C_1 TRANSFER REACTIONS FOR BIOSYNTHESIS IN METHANOGENS

BY

ZHAOSHENG LIN

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C_1 TRANSFER REACTIONS FOR BIOSYNTHESIS IN METHANOGENS

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ZHAOSHENG LIN

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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Abstract

Methanosphaera stadtmanae derives its energy from the reduction of CH_3OH, with H_2, to CH_4. CO_2 and acetate are required, however, for growth. Both $^{14}CH_4$ and $^{14}CO_2$ production were detected in whole cells using ^{14}C-formaldehyde or ^{14}C-serine plus H_2. Both formate and serine blocked the formation of $^{14}CO_2$ from formaldehyde in whole cells. These results implied that enzymes below the level of methylene-tetrahydromethanopterin in the common methanogenic pathway and a tetrahydromethanopterin (H_4M) dependent serine hydroxymethyltransferase (SHMT) were present in this organism. $^{14}CO_2$ was also detected from ^{14}C-formate in both whole cells and-cofactor-depleted cell-free extracts. However, no $^{14}CH_4$ could be detected from $^{14}CO_2$ or ^{14}C-formate plus H_2. Further investigations showed a low level of NADP dependent formate dehydrogenase activity. Long-term labelling results showed that ^{14}C-formate was incorporated specifically into histidine and RNA. These results suggested that formate might enter a C_1 pool distinct from the C_1-H_4M pool.

The activity of partially purified SHMT from several methanogens was tested using H_4M purified from *Methanobacterium thermoautotrophicum* Marburg, tetrahydrosarcinapterin purified from *Methanosarcina barkeri* and tetrahydrofolate (H_4F) as the potential C_1 carriers. H_4M dependent activities were found in *Mb. thermoautotrophicum* Marburg and *Msph. stadtmanae,* members of the *Methanobacteriales*. In members of the *Methanococcales,* the enzyme activity in *Methanococcus thermolithotrophicus* was H_4M dependent,

however, no activity was detected in *Methanococcus voltae*. In the four members of the *Methanomicrobiales* tested: *Methanolobus tindarius, Methanosaeta concilii, Methanospirillum hungatei* GP1 and *Ms. barkeri* Fusaro, the enzyme was strictly H_4F dependent. For all methanogens tested, the SHMT was air-stable.

The H_4F dependent SHMT in *Msp. hungatei* was purified to apparent homogeneity and characterized. The enzyme was found to be a 94 KDa homodimer. The optimal pH was found to be about 8.1 for serine and glycine synthesis. The Kms of the enzyme for H_4F, L-serine and glycine were 0.06 mM, 0.29 mM and 0.62 mM. The enzyme required pyridoxal phosphate for maximum activity. The N-terminal sequence and amino acid composition were also analyzed.

An air-sensitive, NADP-dependent secondary alcohol dehydrogenase was detected in *Msph. stadtmanae*. The enzyme converted 2-propanol and 2-butanol into the relevant ketones in the presence of NADP or vice versa with NADPH. Both 2-propanol and 2-butanol oxidation supported methanogenesis but not growth. Cell free extract activity increased when cells were grown in the presence of 2-propanol and 2-butanol as well as under H_2-limited conditions. Kinetic studies showed that Kms for 2-propanol and NADP were about twice as high as those of acetone and NADPH. The pH optimum for 2-propanol oxidation was about 9.2 while that for acetone reduction was about 8.6. It was therefore suggested that a physiological role of this enzyme could be for the reduction of acetone. This is consistent with the fact that acetone was much more toxic than 2-propanol to the cells.

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ADH	alcohol dehydrogenase
BCE	boiled-cell extracts
CAPS	3-[cyclohexylamino-]-1-propanesulfonic acid
CDCFE	cofactor-depleted cell-free extracts
CFE	cell-free extracts
CoA	Coenzyme A
CoM	2-mercaptoethanesulfonate
CPM	counts per minute
DSM	Deutsche sammling von mikroorganismen
FDH	formate dehydrogenase
F_{420}	hydroxy-5-deazaflavin
H_4F	tetrahydrofolate
H_4M	tetrahydromethanopterin
H_4S	tetrahydrosarcinapterin
HS-HTP	N-7-mercaptoheptanoyl-*O*-phospho-L-threonine
KDa	kilodalton
Mb	*Methanobacterium*
Mc	*Methanococcus*
MFR	methanofuran
Mg	*Methanogenium*
Ml	*Methanolobus*
Ms	*Methanosarcina*
Msp	*Methanospirillum*
Msph	*Methanosphaera*
Mst	*Methanosaeta*
MV	methyl viologen
NMR	nuclear magnetic resonance
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced NADP
PLP	pyridoxal 5′-phosphate
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHMT	serine hydroxymethyltransferase
TEMED	N, N, N′, N′-tetramethylethylene diamine
Tris	trihydroxymethylaminomethane
Tris-Cl	tris hydrochloride

Chapter I

Literature Review

The discovery that methane, the "combustible air", was produced in the sediments of streams, bogs and lakes where there was rich decaying vegetation has led to the subsequent extensive studies of methanogenic bacteria (Balch et al. 1979, Wolfe 1993). It was later found that methanogenic bacteria play an important role in the process of anaerobic degradation as consumers of hydrogen which is generated by the metabolic activities of syntrophic and fermentative bacteria. The phenomenon was named 'interspecies hydrogen transfer' (Ianotti et al. 1973). The activities of methanogenic bacteria form a common end product, methane, which is not only an important fuel source, but also contributes significantly to one of today's environmental concerns (Garcia 1990), the "greenhouse effect". Early stimulus for the interest of studying methanogens originated from this environmental significance of the organisms and from the later-found distinctive and diversified biochemical and physiological characteristics of methanogens (Jones et al. 1987, Boone et al. 1993).

I.1 Methanogens - a unique group of microorganisms

In late 1970's, Woese and associates developed a method of classifying living organisms based on homologies of partial sequences of the 16S ribosomal ribonucleic acid (rRNA) of many

diverse procaryotes and the corresponding small-subunit rRNA of eucaryotes (Fox et al. 1980, Woese and Fox 1977, Woese et al. 1978). Soon after that, the traditional morphological and physiological classification of methanogens was discarded in favour of this new taxonomic approach (Balch et al. 1979). The revolutionary method reclassified all living organisms into three domains: eucarya, bacteria, and archaea (Woese et al. 1990). This classification divided methanogens into three orders: the *Methanobacteriales*, the *Methanococcales* and the *Methanomicrobiales*.

As a group of archaea, methanogens have distinctive physiological and biochemical properties: a). They contain unique one-carbon unit carriers, such as methanofuran (MFR), tetrahydromethanopterin (H_4M), and CoM (2-mercaptoethanesulfonic acid) (Leigh et al. 1984, Daniels et 1984, Jones et al. 1985, Jones et al. 1987, DiMarco et al. 1990, Thauer et al. 1993). CoM has been known to be an essential component in the methanogenic pathway from all substrates (Taylor and Wolfe 1974, Balch and Wolfe 1979, Daniels et al. 1984, Lovley et al. 1984, Jones et al. 1987, DiMarco et al. 1990, Wolfe 1993); b). Methanogens contain novel electron carriers [such as coenzyme F_{420} (7,8-didemethyl-8-hydroxy-5-deazariboflavin 5'-phophate) and HS-HTP (7-mercaptohaptanoylthreonine phosphate)] (Eirich et al. 1978, Eirich et al. 1979, Diekert et al. 1980, Diekert et al. 1981, Eirich et al. 1982, Pfaltz et al. 1982).

Although metabolically restricted to strict anaerobic

environments, methanogens exhibit a very broad habitat diversity. Species have been isolated from virtually every habitat including freshwater and marine sediments, digestive and intestinal tracts of animals, anaerobic waste digesters, geothermal springs and both shallow and deep-sea hydrothermal vents (Smith and Hungate 1958, Zeikus and Wolfe 1972, Stetter et al. 1981, Huber et al. 1982, Miller et al. 1982, Jones et al. 1983a, Jones et al. 1983b, Whitman 1985, Zinder 1993).

To reflect this habitat diversity, individual methanogen species have displayed distinctive physiological and biochemical characteristics. One example is the substrate diversity of methanogens. So far, different methanogens have been found to grow on various substrates including H_2/CO_2, formate, methanol, acetate, and methylamines (Hippe et al. 1979, Jones et al. 1987, Ferry 1993, Keltjens et al. 1993). Some methanogens can also utilize both primary (Frimmer and Widdel 1989) and/or secondary alcohols as the electron donor for methanogenesis and support cell growth (Widdel 1986, Zellner and Winter 1987). The diverse physiological and biochemical characteristics of different methanogens are probably one of the the important features that has permitted them to live in such a broad range of anaerobic habitats.

To sum up, methanogens have distinct characteristics from other living organisms and also possess great diversity within the group in habitats, physiological and biochemical properties.

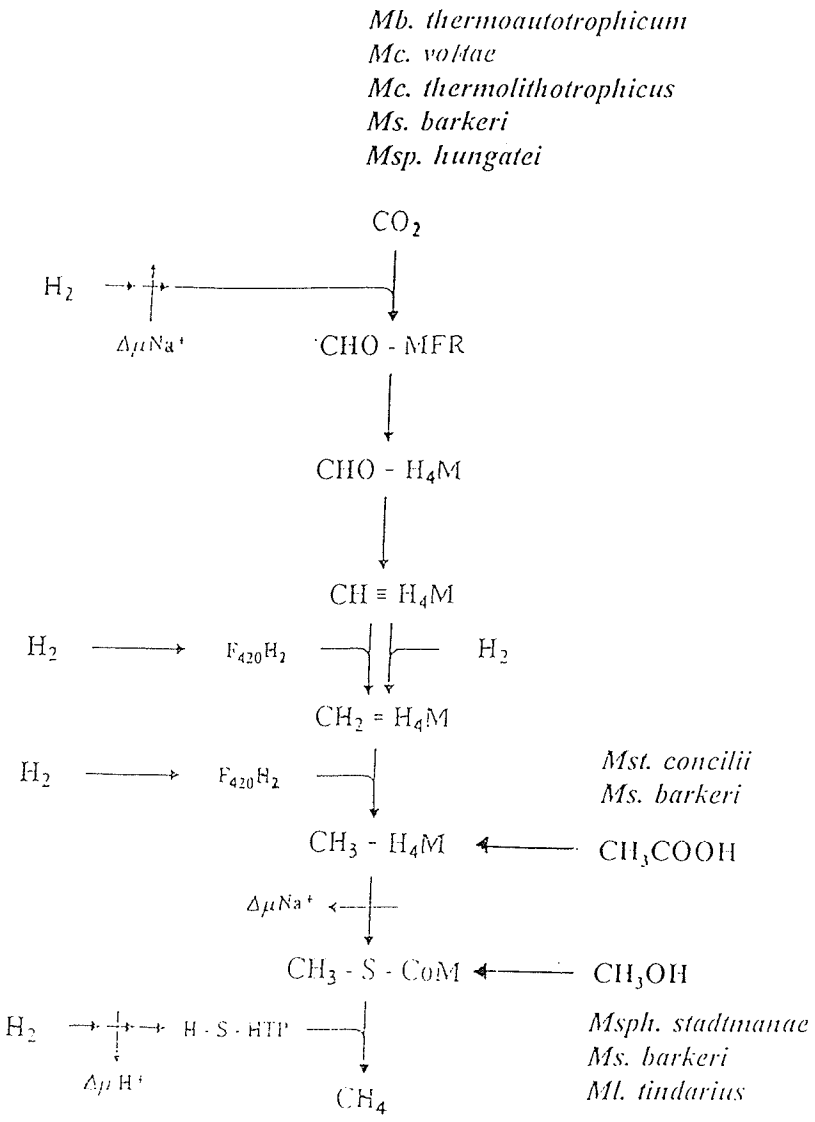
I.2 Methanogenesis and methanogenic pathway

Although some anaerobic eubacteria are capable of producing small amounts of methane under their regular growth conditions (Postgate 1969), methanogens are the only group of organisms known to couple methane synthesis to an energy conservation process (Thauer 1990, Deppenmeier et al. 1996). The methanogenic pathway has been extensively studied and elucidated in some methanogens. Two species of methanogens that have attracted the most attention of researchers for the elucidation of methanogenic pathways are *Methanobacterium thermoautotrophicum*, which is an autotrophic thermophile that requires no vitamin for growth (Zeikus and Wolfe 1972), and *Methanosarcina barkeri*, a methanogen that can grow on H_2/CO_2, methanol, acetate and methylamines (Hippe et al. 1979). The central one-carbon group carrier of the methanogenic pathway, H_4M or tetrahydrosarcinapterin (H_4S), has been identified and characterized from these two organisms respectively (Gunsalus and Wolfe 1978, van Beelen et al. 1984a, van Beelen et al. 1984b). Most of the enzymes that are involved in the methanogenic pathway in these two organisms have also been purified and characterized (Thauer et al. 1993). One of these enzymes, methylene-H_4M dehydrogenase, an enzyme that has been found to be essential for methanogenesis from CO_2, has also been used widely to identify and quantitate H_4M (Keltjens et al. 1986, Mukhopadhyay and Daniels 1989) and to assay other enzyme activities which form methylene- or methenyl-H_4M as the reaction product (Hoyt et al. 1986) because of

the spectral characteristics of methenyl-H_4M. Figure I.1 is given as a summary of methanogenic pathway originating from different substrates. The evolution of our understanding of the pathway can be followed in the following reviews (Wolfe and Higgins 1979, Daniels et al. 1984, Wolfe 1985, Jones et al. 1987, DiMarco et al. 1990, Thauer 1993, Deppenmeier et al. 1996). Only some or all of the cofactors and enzymes listed in the figure are involved in the methanogenic process, depending on the organisms and substrates used. The process of CO_2 reduction in the methanogenic pathway involves several C_1 carriers in the following orders: methanofuran (MFR), which is the C_1 carrier that reacts with CO_2 and forms a formyl-MFR; The next C_1 carrier involved is H_4M, which then carries several oxidation levels of C_1 derivatives; The methyl group is then transferred to CoM and subsequently reduced to methane. Although the methanogenic substrates may vary from species to species, the pathway of methanogenesis always involves the reduction process of a one-carbon unit (e.g., carbon dioxide, formate, methanol, methylamine, C_1 of acetate and trimethylamine) to methane as the cell's energy source.

The formation and transfer of various C_1-carrying H_4M derivatives and enzymes involved in the CO_2 methanogenic pathway are very similar to the C_1-carrying tetrahydrofolate (H_4F) and related enzymes of the acetogenic pathway from CO_2 in acetogens (Fuchs 1986, Ljungdahl 1986, Jones et al. 1987), although the electron carriers used are different. The pathway of acetate formation in autotrophic methanogens has been studied in *Mb*.

Figure I.1. Methanogenic pathways from various substrates (Thauer et al. 1993).


