxhydroxyphosphinyl]-L-lactyl-γ-L-glutamyl]-L-glutamic acid [50]. It functions as a low potential electron carrier, passing electrons from hydrogen or formate to some other molecule. In this specific instance, we are interested in the enzyme-mediated reduction of NADP to NADPH with electrons from F₄₂₀. The structure of F₄₂₀ is shown in Figure 9.

It is suspected that F₄₂₀ is important in several other chemical processes in methanogens. It has been suggested that it plays a role in the methyl reductase enzyme complex, and it has been shown to work with NADP and formate dehydrogenase to catalyze the production of carbon dioxide and hydrogen from formate [24]. It has been demonstrated to contribute electrons to the reaction by which methanogens convert acetyl-CoA and CO₂ to pyruvate, or succinate and CO₂ to α-ketoglutarate [51].

NADPH is an important source of reduction equivalents for

biosynthetic processes in the eubacteria, and evidence suggests that it plays a similar role in the methanogens. Examples of proven NADPH contribution include its inclusion in active glycer-aldehyde-3-phosphate dehydrogenase. It is also apparently involved in the enzymatic conversion of 1,3-diphosphoglycerate to glycer-aldehyde-3-phosphate [51]. Further study should uncover many more instances where NADPH is required. Consequently, the insurance of an adequate supply of NADPH is very important. The NADP reductases present in methanogens perform this function.

MATERIALS AND METHODS

Materials

All chemicals were of reagent grade. Blue Sepharose (Cl-6B) and Sephacryl S-200 were from Pharmacia. The molecular weight standardization proteins were from Sigma, with the exception of ovalbumin (Mann). NADP was from Sigma. All gases were supplied by Airco.

Growth of Methanobrevibacter

Methanobrevibacter smithii strain DE1 was isolated from sewage sludge in the laboratory of Dr. L. D. Eirich. It was maintained [52] and mass cultured [53] by methods previously described. The organism was sub-cultured and maintained in crimp-sealed 100 ml serum bottles containing 20 ml of medium #1, which had been pressurized with a mixture of 80% H₂ and 20% CO₂. The medium contained K₂HPO₄, KH₂PO₄, (NH₄)₂SO₄, NaCl, MgSO₄·7 H₂O, CaCl₂·2 H₂O, trace minerals, trace vitamins, FeSO₄·7 H₂O, Na₂CO₃, sodium acetate,

sodium formate, yeast extract, Bacto-Tryptone, and resazurin in amounts suggested by Balch, et al, [4] with slight modifications. The organism was mass cultured in a 12 liter New Brunswick fermenter. A H_2/CO_2 mixture (80:20) gas mixture was continuously bubbled through the medium with constant stirring at 37°C. The culture was grown for five or six days, and the output of methane was monitored by drawing off 0.25 ml of effluent gas and analyzing it with a Varian Aerograph 90-P gas chromatograph equipped with a Poropak-Q column.

The cells were harvested using a Ceba-Schnell continuous flow centrifuge, yielding 40-60 grams of cells in the form of a moist paste. The cells were then suspended in 0.1 M sodium phosphate buffer (2ml per gram of cells), pH 7.5, flushed with hydrogen, and stored at -20°C until ready for further use.

Preparation of crude extract

The frozen cells were thawed, and lysed by passage 4-5 times through a french pressure cell at 16,000 psi. The slurry was then centrifuged at 40,000 x g for 30 minutes to remove cellular debris. The process yielded a dark brown crude extract which was then stored at -20°C under hydrogen until ready for further use.

Assay procedure

The NADP reductase was assayed according to the method of Eirich, et al[50]. The assay mixture contained 3 ml of 0.1 M sodium phosphate buffer (pH 7.5), 240 nmol NADP, 64 nmol reduced F_{420} , and enzyme as indicated. The reactions were carried out in

Bausch and Lomb spectrophotometer tubes (1.0 x 10.0 cm) which had been sealed with butyl rubber stoppers and flushed with either H₂ or N₂ as indicated. The reaction was monitored with a Turner spectrophotometer at 350nm. The reactions were carried out at 45°C, and were started by the addition of NADP. One unit of activity is defined as the amount of enzyme required to reduce one μ mol of NADP/min at pH 7.5 and 45°C.