Mb. thermoautotrophicum
Mc. voltae
Mc. thermolithotrophicus
Ms. barkeri
Msp. hungatei
CO2
H2
ΔμNa+
CHO - MFR
CHO - H4M
CH ≡ H4M
H2
F420H2
H2
CH2 = H4M
H2
F420H2
Mst. concilii
Ms. barkeri
CH3 - H4M
CH3COOH
ΔμNa+
CH3 - S - CoM
CH3OH
H2
H - S - HTP
Msph. stadtmanae
Ms. barkeri
Ml. tindarius
ΔμH+
CH4

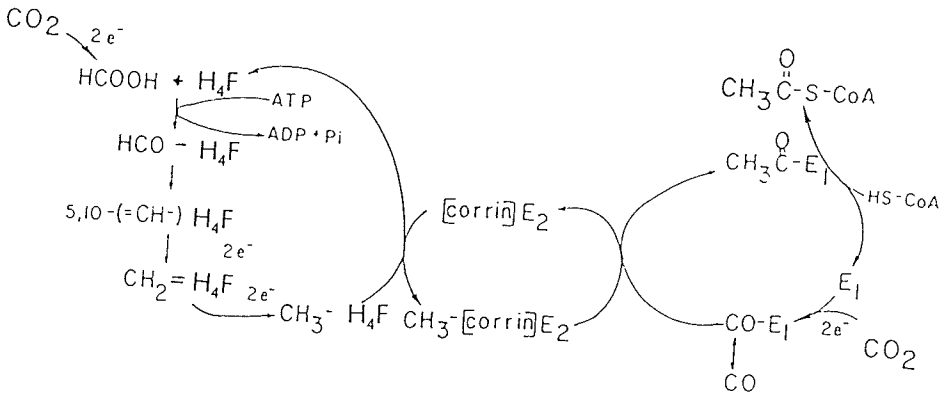
thermoautotrophicum (Stupperich and Fuchs 1984a, 1984b). The process involves a carbon monoxide dehydrogenase and a corrinoid-methyltransferase, which is similar to the acetogenic pathway in eubacteria (Jones et al. 1987). Figure I.2 presents a comparison of CO_2 fixation pathway between acetogens and autotrophic methanogens. In both cases, a pterin derivative (H_4M in methanogens and H_4F in acetogens) is involved as a C_1 carrier. The structural difference between folate and methanopterin will be discussed in section I.3.2. Besides the formation of methane and acetate, the synthesis of pyruvate and oxaloacetate also requires CO_2 in methanogens (Jones et al. 1987). Incomplete TCA cycles were found to be present in methanogens, operating mainly in the reductive direction. The synthetic pathways of various carboxylic acids varies among species (Figure I.3).

I.3 C_1-group transfer for the biosynthesis of amino acids, purines and thymidine

The biosynthesis of some amino acids (eg. methionine and histidine), purines and thymidylate also requires the process of a C_1-group transfer. It is well known that in eubacteria and eucaryotes, H_4F is involved in the C_1-group transfer for the synthesis of the aforementioned compounds (figure I.4) (Walsh 1979, Scrimgeous 1977). The synthesis of histidine and purines requires formyl-H_4F and methenyl-H_4F, while the synthesis of methionine and thymidylate is involved with methyl-H_4F.

Figure I.2. Pathway of acetate synthesis in acetogenic eubacteria (A) and autotrophic methanogens (B) (Jones et al. 1987). E_1 represents the nickel-containing CO dehydrogenase, E_2 (corrin) is the cobamide-containing protein involved in C_1 transfer.

A


CO2
2e⁻
HCOOH + H4F
ATP
ADP + Pi
HCO - H4F
5,10-(=CH-) H4F
2e⁻
CH2= H4F
2e⁻
CH3- H4F
[corrin] E2
CH3-[corrin] E2
CH3 C-S-CoA
CH3 C-E1
HS-CoA
E1
CO-E1
2e⁻
CO2
CO

B

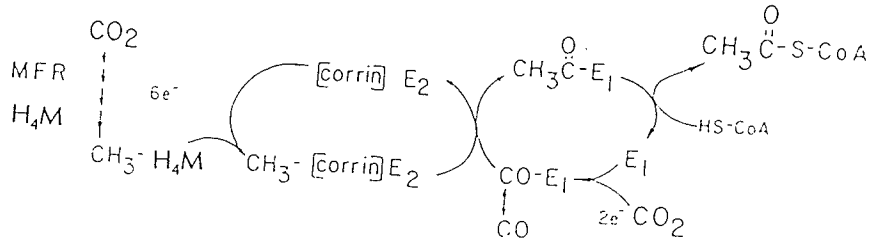

CO2
MFR
H4M
6e⁻
CH3- H4M
[corrin] E2
CH3-[corrin] E2
CH3 C-E1
CH3 C-S-CoA
HS-CoA
CO-E1
E1
CO
2e⁻ CO2

Figure I.3. The incomplete TCA cycle in methanogens (Jones et al. 1987). Solid lines, cycle found in *Mb. thermoautotrophicum*; Broken lines, cycle found in *Ms. barkeri*.

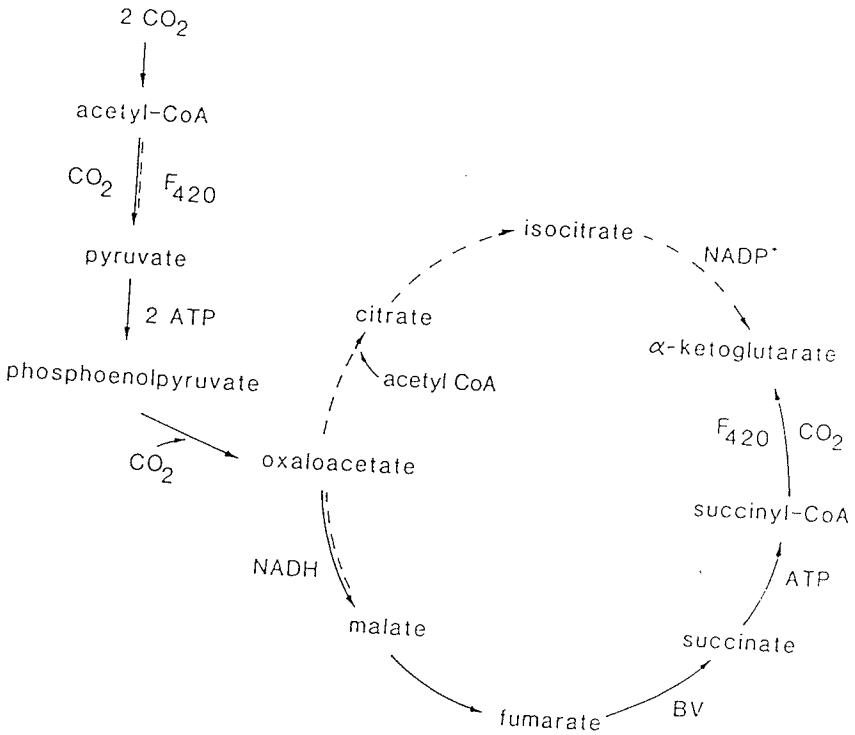
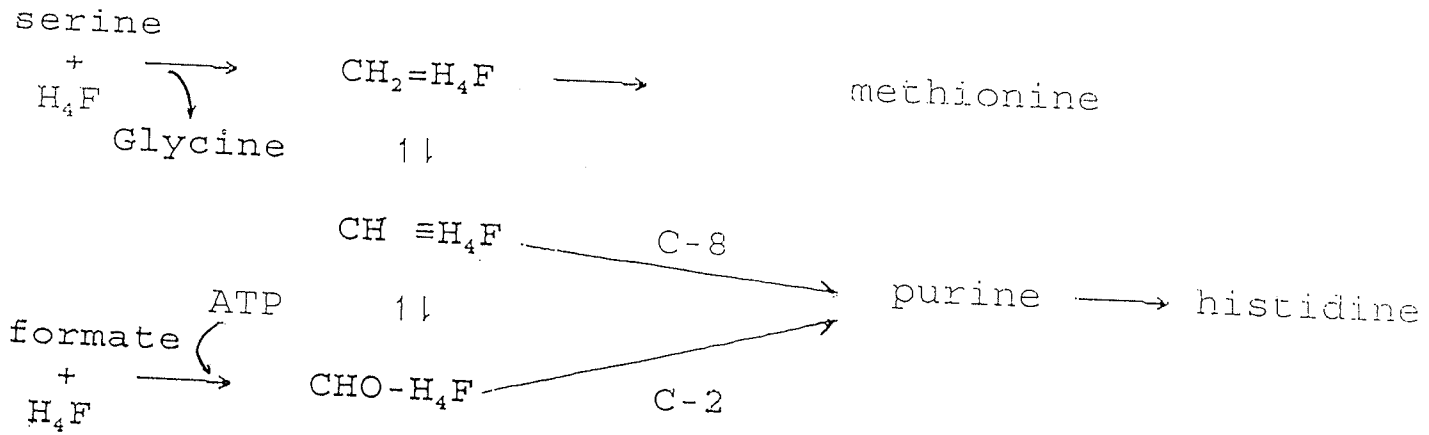

2 CO2
acetyl-CoA
CO2
F420
pyruvate
2 ATP
phosphoenolpyruvate
CO2
oxaloacetate
citrate
acetyl CoA
isocitrate
NADP+
α-ketoglutarate
F420
CO2
succinyl-CoA
ATP
succinate
BV
fumarate
NADH
malate

Figure I.4. Some C_1 transfer reactions in eubacteria (Scrimgeous 1977).


serine
+
H_4F
Glycine
$CH_2=H_4F$
methionine
$CH \equiv H_4F$
C-8
ATP
formate
+
H_4F
$CHO-H_4F$
C-2
purine
histidine

In methanogens, structural analogues of H_4F, H_4M (Blakley 1969, Keltjens et al. 1983), and H_4S (van Beelen et al. 1984a) are found present and functioning as the C_1-group carrier in the methanogenic pathways as mentioned above. In *Msp. hungatei*, a structurally undetermined pterin derivative yet known to be different from both methanopterin and sarcinapterin was found to be present as the major pterin which may function as the C_1 carrier in the methanogenic pathway (Gorris and van der Drift 1986, Keltjens and Vogels 1988). So far, there is only indirect evidence that methanopterin or sarcinapterin might be involved in the C_1-required biosynthesis in methanogens in the same way as folic acid in eubacteria and eucaryotes. The following sections will summarize results concerning C_1-group transfer for biosynthesis in methanogens and the potential cofactors which may be involved.

I.3.1 Sources of one-carbon units for the synthesis of purine, histidine and methionine in methanogens

Most of the information pertaining to one-carbon transfer for the biosynthesis of some amino acids and purine in methanogens comes from ^{14}C long-term labelling and ^{13}C-NMR (nuclear magnetic resonance) techniques. Several methanogens were used for the studies including *Mb. thermoautotrophicum* (Taylor et al. 1976, Holder et al. 1985), *Msph. stadtmanae* (Choquet et al. 1994, Miller et al. 1995), *Methanosaeta concilii* (Ekiel et al. 1985), *Methanosarcina barkeri* (Ekiel et al. 1985, Choquet et al. 1994,

Methanococcus voltae (Choquet et al. 1994) and *Msp. hungatei* (Ekiel et al. 1983, Choquet et al. 1994). The results can be summarized as the following (Table I.1): a). For the synthesis of methionine, the methyl group of methionine comes from the methanogenic carbon sources, either CO_2 (for *Mb. thermoautotrophicum*, *Mc. voltae*, *Ms. barkeri* and *Msp. hungatei*), C-2 of acetate (for *Mst. concilii*) except in *Msph. stadtmanae*; In *Msph. stadtmanae*, C-2 of acetate or C-3 of serine was the major source while methanol was the minor source. b). For the synthesis of purine, the sources of C-2 and C-8 vary depending on the organisms and the contents of media. In *Mc. voltae*, C-2 and C-8 of purine were from CO_2. In *Mb. thermoautotrophicum*, CO_2 is the only one-carbon source in the medium that does not contain formate. However, when [^{14}C] formate was added to the medium, purine but not methane was labelled by formate. In *Msph. stadtmanae*, C-8 of purine was labelled by C-2 of acetate in both media with or without formate. In cultures without formate added into the medium, C-2 of purine was labelled by C-2 of acetate and methanol. In cultures with formate added, C-2 of purine was labelled mainly by formate. In *Ms. barkeri* and *Msp. hungatei*, both C-2 and C-8 of purine were from C-2 of acetate but not from CO_2, the methanogenic carbon source of both organisms.

The one-carbon source for one-carbon transfer in the biosynthesis of thymidylate in methanogens has not been reported so far. It would be interesting to see whether or not there are differences in terms of labelling patterns and cofactors involved in thymidylate synthesis among various methanogens. Since

dihydrofolate reductase is one of the key enzymes that are involved in the biosynthesis of thymidylate in eubacteria and eucaryotes, it would be informative also to see if a dihydromethanopterin reductase is present and involved in the synthesis of thymidylate if H_4M is the one-carbon carrier in the one-carbon transfer process.

Based on the results summarized above, the following conclusions can be made: a). H_4M is involved in some biosynthetic one-carbon transfer reactions in methanogens, such as the synthesis of methionine. This is consistent with the fact that the methyl group of methionine comes from the methanogenic substrates in various methanogens (table I.1). It is also supported by the fact that in *Mb. thermoautotrophicum*, the serine hydroxymethyl transferase (SHMT) was H_4M dependent (Hoyt et al. 1986). It has also been demonstrated that C_1-H_4M (such as methylene-H_4M) from serine would be greatly diluted by the C_1-H_4M pool of methanogenesis (Holder et al. 1985). This is also in support of the above conclusion. b). Other one-carbon carriers may be involved in the aforementioned C_1-required biosynthesis in some methanogens, as indicated by the labelling patterns in several methanogens tested. In *Ms. barkeri* and *Msp. hungatei* (Ekiel at al 1983, 1985, Choquet et al. 1994), the C-2 of acetate, which is the precursor of C-3 of serine, labelled C-2 and C-8 of purine, while CO_2 was the source of methyl group of methionine. It can therefore be concluded that a non-H_4M dependent SHMT might be involved in the biosynthetic process, because all C_1-H_4M from a non-methanogenic carbon source

Table I.1 Origin of C_1 in methane, methyl group of methionine, C-2 and C-8 of purine in methanogens

Organism	CH_4	methyl group of methionine	C-2 of purine	C-8 of purine
Order Methanobacteriales				
Fam. *Methanobacteriaceae*				
Methanobacterium thermoauto-trophicum[a]	CO_2	CO_2	CO_2/formate[a]	CO_2/formate[a]
Methanosphaera stadtmanae[b]	MeOH	C-2 of Acet[g]., MeOH[h]	C-2 of Acet., MeOH/formate	C-2 of Acet.
Order Methanococcales				
Fam. *Methanococcaceae*				
Methanococcus voltae[c]	CO_2	CO_2	CO_2	CO_2
Order Methanomicrobiales				
Fam. *Methanomicrobiaceae*				
Methanospirillum hungatei[d]	CO_2	CO_2	C-2 of Acet.	C-2 of Acet.
Fam. *Methanosarcinae*				
Methanosarcina barkeri[e]	CO_2	CO_2	C-2 of Acet.	C-2 of Acet.
Methanosaeta concilii[f]	C-2 of Acet.	C-2 of Acet.	C-2 of Acet.	C-2 of Acet.

a. Taylor et al. 1976. The position of labels was not specified.
b. Miller et al. 1995, Choquet et al. 1994.
c. Choquet et al. 1994.
d. Choquet et al. 1994, Ekiel et al. 1983.
e. Choquet et al. 1994, Ekiel et al. 1985.
f. Ekiel et al. 1985.
g. Acet, acetate.
h. MeOH, methanol.

Table I.2. Kinetic constants of SHMT from various sources

will be greatly diluted by the methanogenic one-carbon pool carried by H_4M and therefore would be difficult to detect as the major label in certain compounds of cell materials. In *Mb. thermoautotrophicum* and *Msph. stadtmanae*, formate can not enter the methanogenic pathway (Taylor et al. 1976, Miller and Wolin 1985) yet it labelled purine (Taylor et al. 1976, Choquet et al. 1994). The involvement of a one-carbon carrier other than H_4M would be suggested based on these findings in both organisms. In favour of this hypothesis, the formyl-H_4F synthetase level found in *Mb. thermoautotrophicum* was more than 100-fold higher than that of other methanogens tested (Ferry et al. 1976), and a formate auxotroph of *Mb. thermoautotrophicum* was found which contains a low level of formate dehydrogenase (Tanner et al. 1989). c). The labelling pattern in *Mst. concilii* can not be explained clearly yet, because C-2 of acetate is also the methanogenic substrate. It is impossible to differentiate the C_1 labellings between the C_1 pool of methanogenic pathway (which is carried by H_4M) and the C_1 source derived from reactions catalysed by SHMT (the C_1 carrier for the reaction was unknown). Enzymatic studies of SHMT in this organism are needed to understand the situation.