Purification of F₄₂₀ and preparation of reduced F₄₂₀

F₄₂₀ was purified from Methanobacterium thermoautotrophicum [50] and reduced as previously described [16]. Approximately 2.0 milligrams of purified F₄₂₀ (in 2 ml of buffer, pH 7.5) was diluted by the addition of 2.0 ml of 0.1 M sodium phosphate buffer (pH 7.5), and placed in a 10 ml crimp-sealed serum bottle. Crude extract (50 μ l) from Methanobacterium thermoautotrophicum was added to the F₄₂₀ solution and continuously flushed with H₂ in a water bath at 65°C. After five minutes, the vial was pressurized with H₂ to 30 psi, and the reaction was allowed to continue for ten more minutes. During the reduction, the color of the solution changed from a bright fluorescent chartreuse to a dull light green. This preparation was then placed in a boiling water bath for ten minutes in order to destroy all enzymatic activity.

Polyacrylamide gel electrophoresis

Slab gel electrophoresis was performed using the gel composition and buffers described by Laemmli [54], with slight modifications. Samples (25 μ l) containing NADP reductase activity were

subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 60 mA. The gels were stained with Coomassie Brilliant Blue-G (0.01% in destaining solution) for two hours, and destained overnight in methanol/acetic acid/water (5/1/5).

Protein determination

Protein concentrations of the pools containing NADP reductase activity were determined by the Coomassie Blue binding assay of Bradford [55]. Serial dilutions of bovine serum albumin in assay solution were monitored for absorbance at 600nm, and a standard curve was obtained. The protein content of various NADP reductase pools was determined from this standard curve.

RESULTS

Partial purification

Crude extract (46 ml) was applied to a Blue Sepharose (Cl-6B) affinity column (2.5 x 15.5 cm) which was previously equilibrated with 0.05 M sodium phosphate buffer + 10 mM cysteine (pH 7.0) at a flow rate of 30 ml/hr. This buffer, which will hereafter be simply designated as phosphate buffer, also contained 5 mM sodium azide to prevent bacterial growth. Though the column was in aerobic surroundings, the purification was kept relatively anaerobic by pumping buffer and extract from inside a Freter-type anaerobic chamber [56] to the column, and then back into the chamber for fraction collection. The enzyme was eluted by the addition of a 0-2.0 M NaCl gradient (500 ml) in phosphate buffer. The fractions were assayed

as previously described, and the approximate relative protein content of each fraction determined by measuring absorbance at 280nm with a Beckman spectrophotometer. Those fractions with highest activity and lowest relative protein content were pooled and stored at 4°C under nitrogen. Figure 10 shows a typical elution profile. The pool was concentrated by passage through an Amicon PM-10 ultrafiltration membrane.

The concentrated, partially purified enzyme was applied to a Sephacryl S-200 column (1.6 x 95 cm) and the column flushed with phosphate buffer at 30ml/hr. Fractions were assayed for NADP reductase activity and absorbance at 280nm (Figure 11), and the fractions with highest activity were pooled and concentrated. In this case, the enzyme was seen to elute in two distinct peaks, the first (Peak 1) being larger than the second (Peak 2). Since Peak 1 was determined to be a protein of much greater molecular weight, it was assumed that Peak 2 contained the physiological form of the NADP reductase, while Peak 1 was thought to consist of aggregates of the native enzyme.

The Peak 2 concentrate was then applied to a smaller (0.9 x 15 cm) Blue Sepharose column, and the enzyme eluted by employing an NADP gradient (0-2.0 mM) in phosphate buffer, followed by a 2.0 M NaCl wash (200 ml in phosphate buffer). The most active fractions, which were recovered in the NaCl wash, were pooled and concentrated, yielding 6 ml of concentrate. Table 1 shows a summary of the partial purification process. The enzyme was purified over 145 fold, and a yield of 6% was obtained.

Molecular weight and purity determination

The molecular weight of the native enzyme was determined by molecular sieve chromatography with Sephacryl S-200. The void volume of the column was determined with Blue Dextran, and a mixture of standard proteins was applied to the column. The elution peaks for the standard proteins were determined by absorbance at 280nm, and their elution volumes calculated. The elution volume/void volume value for each protein was then plotted against the log of the molecular weight to obtain a standard curve. The elution volumes of the two Sephacryl S-200 peaks were then determined, and their elution volume/void volume values plotted. Peak 2 was found to be a protein with a molecular weight of 120,000 (Figure 12). Peak 1 was found to have a molecular weight of greater than 200,000, and was probably an aggregate of two or more of the Peak 2 native enzyme molecules.

Samples (25 μ l) from each tube containing NADP reductase activity from the Sephacryl S-200 purification step were subjected to SDS-PAGE (Figure 13). The gel revealed several protein bands (52,500, 44,500, 34,000, 28,200, and 19,000 MW). The darkest band (28,200) was the only band present in all the active Sephacryl fractions, and peaked in intensity in those tubes containing the highest NADP reductase activity. It was therefore assumed to be the F₄₂₀-dependent NADP reductase, while the other proteins were ruled out. This data, along with that from the Sephacryl S-200 molecular sieve chromatography, indicates a tetrameric quaternary structure for the native enzyme.

The final concentrate was also subjected to SDS-PAGE, but no clear bands were revealed, indicating that the final concentrate, although very active enzymatically, contained very little protein. Since no proteins in the final concentrate were present in high enough concentrations to appear on the gel, it is impossible to determine precisely if the final concentrate was pure.

Properties of the NADP reductase

The most purified NADP reductase preparation was employed to determine various characteristics of the enzyme. The pH optimum was determined by varying the pH of the assay solution from 6.0 to 9.0 (Figure 14). For the higher pH values, sodium pyrophosphate buffer was employed rather than monobasic-dibasic phosphate buffer. The pH optimum was found to be 7.5. Apparently, sodium pyrophosphate buffer in some ways inhibits the action of the enzyme.

The temperature optimum for the NADP reductase was found to be approximately 45°C (Figure 15). The presence of a very broad peak is consistent with the fact the Methanobrevibacter smithii is a mesophile. It is interesting to note that the NADP reductase functions about as well at 49°C as it does at 37°C, the near-optimum growth temperature for the organism.

The NADP reductase is somewhat sensitive to oxygen, especially in the absence of reducing agent in the buffer. In order to determine the best long term storage conditions for the enzyme, three reducing agents (2-mercaptoethanol, dithiothreitol, and cysteine) were tested at various concentrations (100 mM, 50 mM, 20 mM, 10 mM,

5 mM, and 2 mM), along with various storage gases (H_2 and N_2), buffer concentrations, and storage temperatures. It was found that storage in 0.05 M phosphate buffer (+ 10 mM cysteine) under nitrogen at $4^\circ C$ was the best combination. Cysteine was by far the best reducing agent, but concentrations above 10 mM did not significantly improve enzyme stability, and presented a precipitation problem at low temperatures. Dithiothreitol completely destroyed the activity of the enzyme, while 2-mercaptoethanol did not significantly improve the stability of the enzyme over that of the unstabilized preparation. The choice of headspace gas was somewhat determined by the stage of preparation of the enzyme; hydrogenase-containing preparations (cells or crude extract) were stored under hydrogen, while the semi-purified preparations (hydrogenase-free) were gassed with nitrogen. Nitrogen was determined to be more suitable for long term storage of semi-purified preparations due to its heavier molecular weight, which makes it less likely to diffuse out of the container than hydrogen.

The reducing agent and headspace gas were able to prevent damage by trace amounts of oxygen, but would not prevent inactivation and degradation of the enzyme if exposed to large amounts of oxygen. A 1:10 dilution of crude extract lost all activity after one week of exposure to oxygen at room temperature, even under optimal storage conditions.