However, how is the C-2 of acetate incorporated into purines in *Ms. barkeri* and *Msp. hungatei*? If it enters the C_1 pool through the serine hydroxymethyltransferase (SHMT), then what is the C_1 carrier in the C_1 transfer reaction? And how does formate enter the C_1 pathway for biosynthesis in the two members of *Methanobacteriaceae* studied in this context? Is H_4F involved in

these reactions? All these questions still remain to be answered.

I.3.2 Structures of tetrahydrofolate (H_4F), tetrahydromethanopterin (H_4M) and tetrahydrosarcinapterin (H_4S)

To further understand the possibility of the involvement of folic acid in the C_1-required biosynthetic reactions in methanogens, it would be helpful to compare the structural and chemical characteristics of both H_4F and H_4M as well as their C-carrying derivatives.

Folic acid was first isolated from several biological materials in the 1940s. Several purification methods of folic acid were devised soon after that, using assays of two responses to the vitamin: the growth of certain bacteria which can not synthesize folic acid and the prevention of anemia in chicks (Scrimgeour 1977). The structure of folate in nature was determined to be a pteroyl triglutamate, while the folic acid commonly used in the laboratory is a pteroyl monoglutamate (Blakley 1969, Baugh and Krumdieck 1971).

H_4F, which is the only reactive folate derivative in $_1C$ transfer reaction, is susceptible to rapid oxidative degradation. Oxidation of the compound can be significantly decreased when mild reducing agents such as 2-mercaptoethanol or ascorbate are added to the H_4F solution (Chippel and Scrimgeour 1970). One-carbon groups at three different oxidation levels can be enzymatically bound to H_4F to form formyl-, methenyl-, methylene- and methyl-H_4F (Brewer

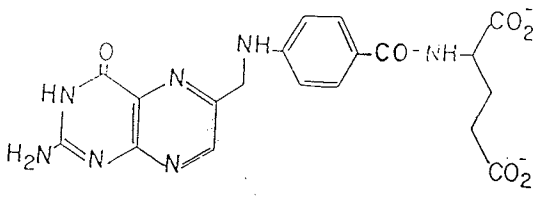
et al. 1970, O'Brien et al. 1973, Clark and Ljungdahl 1984). Rapid nonenzymatic formation of methylene-H_4F can also occur by simply adding formaldehyde to the H_4F solution (Benkovic and Bullard 1973).

Methanopterin was first detected from *Mb. thermoautotrophicum* as a fluorescent compound with an absorbance maximum at 342 nm, and was given the name Factor 342 or YFC (yellow fluorescent compound) (Gunsalus and Wolfe 1978). The chromophoric portion of the compound was later determined to be a pterin, and was named as methanopterin (Keltjens et al. 1983). The complete structure of methanopterin was not elucidated until the identification of several degradation products and the use of two-dimensional nuclear-magnetic-resonance techniques (van Beelen et al. 1984b). The structure of methanopterin is similar to that of folic acid. The major differences between the two compounds are that C-7 and C-11 are methyl substituted in methanopterin, and the side chains consist of different components. Another pterin derivative, analogue of methanopterin, sarcinapterin has so far been found in *Methanosarcina, Methanolobus, Methanosaeta* (formerly named as *Methanothrix)* and a *Methanococcus* sp. (Keltjens and Vogels 1988). The compound contains an additional glutamyl moiety conjugated by an amide linkage to the α-carboxylic group of α-hydroxyglutarate of methanopterin (van Beelen et al. 1984a). Similar to H_4F, H_4M can carry C_1 groups such as formyl-, methenyl-, methylene- and methyl- at the N-5 or N-5 and N-10 positions catalyzed by different enzymes and form H_4M derivatives containing various one-carbon

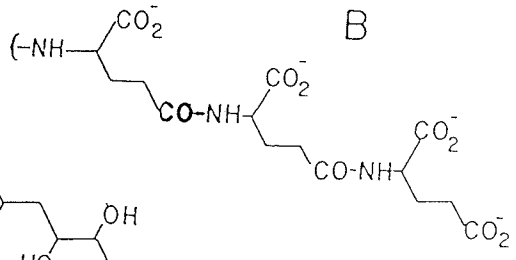
groups (Escalante-Semerena et al. 1984b, Donnelly et al. 1985, Donnelly and Wolfe 1986). Formaldehyde can also react with H_4M chemically and form methylene-H_4M (Escalante-Semerena et al. 1984a, Escalante-Semerena and Wolfe 1984). This characteristic of H_4M has been found very useful for the studies of methylene-H_4M-related reactions, for example, determination of sodium-requiring reactions in the methanogenic pathway using formaldehyde and hydrogen (Kaesler and Schoenheit 1989, Müller et al. 1990). Structures of H_4F, H_4M, and H_4S are shown in figure I.5.

In methanogenic bacteria, whether or not folic acid is present is still not clear. Levels of folic acid in several methanogenic bacteria including *Mb. thermoautotrophicum* and *Ms. barkeri* have been studied using microbiological assay methods (Leigh 1983, Worrel and Nagle 1988). The levels of folate in both organisms were found to be about 10-fold less than those of eubacteria. However, acid-hydrolysed methanopterin could also stimulate the growth of the folate-requiring bacteria (Worrel and Nagle 1988). This suggested that the reported levels of folate in methanogens might not accurately reflect the actual situations, since the heat-treated extracts of methanogens might cause oxidative degradation of methanopterin (or sarcinapterin) which may have similar effects on the bacterial growth as the acid-hydrolysed methanopterin. More accurate and specific assay methods may be needed to further investigate the presence and levels of folate in methanogens.

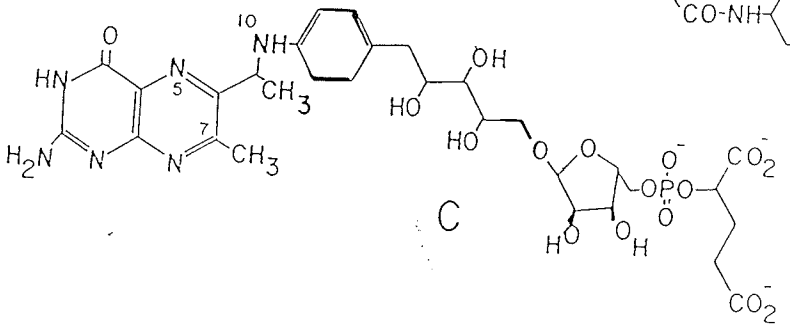
Figure I.5. Structures of folate and methanopterin derivatives (Jones et al. 1987). A, folic acid; B, the pteroyl triglutamate; C, methanopterin; D, sarcinapterin; E, H_4M.



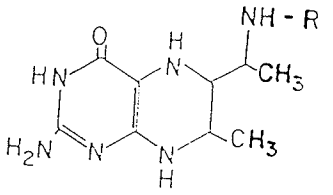
A



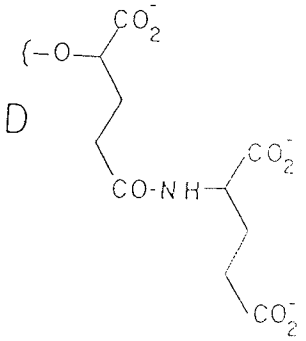
B



C



E



D

I.4 Serine hydroxymethyl transferase

Serine hydroxymethyl transferase (SHMT) is one of the key enzymes that is responsible for C_1-group transfers in eubacteria and eucaryotes. It catalyzes the reversible conversion of L-serine to glycine and a one-carbon group at the oxidation level of formaldehyde (Schirch and Mason 1963, Schirch and Diller 1971 O'Connor and Hanson 1975, Schirch and Peterson 1980). This reaction is in many organisms the major means of generating one-carbon groups required for other biosynthetic pathways. The enzyme has been purified and well characterized in many eubacteria (O'Connor and Hanson 1975, Hopkins and Schirch 1985, Schirch et al. 1985, Miyazaki et al. 1987a, Miyazaki et al. 1987b) and eucaryotes (Schirch and Peterson 1980, Schirch et al. 1986, Strong et al. 1990, Sukanya et al. 1991, Jagath-Reddy et al. 1995).

In methanogens, the enzyme has been purified and preliminarily characterized in *Mb. thermoautotrophicum* ΔH(Hoyt et al. 1986). The enzyme was found to be H_4M but not H_4F dependent. Earlier work on the purification and characterization of SHMT in *Methanobacillus omelianskii* has also been reported (Wood et al. 1965). The enzyme was determined to be H_4F dependent. However, this methanogenic culture was later found to contain a mixture of two organisms, a methanogen identified as *Mb. bryantii*, and an ethanol-oxidizing and hydrogen-producing eubacterium, the S organism (Bryant et al. 1967).

I.4.1 Requirement of cofactors and cations for SHMT activity

Pyridoxal-5'-phosphate (PLP) was found to be a required component for the SHMT activity early in 1955 (Blakley 1955). It was found that PLP was bound at the active site as a Schiff base to the ε-amino group of a lysyl residue (Schirch and Mason 1962, Schirch and Jenkins 1964, Cheng and Haslam 1972, Schirch 1975). The enzyme-bound PLP (absorbance maximum at 428 nm) is converted into the Gly-PLP Schiff base (425 nm) upon addition of glycine, which is an important intermediate for the hydroxymethyl transfer reaction.

In most of the investigations of SHMT carried out so far in eubacteria and eucaryotes, an absolute requirement of PLP has been demonstrated. One exception was reported in Mung Bean (*Vigna radiata*) (Sukanya et al. 1991). The SHMT from Mung bean displayed non-PLP-dependent enzyme activity, and lacked the characteristic visible absorbance spectrum of a PLP-enzyme complex. Furthermore, the SHMT activity was not inhibited by the addition of penicillamine, an inhibitor of PLP-dependent enzymes (Sukanya et al. 1991). It was suggested that a covalently bound carbonyl group might replace PLP in the catalytic process of the hydroxymethyl transfer in Mung bean. In *Mb. thermoautotrophicum* (Hoyt et al. 1986), PLP was reported to be required for maximal SHMT activity. The addition of 0.3 mM PLP stimulated an activity increase of 23%.

Another required cofactor for SHMT activity found in eubacteria and eucaryotes is tetrahydrofolate (H_4F) (Schirch and

Mason 1962, Schirch and Jenkins 1964, Miyazaki et al. 1987). In *Mb. thermoautotrophicum* (Hoyt et al. 1986), the H_4F analogue, H_4M, was required. The purpose of H_4F in the hydroxymethyl transfer reaction is to carry the methylene group for either the synthesis of serine or glycine (Chen and Schirch 1973). H_4F interplays with the PLP-enzyme complex by presenting the methylene group in the correct orientation. For the H_4M-dependent SHMT, although no catalytic mechanistic studies have been performed, the function and reaction mechanism of H_4M in the process of hydroxymethyl transfer should be similar to those of H_4F in the H_4F-dependent SHMT, since the structure of H_4M is similar to H_4F. H_4M has also been known as a carrier of C_1 units at various oxidation levels: formyl-H_4M (Donnelly and Wolfe 1986), methenyl-H_4M (Donnelly et al. 1985), methylene-H_4M and methyl-H_4M (Escalante-Semerena et al. 1984), as was discussed in a previous section (I.3.2), similar to H_4F in eubacteria and eucaryotes.

In other methanogens, whether or not H_4F may function as the hydroxymethyl carrier for the interconversion of serine and glycine is still not clear, although ^{13}C-NMR and ^{14}C-long-term labelling works have shown possible involvement of a non-H_4M one-carbon carrier in the process of one-carbon transfer for biosynthesis (Taylor et al. 1976, Ekiel et al. 1983, 1985, Choquet et al. 1994). To further clarify these possibilities, enzymatic studies should be performed. The development of more specific and sensitive assay methods for the determination of folate and its derivatives may also be very helpful, since in methanogens, folate, if required,

may be required only at a very low level. On the other hand, the levels of methanopterin in many methanogens are very high which may interfere with the detection of folate because of the structural analogy as indicated previously.

Metal ions have also been demonstrated to be required for maximal SHMT activity in eubacteria (Nakamura et al. 1973, O'Connor and Hanson 1975, Schirch et al. 1985), eucaryotes (Schirch and Mason 1963) and a methanogen (Hoyt et al. 1986). Both mono- and divalent cations including K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} have been reported to stimulate SHMT activity in yeast and a facultative methylotrophic bacterium (Nakamura et al. 1973, O'Connor and Hanson 1975). The effect of different cations in SHMT activity of these organisms was shown to vary depending on the growth substrate used (O'Connor and Hanson 1975). In *Mb. thermoautotrophicum* (Hoyt et al. 1986), *E. coli* (Schirch et al. 1985) and some eucaryotic cells (Schirch and Mason 1963), Mg^{2+} was found to stimulate SHMT activity significantly.

I.4.2 Substrate specificity and kinetic constants of SHMT

Besides catalyzing the cleavage of serine into glycine and thus providing the one-carbon group for biosynthetic purposes in the presence of H_4F or H_4M in most eubacteria, eucaryotic cells and other microorganisms, SHMT also catalyzes the reverse direction as the physiological function of the enzyme in some organisms, such as strains of *Hyphomicrobium methylovorum* (Miyazaki et al. 1987). In

these organisms, SHMT plays a key role in the assimilation pathway of one-carbon compounds by synthesizing serine, the main intermediate of the pathway, from formaldehyde and glycine.

Several other amino acids can also be used as substrates of SHMT, forming various products depending on the substrates utilized. In *Euglena gracilis* z (Sakamoto et al. 1991), cytosolic SHMT was found to be able to cleave L-threonine and L-allothreonine into glycine and acetaldehyde. D-threonine and D-allothreonine could not be cleaved by the SHMT. Similar results have also been reported in the cytosolic and mitochondrial SHMT of rabbit liver (Schirch and Ropp 1967, Schirch and Gross 1968, Schirch and Diller 1971, Schirch and Peterson 1980). In the cytosolic and mitochondrial SHMT of rat liver and maize seedlings reported by Masuda et al. (1980, 1987), the enzyme was able to decompose L-allothreonine but not D-allothreonine, L- and D-threonine. Other amino acids that can undergo decomposition by the catalysis of SHMT are phenylserine (Ching and Kallen 1979, Miyazaki et al. 1987) and D-alanine (Schirch and Jenkins 1964, Schirch et al. 1985). Phenylserine can be cleaved to glycine and benzaldehyde while D-alanine reacts with enzyme bound PLP and forms free pyridoxamine phosphate and pyruvate.

The enzyme kinetics have been well studied in different organisms. The affinity for various substrates differs greatly from organism to organism. Table I.2 lists some Km values of several different substrates of various organisms. The Km value for glycine ranges from 0.046 to 0.4 mM, depending on the sources of the

Table I.2. Kinetic constants of SHMT from various sources

Source of enzymes	Km (mM) for Glycine	L-serine	H_4F	References
Mb. thermo-autotrophicum	NA[a]	2.8	1.8[b]	Hoyt et al. 1986
E. coli	0.85	0.8	0.08	Schirch et al. 1985
H. methylo-vorum GM2	0.046	0.15	NA	Miyazaki et al. 1987
Leukemia cell L1210	NA	0.5	0.3	Strong et al. 1990
Sheep liver, cytosolic	NA	1.0	0.82	Jagath-Reddy et al. 1995
Rabbit liver, cytosolic	0.4	0.6	0.015	Schirch and Diller 1971
Rabbit liver, mitochondrial	0.13	0.6	0.025	Schirch and Peterson 1980

a. Not available.
b. H_4M was used instead.

enzyme. The Kms for serine and H_4F also differ greatly, ranging from 0.15 to 2.8 mM and 0.025 to 0.82 mM respectively. Differences in affinity of the enzyme for glycine and serine depend on the physiological role of SHMT in the organism. In *H. methylovorum*, a organism that relies on the synthesis of serine from glycine, the affinity of SHMT for glycine was much higher than the affinity for serine (Miyazaki et al. 1987). In many other cases (Schirch and Peterson 1980, Schirch et al. 1985), the affinities of the enzyme for glycine and serine were similar, indicating that the physiological roles might be to synthesize either glycine or serine depending on the growth requirement of the cells under specific conditions. In *E. coli* (Schirch et al. 1985), synergistic binding of SHMT to H_4F and L-serine has been found. The Km values for serine or H_4F decreased in the presence of increasing concentrations of the alternate substrate.