Kinetic values for the NADP reductase were determined at pH 7.5. The apparent K_m for NADP was found to be $41 \mu M$ (Figure 16), while the K_m for reduced F_{420} was determined to be $42 \mu M$ (Figure 17).

DISCUSSION

As shown in the partial purification summary (Table 1), a large percentage of the NADP reductase was lost in the first two purification steps. The Sephacryl S-200 profile (Figure 11) clearly shows two peaks. The larger peak (Peak 1) had a molecular weight of greater than 200,000 (Sephacryl S-200 is designed only for use with proteins of less than 200,00 MW), and was assumed to be an aggregate of the native enzyme. This aggregation problem can possibly be traced to the original lysis of the cells. The crude extract used in the purification described here was passed 4-5 times through a French pressure cell at 16,000 psi. Each passage through the cell increases the contact of the cells with oxygen. Crude extract prepared by passage (2x) through the pressure cell at 24,000 psi produced a much thicker, darker slurry which had higher activity. Aggregation was thus avoided, since a subsequent purification attempt yielded only one activity peak after passage through Sephacryl S-200.

The last purification step proved to be a problem which still requires some study. Originally, Red Sepharose, which has a strong affinity for NADP-utilizing enzymes, was employed as the final purification step. However, it was found that the NADP reductase from Mbv. smithii binds so tightly to Sepharose that 20 mM NADP plus 3.0 M NaCl could not remove it effectively. In subsequent purifications, Blue Sepharose, which has a greater affinity for NAD-utilizing enzymes than NADP-utilizing enzymes,

was employed. This final step yielded a highly active concentrate, but was not concentrated enough at 6 ml, or later at 1 ml, to be detectable on an SDS-PAGE gel. It is likely that further work on the purification employing greater amounts of starting product and careful procedures to avoid aggregation, will produce better yields, allowing a more precise determination of purity.

To date, only two other F_{420} -dependent NADP reductases have been purified and characterized. Eirich and Dugger [16] have purified and characterized the NADP reductase from Methanobacterium thermoautotrophicum, and Yamazaki and Tsai have done the same with the enzyme from Methanococcus vanniellii [49], and several differences exist between the three enzymes. The NADP reductases from Mbv. smithii and M. thermoautotrophicum are quite similar, both in native molecular weight and subunit molecular weight (Table II). In addition, it appears that both enzymes are tetrameric in structure. In contrast, the NADP reductase from Mc. vanniellii has a native molecular weight and subunit molecular weight quite different from the first two, and is a dimer. Preliminary work by Ted Moore in the laboratory of L. D. Eirich has shown that the NADP reductase from Methanogenium wolfei is similar to those of M. thermoautotrophicum and Mbv. smithii in subunit molecular weight, but is a dimer with a native molecular weight of 56,000.

In addition to structure and molecular weight, differences in the properties of the enzymes exist (Table III). All three enzymes that have been characterized have very different temperature

optima. However, it is interesting to note that although Mc. vanniellii and Mbv. smithii are both mesophiles with similar optimum growth temperatures, the NADP reductase isolated from Mbv. smithii functions best more than 25°C above that of the enzyme from Mc. vanniellii. In fact, it is closer to the thermophile, M. thermoautotrophicum in this respect.

The kinetic behavior of the three enzymes differ also. While the NADP reductase from Mc. vanniellii exhibits a very low K_m for both NADP and reduced F_{420} , the K_m values for the enzymes isolated from M. thermoautotrophicum and Mbv. smithii agree more closely. Both M. thermoautotrophicum and Mbv. smithii exhibit nearly the same K_m for NADP, while their K_m values for reduced F_{420} differ by a factor of three. This higher K_m for reduced F_{420} in M. thermoautotrophicum is possibly related to the fact that M. thermoautotrophicum naturally contains about 50 times more F_{420} than Mbv. smithii [57]. It is also possible that the K_m values differ because thermophilic enzymes often have higher K_m values for their substrates.

The NADP reductase from Mbv. smithii differs slightly from the other two enzymes in that its pH optimum is slightly lower. However, all three enzymes function best at slightly higher than neutral pH, findings which are consistent with the optimal growth pH conditions for most methanogenic bacteria.