The affinity of SHMT for L-threonine, L-allothreonine and DL-phenylserine has also been studied in several organisms. In *Euglena gracilis* Z (Sakamoto et al. 1991), the cytosolic SHMT was found to possess about 10-fold higher affinity for L-allothreonine than that of L-threonine. The affinity of the enzyme for L-allothreonine was greatly decreased by the addition of glycine and L-serine, indicating that these amino acids inhibit the enzyme activity competitively. In *E. coli* (Schirch et al. 1985), the Km of SHMT for L-allothreonine was 1.5 mM, which is about 5-fold higher than the Km for serine and glycine but is very close to that for L-allothreonine in *E. gracilis* Z. In *H. methylovorum* (Miyazaki

et al. 1987), Km for phenylserine was determined to be 33 mM, more than 100-fold higher than the Km for glycine and L-serine.

I.4.3 Purification and properties of SHMT

SHMT has been purified to homogeneity from several eubacteria (O'Connor and Hanson 1975, Schirch et al. 1985, Miyazaki et al. 1987) and eucaryotes (Schirch and Peterson 1980, Strong et al. 1990, Sukanya et al. 1991, Jagath-Reddy et al. 1995). The molecular mass of SHMT has been found to vary from source to source. However, the molecular weights of SHMT from eucaryotic cells are usually greater than those of other sources. A molecular structure of SHMT containing four subunits with identical molecular weights has been determined for several cytosolic and mitochondrial enzymes of eucaryotic cells (Schirch and Peterson 1980, Sukanya et al. 1991). The native enzymes' molecular weights are reported to be about 215 KDa in most cases, with subunit molecular weight of 53 KDa. Although there are similarities in quaternary structure and size of eucaryotic SHMT, there are also different characteristics found among enzymes from different sources. One such example is the difference of substrate specificity between the cytosolic and mitochondrial SHMT of rabbit liver (Schirch and Diller 1971, Schirch and Peterson 1980). The enzymes from both sources have similar affinities for both serine and H_4F, however, the affinity for glycine and the rate of cleaving L-threonine and allothreonine are very much different between the two isoenzymes. The difference

in amino acid composition has also been displayed between cytoplasmic and mitochondrial SHMT of rabbit liver (Schirch and Peterson 1980).

In several eubacteria (O'Connor and Hanson 1975, Schirch et al. 1985, Miyazaki et al. 1987) and a methanogen (Hoyt et al. 1986), the enzymes have been found to have molecular weights of 90 to 100 KDa. The eubacterial enzymes all contain two subunits of identical molecular mass.

In both eubacteria and eucaryotes, the enzyme displays similar spectral characteristics (Schirch and Mason 1962, Schirch and Mason 1963, Schirch and Ropp 1967, Schirch and Peterson 1980, Schirch et al. 1985, Miyazaki et al. 1987). The apoenzyme has an absorbance peak at 340 nm. The spectrum changes differently when apoenzyme binds different substrates. The holoenzyme containing PLP usually has a yellow colour and absorbs maximally between 415-425 nm. When glycine is added to the holoenzyme, the absorbance peak shifts to 495 nm. A change in pH can cause changes of the absorbance spectra of enzyme and enzyme-substrate complex. The spectral studies have played an important role in elucidating the enzyme mechanism because they provide information on the structures of enzyme-cofactor and enzyme-substrate complexes.

The SHMT genes from both eubacteria and eucaryotes have been successfully isolated and sequenced (Stauffer et al. 1981, Schirch et al. 1985, Hopkins and Schirch 1986, Jagath-Reddy et al. 1995), and the gene is designated glyA. Specific amino acid residues including arginine, cysteine, histidine and lysine have been found

essential for SHMT activity. A lysyl residue has long been known to be part of the active site, binding PLP to its ε-amino group and forming the Schiff base which is responsible for binding the amino acid substrates (Chen and Schirch 1973, Schirch et al. 1985, Jathga-Reddy et al. 1995). In the *E. coli* enzyme this lysyl residue is at position 229 (Plamann et al. 1983). The Schiff base can be reduced with sodium borohydride, forming a stable secondary amine between PLP and the protein. The isolation of a PLP-containing active site peptide was facilitated by the reduction. The sequence of this active site peptide of both mammalian and *E. coli* SHMT shows that it contains a histidyl residue to the N-terminal side of the active site lysine (Schirch 1982, Bossa et al. 1976). For comparative kinetic studies, this histidyl residue in *E. coli* has been changed to asparagine by site-directed mutagenesis (Hopkins and Schirch 1986). It is shown that histidine 228 is not an essential component for enzyme catalysis, however, it does significantly affect the affinity of the enzyme for its amino acid substrates and H_4F as well as its derivatives. These data are different from what has been found in sheep liver cytosolic SHMT (Manohar and Rao 1984), in which the histidyl residue is essential for enzyme activity.

Cysteine was first found to be implicated as being at the active site of SHMT by using methyl methanethiosulfonate as the active site probe for the enzyme (Gavilanes et al. 1982). Several later reports further demonstrated the involvement of cysteine residues in the active site (Manohar and Rao 1984, Schirch et al.

1985, Miyazaki et al. 1987). In *E. coli,* the addition of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to the enzyme caused the complete loss of enzyme activity. This enzyme has been known to contain 3 sulfhydryl groups, two buried and one exposed, determined by titrating the native enzyme with DTNB which reacted only with the exposed sulfhydryl group. The incubation of the inactive enzyme with dithiothreitol completely restored the enzyme activity (Schirch et al. 1985). Similar results were obtained when the enzyme from a methylotrophic bacterium, *H. methylovorum* GM2, was treated similarly (Miyazaki et al. 1987). In sheep liver (Schirch et al. 1973, Manohar and Rao 1984), the involvement of one or more cysteine residues in the active site of SHMT was postulated based on results of chemical modification of the amino acid residue. In both cytosolic and mitochondrial SHMT of rabbit liver, no cysteine was detected when the enzyme was titrated with DTNB (Schirch and Peterson 1980). However, this is not consistent with the sequence data in rabbit liver cytosol that 9 cysteine residues were present in each of the 4 subunits (GenomeNet accession #p07511).

Another basic amino acid residue, arginine, was also found to be important for the catalytic function of the enzyme from eucaryotic cells, using a chemical modification method (Manohar and Rao 1984, Usha et al. 1992). Further studies using [7-^{14}C] phenylglyoxal as a reagent for chemical modification have discovered that two arginine residues were modified per subunit of SHMT and that the modification of these residues was prevented by pretreating the enzyme with H_4F. A spectrally discernible 495-nm

intermediate, characteristic of amino acid substrates-bound native enzyme, could not be observed in the phenylglyoxal-modified enzyme. There was also no enhancement of the rate of exchange of the α-proton of glycine upon addition of H_4F in the modified enzyme, which has occurred in the non-modified enzyme. Sequencing the tryptic peptides indicated that Arg-269 and Arg-462 were the sites of phenyglyoxal modification (Usha et al. 1992). It was suggested that the function of the arginine residues in the sheep liver cytosolic enzyme is for the binding of H_4F.

I.5 Objectives

Based on the aforementioned information, objectives of this project were to investigate the involvement of H_4M and other potential C_1 carriers (such as H_4F) in the C_1 transfer reactions for biosynthesis in methanogens, with two major approaches.

In the first approach *Msph. stadtmanae* was chosen as a model organism to study [^{14}C] short- and long-term labelling patterns of various potential C_1 substrates (based on the current understanding of the common methanogenic pathway), because this organism possesses most of the enzymes (Schwörer and Thauer 1991) and H_4M for methane production but is unable to generate methane from CO_2 (Miller and Wolin 1985). The outcome was expected to answer the following questions: **(1)** Where is the break of the methanogenic pathway that causes the inability of the organism to reduce CO_2 to methane? **(2)** Is H_4M involved in biosynthetic reactions? And since

the literature has been confusing with respect to the presence of H_4F as a potential C_1 carrying cofactor in methanogens (Leigh 1983, Worrell and Nagel 1988) along with H_4M, we also looked at **(3)** the use of formate incorporation into potential C_1-requiring compounds. Formate was found to be incorporated into purines and histidine specifically in *Mb. thermoautotrophicum* (Taylor et al. 1976).

The second approach to study the involvement of H_4M or other potential cofactors was enzymatic studies. Since SHMT is an important biosynthetic enzyme requiring a C_1 carrier, and is shown to be variable in terms of the pterin specificity among methanogens (Choquet et al. 1994, Hoyt et al. 1986), this enzyme was targeted in various methanogens. The enzymatic studies included **(1)** investigation of the responsible pterins in different methanogens, and **(2)** further purification and characterization of the enzyme if a non-H_4M specific SHMT could be detected, since a H_4M dependent SHMT has already been characterized (Hoyt et al. 1986).

Chapter II

Oxidation/reduction of methanol, formaldehyde, formate and serine in *Methanosphaera stadtmanae*

II.1 Introduction

Methanosphaera stadtmanae is an archaeum belonging to the *Methanobacteriales*. It is one of the few representatives of this group which can not reduce CO_2 to methane. Rather, it requires methanol and hydrogen for methanogenesis, and CO_2 and acetate for bulk cell biosynthesis (Miller and Wolin 1985). It has also been suggested that methanogenesis from methanol and H_2 follows a similar pathway as in *Methanosarcina barkeri* in which corrinoid proteins are involved in the methyltransfer from methanol to the methanogenic pathway (van der Wijngaard et al. 1991). Interestingly, Schwörer and Thauer (1991) have detected formylmethanofuran dehydrogenase activity in this same organism as well as methylene-H_4M dehydrogenase and reductase activities. Low levels of H_4M but not methanofuran (MFR) have been detected in this organism (van der Wijngaard et al. 1991). Since *Msph. stadtmanae* can not use CO_2 as methanogenic growth substrate, the function of the H_4M detected, as well as the low levels of enzymes corresponding to its use remains unclear.

One possibility would be that these enzymes and cofactors are involved in providing C_1 intermediates for specific biosynthetic

pathways. The biosynthetic pathways of C_1-requiring molecules such as methionine, histidine and purine have been studied using either ^{14}C radioactive labelling or ^{13}C-NMR technique in several methanogens (reviewed in Chapter I). The major results on C_1-requiring biosynthesis in *Msph. stadtmanae* can be summarized as the followings: 1.Methyl group of methionine was from C-2 of acetate and methanol (Miller et al. 1995). 2. C-8 of purine was also from C-2 of acetate. 3. C-2 of purine was from C-2 of acetate and methanol in medium without formate added, and was exclusively from formate in medium containing formate (Choquet et al. 1994). However, it is still not clear if H_4M or other C carriers are involved in these one-carbon group transfering reactions.

In *Msph. stadtmanae,* since CO_2 and the methyl group of acetate can not be used as methanogenic growth substrates, it would be important to determine to what extent various C_1 substrates (methanol, formaldehyde, formate and CO_2) can be oxidized to CO_2 or reduced to CH_4 by using different [^{14}C] C_1 substrates followed by the studies of long- and short-term labelling patterns of cell materials. Results of these experiments should also indicate where the break in the methanogenic pathway is in this organism and which of the C_1 intermediates may provide carbons for biosynthesis as well as the C_1 carriers involved.

II.2 Materials and methods

II.2.1 Materials.

[^{14}C] formaldehyde (10 mCi/mmol), [^{14}C] formate (Na+, 43 mCi/mmol), [U-^{14}C] serine (108 mCi/mmol), [^{14}C] Na_2CO_3 (8 mCi/mmol) and [^{14}C] methanol (3.8 mCi/mmol) were from NEN Research Products. Scintillation cocktail used in this study was BCS-104 from Amersham. TLC plates (0.1 mm cellulose, 20 x 20 cm) were from Whatman.

II.2.2 Growth of cells and preparation of cell suspension.

Cells of *Msph. stadtmanae* (DSM 3091) were grown at 37°C under H_2/CO_2 (80/20, v/v) in a medium described by Miller and Wolin (1985) with some modification (Sparling et al. 1993):

Tryptone/Trypticase	2.0g/L
Yeast Extract	2.0g/L
K_2HPO_4	0.3g/L
KH_2PO_4	2.8g/L
$(NH_4)SO_4$	0.3g/L
NaCl	0.61g/L
$MgSO_4 \cdot 7H_2O$	0.13g/L
$CaCl_2 \cdot 2H_2O$	0.08g/L
Na Acetate	0.50g/L
Na_2SeO_3	0.086mg/L

Na_2CO_3	1.7g/L
$Na_2S \cdot 9H_2O$	0.43g/L
NH_4Cl	1.0g/L
Resazurin	0.0005g/L
Vitamin Supplement	15.0 mL
Mineral Elixir	15.0 mL
Methanol	0.4% (v/v)

$Na_2S \cdot 9H_2O$ was added into the medium from a stock solution after the medium was made anaerobic with 3 cycles of evacuating (10 min each) and gasing (3 min each). Methanol was added after the medium was autoclaved. The mineral elixir and vitamin supplement solutions were made using the following formula:

Mineral elixir (Daniels et al. 1984a):

Trisodium Nitriloacetate	2.02g/L
$FeCl_3x6H_2O$	0.21
$CoCl_2x6H_2O$	0.20
$MnCl_2x4H_2O$	0.10
$ZnCl_2$	0.10
$CaCl_2x2H_2O$	0.05
$CuSO_4x2H_2O$	0.05
$Na_2MoO_4x2H_2O$	0.05
$NiCl_2x6H_2O$	0.10

Vitamin Supplement (Wolin et al. 1963):

Pyridoxine-HCl	10mg/L
Riboflavin	5
Thiamine	5

Nicotinic Acid	5
p-Aminobenzoic Acid	5
Lipoic Acid (Thioctic Acid)	5
Biotin	2
Folic Acid	2
Cyanocobalamin	1

Cells were harvested in the exponential growth phase, washed three times with 40 mM potassium phosphate pH 6.9, and suspended in the same buffer. When NaCl was added, 90 mM was used unless otherwise specified. All the procedures above were performed anaerobically in a Coy anaerobic chamber furnished with an-oxygen-detector.

II.2.3 Effect of propyl iodide on methanogenesis from methanol

To each stoppered 25-ml tube containing 9.5 ml anaerobic potassium phosphate buffer (40 mM, pH 6.9), 0.5 ml of cell suspension was added. The gas phase was pure hydrogen. NaCl and propyl iodide were added from stock solutions. Methanogenesis was started by the addition of 0.4% (v/v) methanol.

II.2.4 Preparation of cell-free extracts (CFE) and cofactor-depleted-cell-free extracts (CDCFE).

Washed cells were lysed after 3 anoxic passages through a

French pressure cell (120,000 kPa). CFE was obtained after anoxic centrifugation at 48,000 x g at 4°C for 30 min. CDCFE was obtained by precipitating proteins with 95% $(NH_4)_2SO_4$ (w/v) in CFE, discarding supernatant and resuspending the pellet in the same buffer, then repeating these procedures twice more.

II.2.5 NAD(P)-dependent Formate dehydrogenase (FDH) assays.

The activity of FDH was assayed in 20 mM MES [2-(N-morpholino)ethanesulfonic acid] (pH6.5) (Tanner et al. 1989) and Tris-HCl (pH 7.5 and 8.0) (Sparling and Daniels 1986). Sodium formate was added with the final concentration of 22 mM in non-radioactive assays. In assays using [^{14}C] formate as the substrate, a lower concentration (9.62 mM) was used. For the various electron acceptors tested, NAD, NADP, F_{420}, methyl- and benzyl-viologen were all added to the final concentration of 0.5 mM.