There are two possible reasons why the NADP reductases from these enzymes differ. The first is that these variations are the

result of phylogenetic differences. Both M. thermoautotrophicum and Mbv. smithii are in the order Methanobacteriales, while Mc. vanniellii is in the order Methanococcales [4]. It does appear that the NADP reductase from Mbv. smithii is structurally more similar to that of M. thermoautotrophicum than Mc. vanniellii. Thus, this difference in the enzymes may be simply reflecting the lack of genetic similarity between the Methanobacteriales and the Methanococcales.

The second possibility is that the differences in the NADP reductases stem from environmental influences such as extreme vs. moderate growth temperatures. While our evidence indicates that the environment probably does not play a large role in the structural arrangement and size of the enzyme, it could be having an effect on the kinetic behavior of the enzyme. In M. thermoautotrophicum, a more heat stable, tetrameric structure is favored. It is possible that the tetrameric structure of the NADP reductase from Mbv. smithii functions at lower temperatures because of small structural modifications which are reflected in its lowered K_m value for reduced F_{420} .

From the data accumulated thus far, it is likely that phylogenetics plays a greater part in the structural characteristics of these enzymes. Preliminary data on the F_{420} -dependent NADP reductase from Methanogenium wolfei, a representative of the third order of methanogens, the Methanomicrobiales, lends support for this idea, since this enzyme has a subunit molecular weight similar to those

from the order Methanobacteriales; however, unlike the NADP reductases from the Methanobacteriales, that from Methanogenium wolfei is a dimer. As more F_{420} -dependent NADP reductases are characterized, the relationship between phylogenetics and structural differences in these enzymes will be more clearly understood.

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FIGURE 1. Balch's classification scheme for the
methanogens known as of 1979 [4]

ORDER	FAMILY	GENUS	SPECIES
Methanobacteriales	Methanobacteriaceae	Methanobacterium	M. formicum
			M. bryantii
			M. thermoautotrophicum
		Methanobrevibacter	M. ruminantium
Methanococcales	Methanococcaceae	Methanococcus	M. arboriphilus
			M. smithii
			M. vannielii
			M. voltae
Methanomicrobiales	Methanomicrobiaceae	Methanomicrobium	M. mobile
		Methanogenium	M. cariaci
			M. marisnigri
		Methanospirillum	M. hungatei
	Methanosarcinaceae	Methanosarcina	M. barkeri

FIGURE 2. Current classification scheme for the known methanogens

ORDER	FAMILY	GENUS	SPECIES
Methanobacteriales	Methanobacteriaceae	Methanobacterium	thermoautotrophicum formicum 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 marisnigri olentangyi thermophilicum tatii wolfei
	Methanoplanaceae	Methanospirillum	lungatei
		Methanoplanus	limnicola
	Methanosarcinaceae	Methanosarcina	barkeri mazei acetovorens
		Methanotherix	solongenii
		Methanolobus	indarius
		Methanococcoides	methylutens

FIGURE 3. Structure of peptidoglycan (A) and pseudopeptidoglycan (B).

(A)

(B)

FIGURE 4. Levels of the oxidation-reduction couples involved in the production of methane from CO₂

FIGURE 5. Structure of CDR factor (A) and
methanopterin (B) [25, 26, 27]

Ⓐ

Ⓑ

FIGURE 6. Proposed tricyclic pathway utilized
by the methanogens during the reduction
of CO₂ to methane

FIGURE 7. The incomplete reductive carboxylic acid cycle, and pathway from CO_2 to acetyl-CoA

- ⊕ $\text{CH}_3\text{-CoM}$ (RPG effect)
- ⊖ $\text{CHCl}_3, \text{Br-CoM}, \text{etc}$

FIGURE 8. Carbohydrate synthesis pathway for
the methanogens

FIGURE 9. Structure of F_{420} [50]

FIGURE 10. Elution profile of Methanobrevibacter smithii strain DE1 crude extract after elution from the Blue Sepharose (Cl-6B) affinity column. Both enzymatic activity (○—○) and relative protein content (●—●) are shown, as well as the NaCl gradient (.....). The points at the far left (□—□) are absorbance at 280nm values on a scale 10 times that of the far right scale, indicating that a large amount of protein passed unbound through the column.