II.2.6 Short-term labelling experiments.

All experiments with cell suspensions were performed at 37°C in 25 ml-tubes containing 0.5 ml cell suspension and 9.5 ml potassium phosphate buffer pH 6.9. The gas phase was 100% H_2 unless otherwise indicated. The reaction was started by the addition of cells. The tubes were shaken on their side during incubation in a reciprocal shaker (Haake). The measurement of $^{14}CH_4$ or $^{14}CO_2$ was conducted following the procedures described by Zehnder et al.

[1979] with the following modifications: 1 ml of gas sample was injected into the 25 ml vial containing 1.5 ml 0.2 N KOH to trap $^{14}CO_2$. The vial was shaken vigorously at room temperature for 1 hr. 2 ml of gas sample was then taken out and injected into another 25 ml vial containing 22 ml scintillant and shaken vigorously at 0°C for 1 hr. By using this method, 100% $^{14}CO_2$ was trapped and 80% to 85% of $^{14}CH_4$ was absorbed in the scintillant of controls. The radioactive counting was performed in a liquid scintillation counter (Beckman LS-230). Samples with counts less than 50 cpm were considered as having only background levels and are reported as such in the result section.

II.2.7 Long-term labelling experiments.

In the formate-labelled cultures, cells were grown in the aforementioned medium and condition except that 0.1 mmol of [^{14}C] formate (specific radioactivity was 555 dpm/nmol) was added to 50 ml of cultures in 100-ml serum bottles. In the methanol-labelled cultures, 5 mmol of [^{14}C] methanol (specific radioactivity was 22.4 dpm/nmol) was added to 50 ml of cultures. When propyl iodide was added, it was added to 500 μM (final concentration) 43 hr post inoculation (5 hours prior to cell harvest). It was reported that propyl iodide inactivated the methylation of corrinoid proteins in methanogens (Kenealy and Zeikus 1981). Measurement of $^{14}CO_2$ and $^{14}CH_4$ was as mentioned above. Radioactivity of the macromolecules within the cells was measured after washing the cells trapped on

a 0.45 μm filter (Gelman) with 10 ml 5% trichloroacetic acid (Sparling et al. 1993). Cytoplasmic proteins were collected and hydrolysed by following procedures of Ekiel and coworkers (Ekiel et al. 1983). Amino acids were separated by two-dimension thin-layer chromatography, using the solvent system n-butanol/acetic acid/water (80:20:20 [vol/vol]) and phenol/water (75:25 [w/w])(Brenner et al. 1965). Spots corresponding to the amino acids were visualized by ninhydrin reagent and identified by comparing R_f values to known standards. Radioactivity of amino acids on thin-layer plates was determined by carefully scraping the cellulose off and counting it in scintillation cocktail. Ribosomal RNA was separated using the phenol method (Hespell et al. 1975).

II.2.8 Protein determinations.

Protein concentration was determined by the method of Bradford (1976), using Coomassie brilliant blue G and bovine serum albumin as a standard.

II.3 Results

II.3.1 Effect of propyl iodide on methanogenesis from methanol/H_2 in cell suspensions of *Msph. stadtmanae*.

Corrinoid proteins have been reported to be involved in the methanogenic pathway from methanol in *Msph. stadtmanae* (van der

Wijngaard et al. 1991). To confirm this, propyl iodide, an inhibitor of corrinoid protein (Kenealy and Zeikus 1981) was added into the assay buffer (40 mM phosphate buffer, pH 6.9) containing 0.5 ml of cell suspensions. Methane formation from methanol was completely inhibited by the addition of 160 μM propyl iodide, while the concentration of NaCl in the buffer did not affect the rates of methanogenesis (figure II.1). This latter phenomenon had been observed previously (Sparling et al. 1993). The results of figure II.1 indicated that methanogenesis from methanol in *Msph. stadtmanae* involved the methylation of a corrinoid-protein which was not affected by NaCl but was inhibited by propyl iodide.

Contrary to methanogenesis from methanol, the rate of methane formation from L-serine (donating C_1 units at the formaldehyde level) was significantly affected by the concentration of NaCl in the buffer (figure II.2). It was reported that methanogenesis from the level of formaldehyde is a sodium-driven process and therefore requires the presence of sodium ions (Müller et al. 1988). Sodium ion could also affect the entry of serine into cells. However, this need to be confirmed with further experiments.

II.3.2 Formation of $^{14}CO_2$ and $^{14}CH_4$ from different C_1 substrates in cell suspensions of *Msph. stadtmanae*.

To determine where the break of the general methanogenic pathway is in *Msph. stadtmanae*, several potential substrates which were known to enter the methanogenic pathway in different methanogens were used. The production of $^{14}CO_2$ was detected from

Figure II.1. Methanogenesis from methanol under hydrogen in cell suspensions of *Msph. stadtmanae*. Methanol was added to the final concentration of 0.4% (v/v). ■, 90 mM NaCl; ▲, 0.35 mM NaCl; ●, 90 mM NaCl + 160 μM propyl iodide.

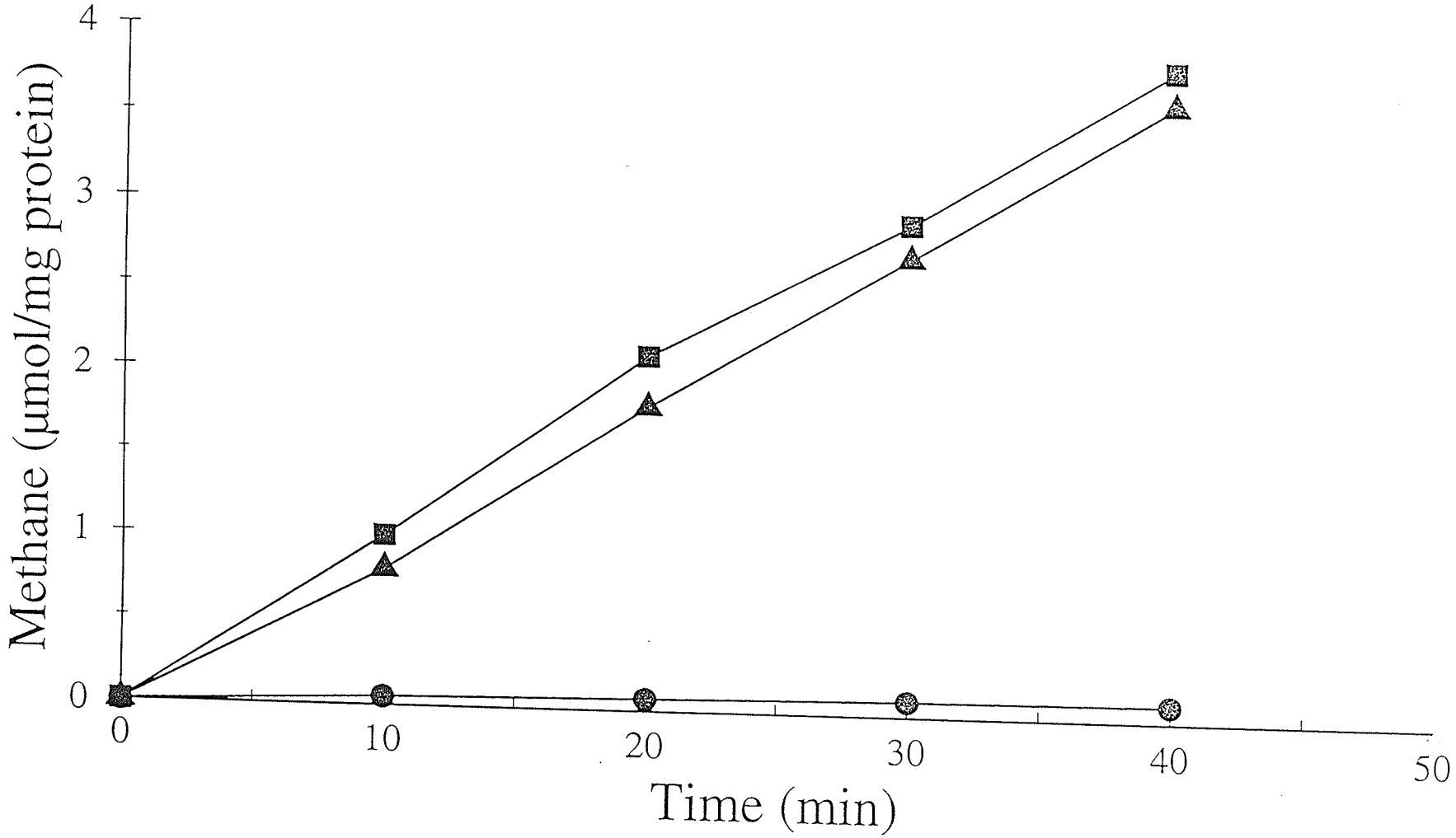
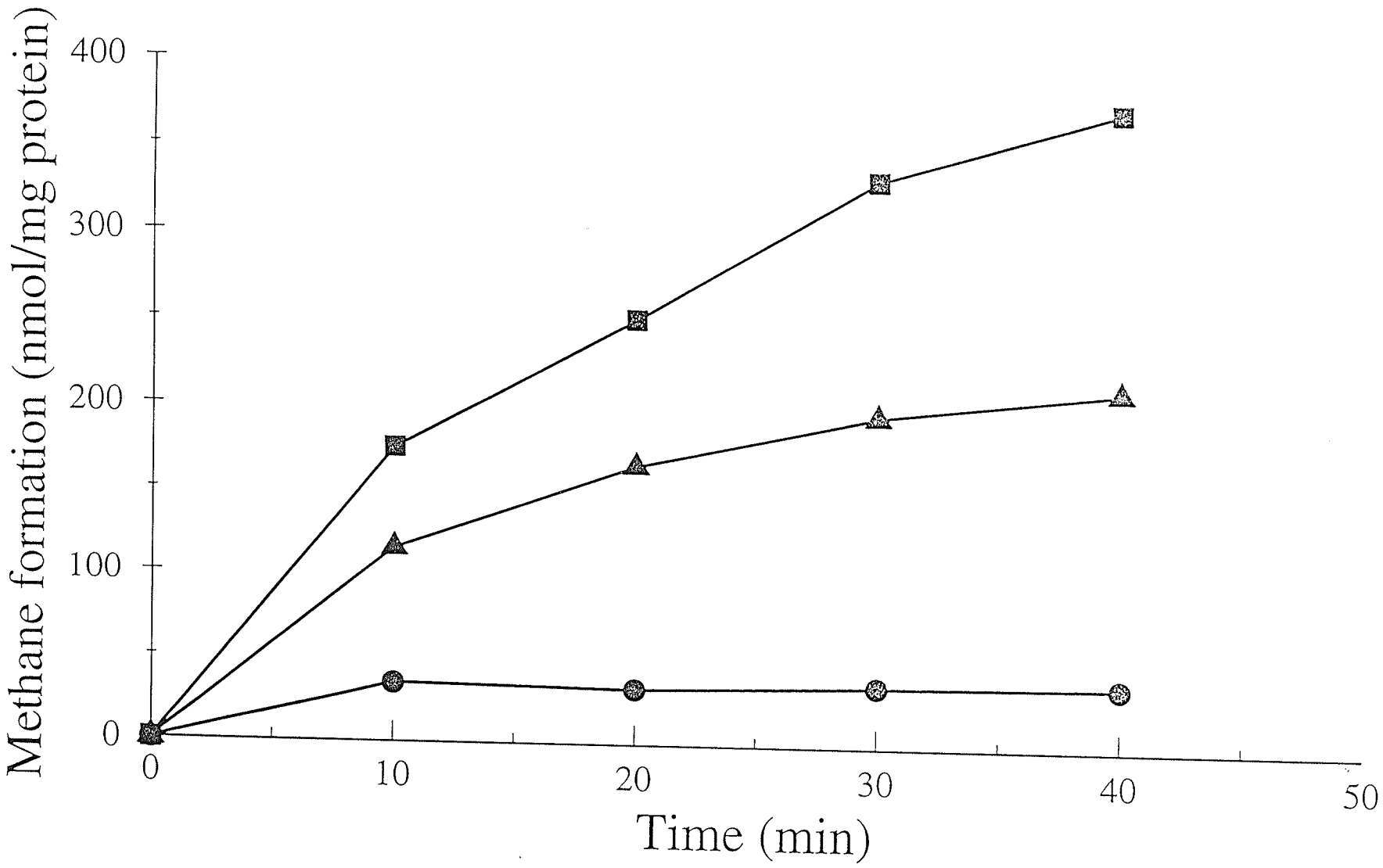

Methane (µmol/mg protein)
4
3
2
1
0
0
10
20
30
40
50
Time (min)

Figure II.2. Effect of [NaCl] on methanogenesis from L-serine plus H_2 in cell suspensions. ■, 90 mM NaCl; ▲, 30 mM NaCl; ●, 0.35 mM NaCl.


Methane formation (nmol/mg protein)
400
300
200
100
0
0
10
20
30
40
50
Time (min)

[U-^{14}C] serine, formaldehyde and sodium formate but not methanol, while $^{14}CH_4$ was formed from [^{14}C] methanol, formaldehyde and serine in the presence of hydrogen gas. Neither [^{14}C] sodium carbonate nor [^{14}C] sodium formate could be incorporated into $^{14}CH_4$ (table II.1). The aforementioned results indicated that: 1. a H_4M-dependent serine hydroxymethyltranferase (Hoyt et al. 1986) might be present in *Msph. stadtmanae*, converting serine into glycine and-methylene-H_4M. The latter product would then have been converted into CH_4 by the enzymes of the methanogenic pathway. 2. Enzymes below the level of methylene-H_4M in the common methanogenic pathway were all present in *Msph. stadtmanae* because both formaldehyde and L-serine could be used as methanogenic substrates. Some enzymes above the level of-methylene-H_4M were absent or inactive since cells of *Msph. stadtmanae* failed to oxidize [^{14}C] methanol into CO_2 or reduce CO_2 into CH_4. 3. A formate dehydrogenase might be present.

Attempts to couple the oxidation of formaldehyde or formate to the reduction of methanol were unsuccessful. $^{14}CO_2$ could not be incorporated into $^{14}CH_4$ even in the presence of^{12} [C] methanol, indicating that the previous failure to detect CO_2 reduction with H_2 (Miller and Wolin 1985) was not due to the absence of an RPG effect (i.e. methane production dependent stimulation of CO_2 reduction) (Gunsalus and Wolfe 1977) when methanol is not present.

Table II.1. Formation of $^{14}CO_2$ and $^{14}CH_4$ from different substrates under hydrogen [a]

substrate	amount added (nmol)	[^{14}C] in CO_2 nmol	[^{14}C] in CH_4 nmol
[^{14}C] methanol	10	ND[b]	5.15
[^{14}C] serine	10	0.003	0.11
[^{14}C] formaldehyde	10	0.012	1.97
[^{14}C] sodium carbonate	125	17.17	ND
[^{14}C] sodium formate	23	0.009	ND

a. 0.405 mg protein added in 10 ml assay buffer. Samples were taken at 60 min. All data represent averages of duplicates with less than 10% difference.
b. ND = not detected, cpm<50.

II.3.3 Determination of a NADP-dependent formate dehydrogenase (FDH) activity in *Msph. stadtmanae*.

FDH activity was detected in the $(NH_4)_2SO_4$-precipitated CFE (cofactor depleted). The enzyme activity was NADP (0.3 nmol/min.mg protein) or NAD (0.2 nmol/min.mg protein) dependent but not methyl viologen, benzyl viologen or F_{420} dependent (table II.2). To confirm the presence of FDH activity, further experiments were performed, using [^{14}C] formate to detect the production of $^{14}CO_2$ in $(NH_4)_2SO_4$-precipitated CFE. When NADP was added to the assay buffer, $^{14}CO_2$ was produced to 0.08 nmol/min.mg protein, which is about-10-fold higher than the control without NADP addition. The lower rate in $^{14}CO_2$ production might have been due to the lower formate concentration added (9.62 mM ^{14}C formate for radioactive detection and 22 mM formate for non-radioactive enzyme assays).

II.3.4 Inhibition of $^{14}CO_2$ and $^{14}CH_4$ formation from [^{14}C] formaldehyde by [^{12}C] serine and formate.

Formation of $^{14}CO_2$ from [^{14}C] formaldehyde was confirmed in cell suspensions of *Msph. stadtmanae* (figure II.3) in the presence of hydrogen and methanol. The rates of CO_2 and CH_4 formation from formaldehyde were 0.08 and 0.33 nmol/min.mg protein, respectively. Production of $^{14}CO_2$ was reduced by the addition of [^{12}C] serine or formate while [^{14}C] methane formation was only inhibited by serine but not by formate (figure II.4). These results indicated that

Table II.2. Activities of Formate dehydrogenase in *Msph. stadtmanae* with various electron acceptors

electron acceptor	activity (nmol/min.mg protein)		
	pH 6.5[a]	pH 7.5[b]	pH 8.0[b]
NADP	0.3	---[c]	---
NAD	0.2	---	---
F_{420}	ND[d]	ND	ND
MV	ND	ND	ND
BV	ND	ND	ND

a. MES buffer used for assaying NADP-dependent formate dehydrogenase (Tanner et al. 1989).
b. Tris-HCl buffer used for assaying F_{420}-dependent (pH 7.5) and MV-dependent (pH 8.0) formate dehydrogenase (Sparling and Daniels 1986).
c. Not determined.
d. Not detected.

Figure II.3. Formation of $^{14}CO_2$ (●) and $^{14}CH_4$ (■) from [^{14}C] formaldehyde in the presence of hydrogen and methanol. To each assay tube, 0.5 mL of cells (2.1 mg protein) and 5.05 μmol formaldehyde (220 dpm/nmol) were added at time 0. Data are averages of duplicates (±10%).

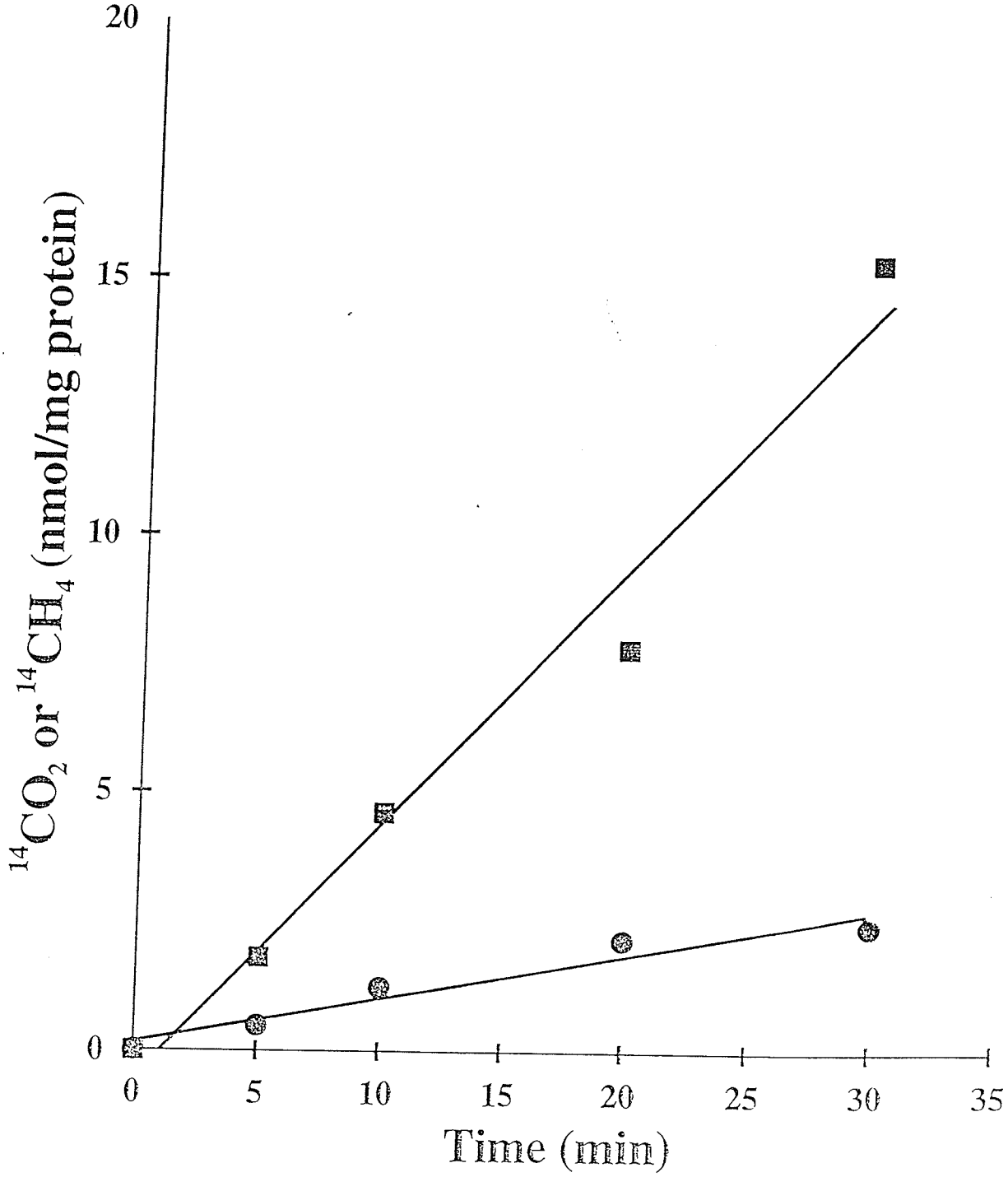
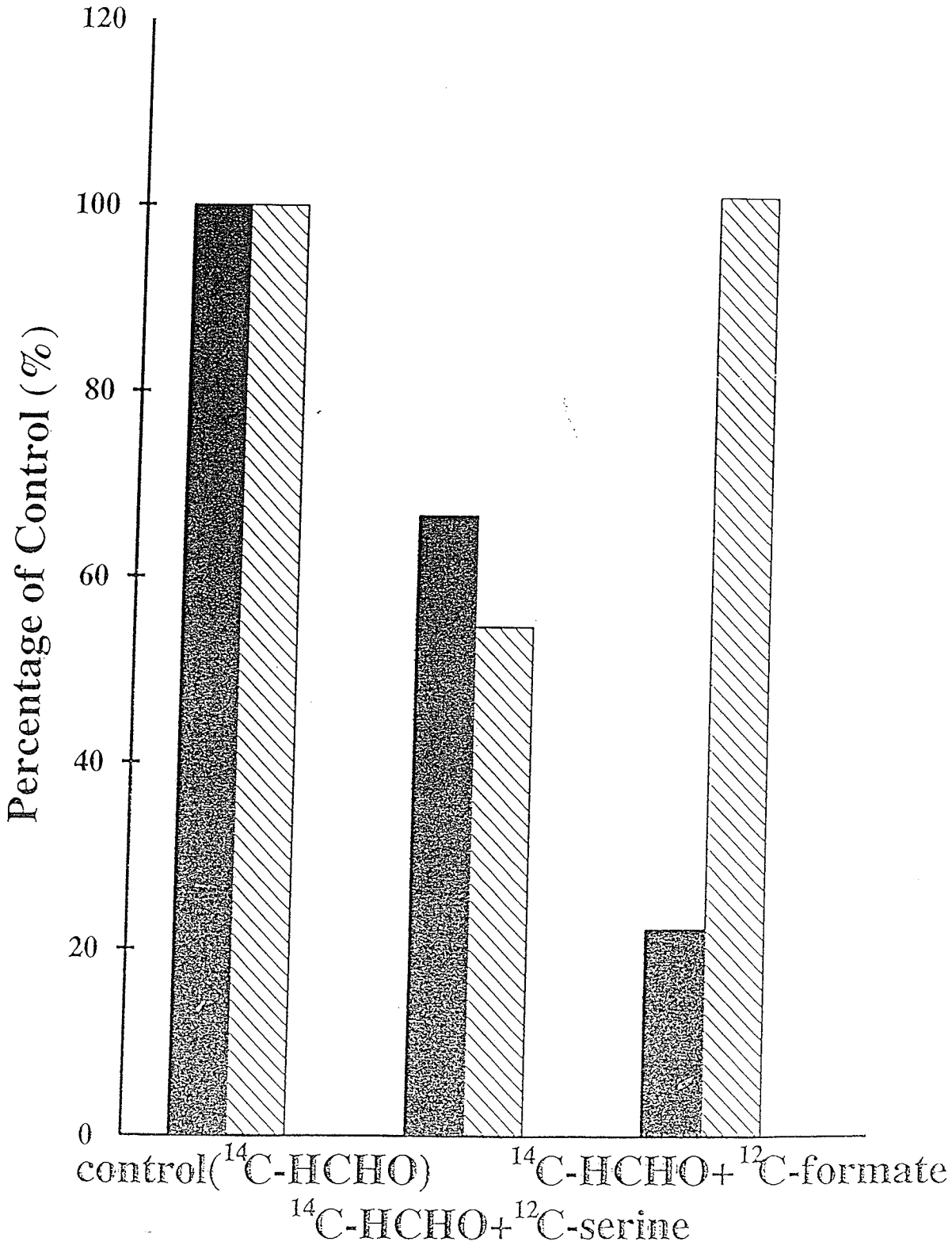

20
15
10
5
0
$^{14}CO_2$ or $^{14}CH_4$ (nmol/mg protein)
0
5
10
15
20
25
30
35
Time (min)

Figure II.4. Inhibition of $^{14}CO_2$ (■) and $^{14}CH_4$ (▨) from [^{14}C] formaldehyde by [^{12}C] serine and formate. Data are averages of duplicates (±5%). 100% of $^{14}CO_2$ was 1998 dpm. 100% of $^{14}CH_4$ was 3.26 x 10^5 dpm. Formaldehyde was added to a final concentration of 10 μM (22,200 dpm/nmol), serine and formate were added to a final concentration of 25 mM. Each assay tube contained 0.41 mg protein. Samples were taken after 60 min of incubation.


120
100
80
60
40
20
0
Percentage of Control (%)
control(14C-HCHO)
14C-HCHO+12C-formate
14C-HCHO+12C-serine

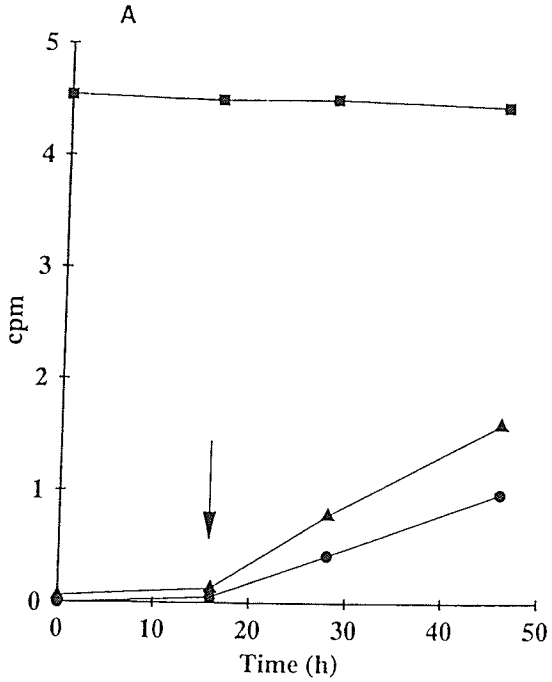
there was competition between formate and formaldehyde in the process of $^{14}CO_2$ formation from formaldehyde and between serine and formaldehyde in the process of $^{14}CH_4$ formation from formaldehyde.

II.3.5 [^{14}C] long-term labelling studies.

The presence of enzymes above the level of formaldehyde of the methanogenic pathway in the cells of *Msph. stadtmanae* implied that methanol might be the C_1 donor for certain C-requiring biosynthetic processes, through part of the methanogenic pathway. The presence of a NADP-dependent FDH and the competition between formate and formaldehyde in the process of CO_2 formation indicated that formate might be a C_1 precursor for some biosynthetic compounds. To examine these possibilities, long-term labelling with [^{14}C] methanol or formate as substrates was studied. In cultures supplemented with [^{14}C] methanol, most of the label went to CH_4 and a small amount in the cell pellets. No $^{14}CO_2$ was produced during the growth period (figure II.5a).

In cultures supplemented with [^{14}C] formate, most of the label remained in the supernatant, but a small amount of formate was incorporated into the cell pellets and CO_2 (figure II.5b). At late exponential phase of cell cultures (46 hr), cells labelled with either [^{14}C] methanol or formate were harvested and fractionated. Both methanol and formate were found to label ribosomal RNA and cytoplasmic proteins (table II.3). Since a portion of the observed

Figure II.5. Incorporation of [^{14}C] formate (A) and [^{14}C] methanol (B) into cells. Arrow indicates the start of culture shaking. All data are averages of duplicates with less than 5% difference. ■: cpm (x10^7) in supernatant; ▲: cpm (x10^6) in cell material remaining on membrane filter after cold-TCA wash; ●: cpm (x10^5) in CO_2; *: cpm (x10^7) in CH_4.


A
5
4
3
2
1
0
cpm
0
10
20
30
40
50
Time (h)

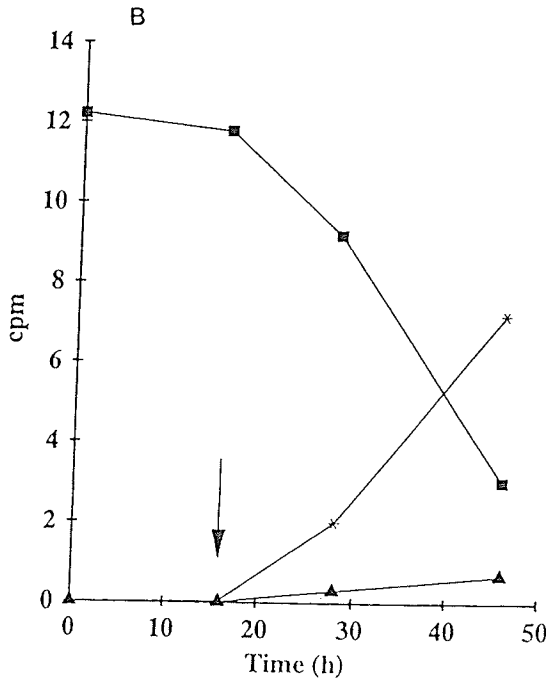

B
14
12
10
8
6
4
2
0
cpm
0
10
20
30
40
50
Time (h)

Table II.3. Long-term Labelling of [^{14}C] substrates in *Msph. stadtmanae*[a]

[^{14}C] substrate (mmol)	^{14}C in supernatant mmol	%	^{14}C in cell material[b] μmol	%	^{14}C in CH_4[c] mmol	%	^{14}C in CO_2[c] μmol	%	^{14}C in ribosomal RNA (μmol)	^{14}C in cytoplasmic protein (μmol) -PI[d]	+PI
methanol 5	1.35	27	33	0.66	3.17	63.5	ND	ND	0.02	0.15	0.01
formate 0.1	0.097	97	2.5	2.5	ND[e]	ND	0.19	0.19	0.23	0.22	---[f]

a. Data shown are averages of duplicates with less than 10% difference.
b. This was measured after washing cells trapped on membrane filter with 10 mL of 5% cold TCA.
c. Not including those dissolved in supernatant.
d. PI = propyl iodide.
e. ND = not detected.
f. Not determined.

radioactive counts could have been from methylated corrinoid proteins involved in methanogenesis from methanol, as is observed in *Ms. barkeri* (Kremer et al. 1993), cultures incubated with $^{14}CH_3OH$ to late exponential phase were also incubated with propyl iodide for 3 h prior to harvesting cells. When these cells were measured, the ^{14}C counts in cytoplasmic proteins were 10-fold less than in the untreated cells (Table II.3) indicating that most of the observed counts in untreated cells were due to the labelling by methanol of corrinoid proteins, rather than biosynthetic end products. The acid hydrolysates of cytoplasmic proteins were separated using thin-layer chromatography (TLC) techniques. No label was detected in amino acids from [^{14}C] methanol-labelled cytoplasmic proteins, while label was found in histidine of [^{14}C] formate-labelled cytoplasmic proteins.