FIGURE 11. Elution profile of partially purified NADP reductase after elution from a Sephacryl S-200 column. Both activity (○—○) and relative protein content (●—●) are shown. Peak 2 was assumed to be the physiological form of the native enzyme, while Peak 1 was thought to be an aggregate of the native enzyme.

TABLE I. Purification summary of the NADP
reductase from Methanobrevibacter
smithii strain DE1

Fraction	Total activity (U)	Protein (mg)	Spec. act. (U/mg protein)	Fold purification	Yield
Crude extract	367.5	333.5	1.10	-----	100%
Blue Sepharose eluate	93.6	3.08	30.38	27.6	25.5%
Sephacryl S-200 eluate Pool 1	83.7	0.99	85.82	78.0	22.8%
Sephacryl S-200 eluate Peak 2	56.9	0.85	67.00	61.0	15.4%
Post Blue Sepharose concentrate	21.5	0.135	159.00	145.0	6.0%

FIGURE 12. Determination of the molecular weight of the native NADP reductase. The following standard proteins were employed in the determination: A, cytochrome C (12,400); B, carbonic anhydrase (29,000); C, ovalbumin (45,000); D, bovine serum albumin (66,000); E, alcohol dehydrogenase (150,000); F, β -amylase (200,000).

FIGURE 13. Determination of subunit molecular weight for the NADP reductase by SDS-PAGE. A 10% polyacrylamide gel used. The following standard proteins were employed in the determination: A, α -lactalbumin (14,200); B, trypsin inhibitor (20,100); C, trypsinogen (24,000); D, carbonic anhydrase (29,000); E, glyceraldehyde-3-phosphate dehydrogenase (36,000); F, ovalbumin (45,000).

FIGURE 14. Effect of pH on the activity of the NADP reductase. Assays were performed in the indicated buffers (0.1 M) at the pH values shown, as described in Materials and Methods.

FIGURE 15. Effect of temperature on the activity of the NADP reductase. Assays were performed at the indicated temperature values as described in Materials and Methods.

FIGURE 16. Effect of NADP concentration on the rate of reduction of NADP. Assays were performed at the specified NADP concentrations as described in Materials Methods. The K_m for NADP was found to be 41 μ M.

FIGURE 17. Effect of reduced F_{420} concentration on the reduction of NADP. Assays were performed at the specified F_{420} concentrations as described in Materials and Methods. The K_m for reduced F_{420} was found to be $42 \mu M$.

TABLE II. Comparison of the native molecular and subunit molecular weights for four NADP reductases

ORGANISM	ORDER	MOLECULAR WEIGHT	SUBUNIT MOLECULAR WEIGHT
<hr/>			
<u>Methanobacterium</u>			
<u>thermoautotrophicum</u>	Methanobacteriales	112,000	28,500
<u>Methanobrevibacter</u>			
<u>smithii</u>	Methanobacteriales	120,000	28,200
<u>Methanogenium</u>			
<u>wolfei</u>	Methanomicrobiales	56,000	28,500
<u>Methanococcus</u>			
<u>vannielii</u>	Methanococcales	83,000	43,000

TABLE III. Comparison of the properties of
three NADP reductases

ORGANISM	GROWTH TEMP. OPTIMUM	ENZYME TEMP. OPTIMUM	ENZYME PH OPTIMUM	K _m NADP	K _m F ₄₂₀
<u>Methanobacterium</u>					
<u>thermoautotrophicum</u>	65°C	60°C	8.0	40 μM	128 μM
<u>Methanococcus</u>					
<u>vannielii</u>	40°C	17-20°C	8.0	10 μM	8 μM
<u>Methanobrevibacter</u>					
<u>smithii</u>	40°C	45°C	7.5	41 μM	42 μM