II.4 Discussion

Short term labelling using $^{14}C_1$-donors has been used previously to look at the use of various methanogenic substrates and at competition between these various substrates (for example, Krzycki et al. 1982). A C_1-donor that has been very useful for the study of intermediate steps in the methanogenic pathway is formaldehyde. Free formaldehyde can chemically react with H_4M (Escalante-Semerena et al. 1984) as it had previously been observed for H_4F (Uyeda and Rabinowitz 1967). Formaldehyde has since been used in several studies of whole cell methanogenesis (Müller et al. 1988).

Our results for methanogenesis from both formaldehyde and L-

serine under a hydrogen gas phase confirmed that enzymes below the level of formaldehyde in the common methanogenic pathway were present in *Msph. stadtmanae*. Consistent results of enzymatic studies have been reported (Schworer and Thauer 1991). The break point in the common methanogenic pathway would be in the formyl-level, either because of the lack of MFR (van der Wijngaard et al. 1991) or the absence of the enzyme that catalyzes the transfer of formyl- units. Whole cell methanogenesis from methanol was inhibited by propyl iodide, indicating that the process involved a corrinoid-specific methyltransferase, similar to what was found in *Methanosarcina barkeri* (Pol et al. 1982, Keltjens and van der Drift 1986). This result is also consistent with the findings that crude cell extracts of *Msph. stadtmanae* could synthesize methane from CH_3-B_{12} in the presence of hydrogen (van der Wijngaard et al. 1991).

Our results of protein incorporation from long-term labelling showed [^{14}C] methanol labelling of proteins but not of amino acids, probably because of the methylation of corrinoid proteins by methanol (Kremer et al. 1993), which then lost their methyl groups and label when hydrolysed. This was confirmed by adding propyl iodide to the cells at the end of growth, which caused a substantial reduction in the counts observed in the protein fraction. However, using a ^{13}C-NMR technique, Miller and coworkers (1995) found that the methyl group of methionine originated mainly from C-2 of acetate, and also from methanol though at a much lower level of incorporation.

L-serine could be used as a methanogenic substrate in whole

cells of *Msph. stadtmanae*, but attempts to grow the cells in L-serine under H_2/CO_2 were unsuccessful. In *Mc. voltae*, an attempt to grow cells using L-serine as the carbon source was not successful (Whitman et al. 1982). However, there was no evidence that cells of *Mc. voltae* could synthesize methane from serine. Methanogenesis from serine plus H_2 was also demonstrated by Romesser and Wolfe (1982) using CFE of *Mb. thermoautotrophicum*. The means by which C_1 units from serine enter the methanogenic pathway has been resolved in *Mb. thermoautotrophicum* where a H_4M-dependent serine hydroxymethyl transferase has been found (Hoyt et al. 1986), forming glycine and methylene-H_4M from L-serine. This is consistent with the data in *Msp. hungatei* that glycine is formed from serine (Ekiel et al. 1983). The function of this activity in *Msph. stadtmanae* may be to provide glycine for protein synthesis and methylene-H_4M for C_1-required biosynthesis. This is supported by the data presented by Miller and coworkers (1995) that the C-3 of serine, derived from the C-2 of acetate, was the source of the methyl group of methionine.

The data presented with respect to formate use are consistent with data observed in *Mb. thermoautotrophicum* Marburg. Indeed, formate was shown to be incorporated specifically into purines and histidine in *Mb. thermoautotrophicum* (Taylor et al. 1976), an organism for which formate is not a methanogenic substrate. Subsequently, an auxotroph of *Mb. thermoautotrophicum* Marburg was found to require formate for growth (Tanner et al. 1989). Extracts of the wild-type strain were shown to possess low levels of a-NADP-

dependent FDH activity. Although formate was not added to the growth medium, a FDH activity was observed in *Msph. stadtmanae* that was similar to that described by Tanner et al. (1989) in *Mb. thermoautotrophicum*. Both have been shown to be NAD/NADP dependent, F_{420} independent, and viologen dye insensitive. The activities measured were also very low in both cases (0.3 vs 7.5 nmol/min. mg protein), as would be expected from enzymes involved in biosynthetic pathways. Our further results of long-term labelling showed that both [^{14}C] methanol and formate labelled RNA. This is consistent with a recent report that both formate and methanol can be incorporated into the C-2 of purine (Choquet et al. 1994). H_4M is probably the C_1-carrier for the incorporation of methanol into purine. This is supported by the facts that both L-serine and formaldehyde are methanogenic substrates. However, how formate is incorporated into purine in *Msph. stadtmanae* still remains unclear.

One possibility is that H_4M is involved as the C_1 carrier, as was suggested previously (Choquet et al. 1994). Another possibility is that a C_1 carrier other than H_4M , such as H_4F, is involved. This is supported by our data that formate could not be incorporated into methane and that there was competition between formaldehyde and formate in CO_2 formation. A scheme of C_1 metabolism in *Msph. stadtmanae* (figure II.6) which involved a C_1 carrier other than H_4M (such as H_4F) was proposed which would be consistent with the results discussed above. However, whether or not folic acid or other structurally similar C_1 carrier is present in methanogens is still not clear (see section I.3.2). Further enzymatic studies have

to be performed to confirm whether or not H_4F or another C_1 carrier is involved in cell biosynthesis of *Msph. stadtmanae*.

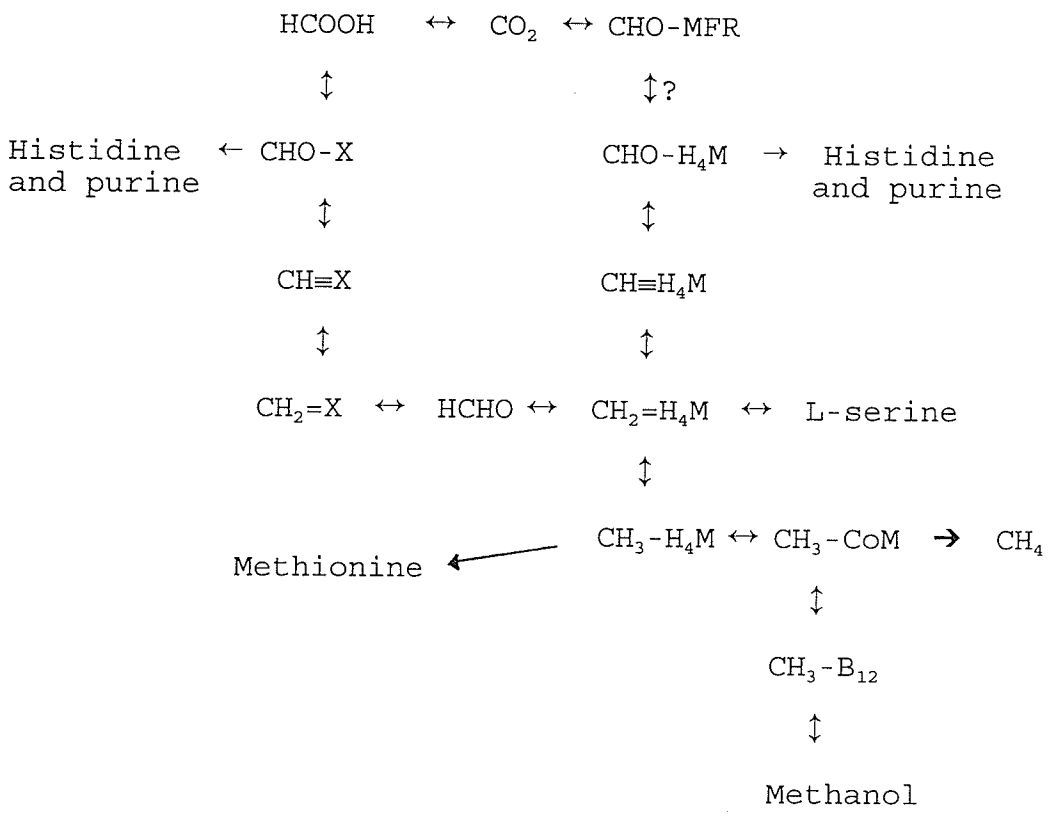


Figure II.6. Proposed scheme for the oxidation and reduction of various substrates in *Msph. stadtmanae*. ? :step known to be present in other members of the *Methanobacteriales* but not yet observed in *Msph. stadtmanae*; X : potential C_1 carrier other than H_4M.

Chapter III

Investigation of serine hydroxymethyl transferase (SHMT) in methanogens

III.1 INTRODUCTION

Serine hydroxymethyl transferase (SHMT) has been shown to be dependent on tetrahydromethanopterin (H_4M) in *Methanobacterium thermoautotrophicum* as the acceptor of the hydroxymethyl group of L-serine (Hoyt et al. 1986). Indeed, methanogenesis observed from serine in *Mb. thermoautotrophicum* confirmed that the C_1 carrier of this hydroxymethyl transfer reaction is H_4M. The enzyme also required pyridoxal-5-phosphate to catalyze the reaction:

L-serine + H_4M ↔ glycine + 5,10-methylene-H_4M

In eukaryotic cells and eubacteria, SHMT has been shown to be dependent on tetrahydrofolate (H_4F) and also required pyridoxal-5-phosphate for maximal activity (Schirch et al. 1985; Schirch 1971). In other methanogens, serine hydroxymethyl transferase activities have not yet been reported. A renewed interest in this enzyme and especially the pterin specificity of this enzyme in various methanogens is based on ^{13}C NMR and ^{14}C labelling work (Chapter II of this thesis, Miller et al. 1995, Choquet et al. 1994). The results (reviewed in chapter I of this thesis) suggested that there might be variation in terms of pterin specificity among SHMT of methanogens. In species of the *Methanobacteriales* and *Methanococcales* tested, the results are consistent with the

presence of a H_4M dependent enzyme while in *Methanospirillum hungatei and Methanosarcina barkeri* (both are members of the *Methanomicrobiales*), ^{13}C-NMR results indicate that the enzyme might be dependent on a cofactor other than H_4M or H_4S, the only pterin so far detected in *Ms. barkeri* (Keltjens and Vogels 1988).

In this study, SHMT activities were tested in several methanogens, including representatives of *Methanomicrobiales (Msp. hungatei* GP1, *Methanosaeta concilii, Ms. barkeri* Fusaro, and *Methanolobus tindarius), Methanobacteriales (Msph. stadtmanae, Mb. thermoautotrophicum* Marburg), and *Methanococcales (Methanococcus thermolithotrophicus* and *Methanococcus voltae)*, using H_4M purified from *Mb. thermoautotrophicum* Marburg, H_4S purified from cells of *Ms. barkeri* Fusaro and H_4F (Sigma) as potential acceptors of the C-3 of L-serine.

III.2 MATERIALS AND METHODS

III.2.1 Chemicals

Formaldehyde, dimedone, glycine, serine, sodium acetate, ammonium sulfate, DEAE-Sephadex A-50 and tetrahydrofolate were obtained from Sigma Chemical Co.. [^{14}C] formaldehyde, and 3-^{14}C-serine were purchased from Amersham. Hydroxylapatite was from BIORAD. Purified H_4S was a gift from Dr. J. Krzycki (Ohio State

University).

III.2.2 Microorganisms and culture conditions

In all the media recipes described in this section, $Na_2S \cdot 9H_2O$ and methanol (when needed) were added as described in section II.2.2 of this thesis. Protocols for anaerobic preparation of the media, based on Daniels et al. (1986), were described in the previous chapter section II.2.2. The recipes of mineral elixer and vitamin supplement used were also given in section II.2.2. In the medium for *Mc. thermolithotrophicus*, $MgCl_2$ was added from a anaerobic sterile stock solution after media were autoclaved.

Msp. hungatei GP1(DSM 1101) was grown at 37°C, pH 6.8-7.2 under H_2/CO_2 (80/20, v/v) with the following medium (Daniels et al. 1986):

NaCl	0.3g/L
$MgCl_2 \cdot 6H_2O$	0.064g/L
NH_4Cl	0.40g/L
$Na_2S \cdot 9H_2O$	0.43g/L
Na Acetate	0.42g/L
K_2HPO_4	0.82g/L
$CaCl_2 \cdot 2H_2O$	0.064g/L
Resazurin	0.0005g/L
Na_2CO_3	1.0g/L
Mineral Elixir	10.0 mL/L
Vitamin Supplement	10.0mL/L

Folate-free medium was prepared by removing folate from the vitamin supplement.

Mst. concilii (DSM 3671) was grown at 35°C, pH 7.0 under N_2:CO_2 (80/20, v/v) with the following medium (Patel 1984):

KH_2PO_4	0.30g/L
NaCl	0.60g/L
$MgCl_2.6H_2O$	0.10g/L
$CaCl_2.2H_2O$	0.08g/L
NH_4Cl	1.00g/L
$KHCO_3$	4.00g/L
Mineral Elixir	10mL/L
Vitamin Supplement	10mL/L
$Na_2S.9H_2O$	0.43g/L
Resazurin	0.0005g/L
Na Acetate	6.80g/L

Ms. barkeri Fusaro (DSM 804) cultures were maintained at 37°C, pH 7.0 under H_2/CO_2 (80/20, v/v) with the following medium (Daniels et al. 1986):

K_2HPO_4	0.35g/L
KH_2PO_4	0.23g/L
NH_4Cl	0.5g/L
Yeast Extract	2.00g/L
$MgSO_4 \cdot 7H_2O$	0.50g/L
$CaCl_2 \cdot 2H_2O$	0.25g/L
NaCl	2.25g/L
$NaHCO_3$	0.85g/L
$Na_2S \cdot 9H_2O$	0.43g/L

Mineral Elixir	10.0 mL/L
Vitamin Supplement	10.0 mL/L

Ml. tindarius (DSM 2278) cultures were maintained at 25°C, pH 6.5 under N_2/CO_2 (80/20, v/v) with the following medium (König and Stetter 1982):

KCl	0.335g/L
$MgCl_2.6H_2O$	4.00g/L
$MgSO_4.7H_2O$	3.45g/L
NH_4Cl	0.25g/L
$CaCl_2.2H_2O$	0.14g/L
K_2HPO_4	0.14g/L
NaCl	18.0g/L
$NaHCO_3$	1.00g/L
$Fe(NH_4)_2(SO_4)_2.7H_2O$	0.002g/L
Resazurin	0.0005g/L
$Na_2S.9H_2O$	0.43g/L
Methanol	0.4% (v/v)
Mineral Elixir	10mL/L
Vitamin Supplement	10mL/L

Mb. thermoautotrophicum Marburg (DSM 2133) was grown at 65°C under H_2/CO_2 (80/20, v/v) in a mineral medium (Daniels et al. 1986):

KH_2PO_4	0.42g/L
K_2HPO_4	0.23g/L
$MgCl_2 \cdot 6H_2O$	0.04g/L
$CaCl_2 \cdot 2H_2O$	0.03g/L
NaCl	0.595g/L
NH_4Cl	0.70g/L
Na_2CO_3	0.16g/L

Resazurin	0.0005g/L
Mineral Elixir	10.0 mL/L
$Na_2S \cdot 9H_2O$	0.43g/L

Msph. stadtmanae (DSM 3091) was grown at 37°C under H_2/CO_2 (80/20, v/v) in a medium described by Miller and Wolin (1985) with some modification (Sparling et al. 1993) (see Materials and Methods of Chapter II).

Folate-free medium was prepared by replacing yeast extract and tryptone with folate-free tryptone (DIFCO) and omitting folate from the regular vitamin supplement.

Mc. voltae (DSM 1537) was grown in the following medium at 35°C, pH 7.0 under H_2/CO_2 (80/20, v/v) (Whitman et al. 1982):

KCl	0.335g/L
$MgCl_2.6H_2O$	4.00g/L
$MgSO_4.7H_2O$	3.45g/L
NH_4Cl	0.25g/L
$CaCl_2.2H_2O$	0.14g/l
K_2HPO_4	0.14g/L
Yeast Extract	2.00g/L
Trypticase	2.00g/L
NaCl	18.0g/L
$NaHCO_3$	5.00g/L
$Fe(NH_4)_2(SO_4)_2.7H_2O$	0.002g/L
Resazurin	0.0005g/L
$Na_2S.9H_2O$	0.43g/L
Na Acetate	1.00g/L
Mineral Elixir	10mL/L

Vitamin Supplement	10mL/L

Mc. thermolithotrophicus (DSM 2095) was grown in the following medium at 62-65°C, pH 6.0 under H_2/CO_2 (80/20, v/v) (Belay et al. 1984):

NaCl	29.2g/L
$MgCl_2 \cdot 6H_2O$	5.1g/L
NH_4Cl	1.0g/L
KCl	0.34g/L
KH_2PO_4	0.28g/L
K_2HPO_4	0.28g/L
$CaCl_2 \cdot 2H_2O$	0.10g/L
Resazurin	0.0005g/L
$Na_2WO_4 \cdot 2H_2O$	0.00007g/L
Na_2SeO_4	0.00004g/L
Na_2CO_3	0.20g/L
$Na_2S \cdot 9H_2O$	0.43 g/L
Mineral Elixir	10.0 mL/L

III.2.3 Preparation of cell-free extracts (CFE) from cultures.

Cells in the exponential growth phase were harvested by centrifugation with rubber O-ring sealed centrifuge tubes (Nalgene), washed three times with 20 mM potassium phosphate pH 7.2, and suspended in the same buffer to form a concentrated cell

suspension. All the above manipulations were performed anaerobically in an anaerobic chamber (Coy Laboratory Prod., MI) furnished with an oxygen-detector. Washed cells were lysed after 3 aerobic passages through a French pressure cell (35,000 psi). CFE was obtained after centrifugation at 25,000 x g at 4°C for 30 min. Anaerobic lysis of cells using the French Press was also performed in earlier experiments. No difference was observed in terms of SHMT activity between the aerobic and anaerobic lysis methods.

CFEs (or ammonium sulfate treated CFE) of all methanogens tested were stored at -60 °C aerobically.

III.2.4 Preparation of methylene-H_4M dehydrogenase-free SHMT in methanogens by ammonium sulfate precipitation.

The following enrichment procedure was performed aerobically at 4 °C: solid ammonium sulfate was added slowly to the cell free extracts to 70% of saturation (43.6 g/100 ml). After stirring for 10 min, the solution was centrifuged at 25,000 x g for 15 min. The pellet was dissolved in 20 mM potassium phosphate buffer, pH 7.2, containing 0.1 mM pyridoxal phosphate and 1 mM EDTA. The remaining ammonium sulfate was removed through ultrafiltration at 60 psi (both the membrane holder and the YM-10 membrane were from Amicon). The ultrafiltration process was repeated at least twice using 10 times volume of the same buffer. Alternatively dialysis was carried out with the precipitated protein against 40 times volume of the aforesaid potassium phosphate buffer to remove the remaining

ammonium sulfate.

III.2.5 Preparation of boiled cell extracts (BCE) and purification of H_4M

To prepare anaerobic boiled cell extracts, cells were harvested and resuspended anaerobically as described previously. Cell suspensions were boiled for 10 min under anaerobic conditions. By use of gassing needles the stoppered tubes or bottles that contained anaerobic cell suspensions was gassed with a gentle stream of oxygen-free hydrogen gas throughout the heat treatment and subsequent cooling period (Gunsalus and Wolfe 1980). Once cooled to room temperature, gassing needles were removed and boiled cell suspensions were transfered to the anaerobic chamber. The tubes or bottles were unstoppered, the extracts transfered to ultracentrifugation tubes and sealed. The boiled cell extracts were obtained by ultracentrifuging the boiled cell suspensions at 100,000 x g for 60 min.

H_4M was purified from the anaerobic BCE in the anaerobic chamber as previously described (Mukhopadhyay and Daniels 1989; Escalante-Semerena et al. 1984) with the following procedures: DEAE-Sephadex A-25-150 (medium) column (2.5 x 15 cm) was packed in 20 mM potassium phosphate buffer, pH 7.0. The column was equilibrated with 20 mM KHP buffer of pH 7.0 containing 10 mM mercaptoethanol. BCE from *Mb. thermoautotrophicum* strain Marburg was loaded into the column, washed with 1 bed volume of equilibrating buffer following by another bed volume of the same

buffer containing 160 mM NaCl. The column was eluted with 4 bed volumes of 20 mM phosphate buffer (pH 7.0) under a NaCl gradient of 160 to 300 mM. H_4M was eluted out with around 200 mM NaCl. Fractions with activity (see next section for the method of H_4M identification) were collected, yielding a yellow solution.

The pooled solution was then loaded to another DEAE-Sephadex A-25 column equilibrated with phosphate buffer, pH 5.0, containing 10 mM mercaptoethanol. The column was then washed and eluted as the first column under the same conditions except all buffers were at pH 5.0. H_4M was eluted at about 270 mM NaCl. A colourless solution containing H_4M was collected, desalted as previously described (Keltjens et al. 1986). Salt-free tetrahydromethanopterin was obtained by use of Sep-Pak C_{18} cartridges (Waters Associates). Prior to use the cartridge was activated with 100% methanol and subsequently washed with 25 mM potassium phosphate buffer (pH 3.0). The H_4M pool from the DEAE-Sephadex column was then loaded into the cartridge. H_4M adhered to the cartridge C_{18} packing materials and was eluted from the cartridge with 50% aqueous methanol acidified to pH 3.0 with acetic acid. H_4M eluted from the cartridge was concentrated and the remaining methanol in the H_4M was removed from the collected pool of H_4M with procedures similar to the preparation of BCE: a stopper-sealed bottle containing the pooled solution was heated for faster evaporation in a hot water bath (70-80°C), and the bottle was flushed with oxygen-free hydrogen gas through needles until the proper volume of pooled solution was obtained and used as the source of H_4M for experiments.

III.2.6 Identification and quantitation of H_4M

H_4M was identified and quantitated enzymatically as described (Escalante-Semerena et al. 1984), including the following procedures:

a). Preparation of the cofactor-free methylene-tetrahydromethanopterin dehydrogenase. An amount of CFE of *Mb. thermoautotrophicum* strain Marburg equal to 5% (v/v) of the bed volume was passed at 4°C through a column packed with Sephadex G-25 anaerobically (in an anaerobic chamber). Sephadex G-25 was preswollen in 50 mM Tris-HCl buffer (pH 7.2) containing 30 mM $MgCl_2$, 1 mM dithiothreitol, and 10% (v/v) glycerol. Equilibration and elution were performed with the same buffer. The enzyme fraction eluted in the void volume separated from the low molecular weight coenzymes. The enzyme was collected and sealed in stoppered tubes, and stored at -20°C to prevent loss of activity.

b). Identification and quantitation of H_4M. Under anaerobic conditions, 10 to 50 μL of H_4M containing samples were added into a round cuvette (Daniels and Wessels 1984) containing 3 mL of anoxic potassium phosphate buffer (20 mM, pH 7.0) which contained 1 mM dithiothreitol and 0.3 mM formaldehyde. After preincubation for 10 min at 60°C, the reaction was initiated by adding 25 μL of Sephadex G-25 treated enzyme by means of a gas-tight syringe. The absorbance at 340 nm was recorded until no further increase occurred. The concentration of H_4M was calculated from the increase of the absorbance, based on the molar extinction coefficient ε_{340} =

20.6 / mM. cm of 5,10-methenyl-H_4M (Escalante-Semerena et al. 1984).

H_4S from *Ms. barkeri* could also be quantitated using the enzyme preparation from *Mb. thermoautotrophicum* following the above procedures.

III.2.7 SHMT assay conditions

Unless specified otherwise, SHMT was assayed aerobically using the following method (Taylor and Weissbach 1965). The complete assay system contained 0.1 μmole 3-^{14}C-L-serine (1.04 x ^{6}10 cpm/umol), 0.1 μmole pyridoxal phosphate, 0.05 μmoles dl-H_4F or H_4S (or 0.1 μmoles H_4M), 4 μmoles 2-mercaptoethanol, 30 μmoles potassium phosphate and enzyme in a total volume of 0.4 ml, pH 7.5. All components except H_4F (or H_4M) were added first and incubated at 37 °C for 5 min. Reactions were then initiated by the addition of H_4F (or H_4M) and terminated 5 to 15 min later with 0.3 ml of 1.0 M sodium acetate, pH 4.5; 0.2 ml 0.1 M formaldehyde and 0.3 ml 0.4 M dimedone (in 50% ethanol) were added in succession and the tubes were heated 5 min in a boiling water bath to accelerate formation of the formaldehyde-dimedone derivative. The tubes were then cooled for 5 min in an ice bath before the dimedone derivative was extracted by vigorous shaking with 5.0 ml toluene at room temperature. Two min of centrifugation at 2,000 rpm separated the water and toluene phases and 3.0 ml of the upper phase (toluene with formaldehyde-dimedone complex extracted in it) were removed

for counting. The entire assay was carried out in a 12-ml conical centrifuge tube. When the enzyme was assayed anaerobically using the same method, similar result was obtained.

The basis of this assay is the exchange of formaldehyde with the [^{14}C] methylene-tetrahydrofolate (or H_4M) formed from the reaction of serine and H_4F (or H_4M). It was demonstrated (Geller and Kotb 1989) that under these assay conditions, 80% of methylene-complex can be exchanged with formaldehyde when the assay solution was incubated for 5 min in the presence of formaldehyde.

III.2.8 Calibration of radioactive counts and calculation of activity

Background counts (consistently between 350 to 450 cpm) were always observed even when no enzyme and no H_4F (H_4M) was added. Therefore, separate controls were prepared for each enzyme assay. All data were corrected by the observed background radioactivity in the controls. The enzyme activity was calculated as the following: after correction for the background counts and the counting efficiency, the amount of [^{14}C] formaldehyde-dimedone observed was considered to be 80% of the methylene- complex produced in the reaction based on what was found by Geller and Kotb (1989).

III.2.9 Protein determination

The protein concentration was determined by measuring the

absorbance at 280 nm or by the method of Bradford (1976).

III.2.10 Radioactivity measurements

Radioactivity was measured using a $^{14}C/^{3}H$ window of the liquid scintillation counter (Beckman LS-230). Count efficiency was determined by using external standards.

III.2.11 Methane formation from L-serine

Cultures of methanogens were prepared and harvested as described in section III.2.2 and III.2.3. Cells were then washed three times in an anaerobic Coy chamber with 40 mM anaerobic potassium phosphate buffer, pH 7.0, and resuspended in the same buffer to form a concentrated cell suspension. Half a milliliter of cells (2 to 5 mg protein) was then added to a 25 ml stoppered tube which contained 9.5 ml of the aforementioned buffer with 90 mM NaCl. To remove the residual methane in the cultures, the tubes were incubated in a reciprocal shaker (Haake) at 37°C for 10 min, and the gas phase replaced through 3 cycles of evacuating (5 min each) and flushing with pure hydrogen (1 min each). L-serine was added to a final concentration of 40 mM at time zero. Methane was measured by gas chromatography using a chromosorb 102 column (Supelco, Oakville, Ont.) and a flame ionization detector (Varian Aerograph series 2100).

III.2.12 Determination of ^{14}C serine level in the cell cytosol

Cell suspensions were prepared as described in section III.2.3. ^{14}C L-serine (final conc. 10 mM) (4400 cpm/μmol) was added into assay tubes containing 9.5 ml assay buffer and 0.5 ml of cell suspension, prepared as described in the last section. Aliquots of 0.5 ml of samples were taken at 3 min after the addition of serine through a 1 ml syringe, and subsequently transfered to a vacuum manifold equipped with 0.45 μm membrane (Sparling et al. 1993). About 10 ml of assay buffer was used to wash the cell material trapped on the membrane. Controls were prepared with cell material washed by 10 ml of 5% TCA. Radioactivity was measured as described in section III.2.10. Before cell pellets were measured for radioactivity, 0.5 ml of 5% TCA was added into each vial containing the membrane with pellet on it and the vial was incubated for 30 min. Radioactive counts observed in control samples were used as background counts. Radioactivity in the cell cytosol was calculated as counts observed in the samples minus the background counts in the controls.

III.2.13 Determination of folate level using a folate assay kit (ICN)

Determination of folate was performed using a folate-SNB radioassay kit [^{125}I] purchased from ICN. The assay procedures

(Chanarin 1969) were described in the information sheet with the assay kit, which contains a folate-binding protein solution and an ^{125}I-label folate solution. The principle of this method is the competitive binding of labelled folate with folate in the sample to the binding protein.

III.3 Results

III.3.1 Investigation of SHMT activity in different methanogens

SHMT activities were investigated using ammonium sulfate precipitated cell-free extracts in all methanogens tested (table III. 1). When H_4M and H_4F were used as the potential C_1 carriers in the hydroxymethyl transfer reaction, H_4F dependent SHMT activities were demonstrated in *Msp. hungatei*, *Ms. barkeri*, *Mst. concilii* and *Ml. tindarius*. No H_4M dependent activity was observed in these organisms. In all the other methanogens tested in table III.1, SHMT activities were found to be H_4M-dependent except in *Mc. voltae*. No SHMT activity was detected in *Mc. voltae* in the aerobic or anaerobic enzyme preparation and assay. A crude cell extract of *Escherichia coli* was used as a control which showed H_4F-dependent and no H_4M-dependent SHMT activity.

To further study the nature of the SHMT activity observed in *Msp. hungatei*, boiled cell extracts (BCE) were prepared using *Msp. hungatei* cells. It has been demonstrated that *Msp. hungatei* contained a pterin derivative which is not identical to H_4M Table

Table III. 1. Comparison of serine hydroxymethyltransferase activity in methanogens (nmol/min.mg protein)[a]

	Cofactor[b]			
	H_4F	H_4M	H_4S	BCE[c] of *Msp. hungatei*
Msph. stadtmanae	ND[d]	3.6	0.3	0.3
Mb. thermoautotrophicum Marburg	ND	1.0	–[e]	–
Mc. thermolithotrophicus	ND	1.3	–	–
Mc. voltae	ND	ND	–	–
Ms. barkeri	1.8	ND	ND	–
Mst. concilii	2.7	ND	ND	–
Ml. tindarius	1.2	ND	ND	–
Msp. hungatei	3.8	ND	ND	ND
E. coli crude cell extracts	5.6	ND	–	–

a. Enzymes from various methanogens were partially purified by precipitating the cell-free extracts (CFE) with 70% saturated ammonium sulfate. SHMT enzyme assay was carried out as described under Materials and Methods.
b. Both H_4F and H_4S were added to the final concentration of 0.125 mM, while H_4M was added with 0.25 mM. H_4F, tetrahydrofolate; H_4S, tetrahydrosarcinaptein; H_4M, tetrahydromethanopterin.
c. 50 μl out of 0.5 ml BCE [made from 2.5g (wet wt) of cells] was added as the cofactor source.
d. ND, not detected.
e. -, not tested.

(Keltjens and Vogels 1988). It was interesting that BCE of *Msp. hungatei* could not act as the source of C_1 carrier (table III.1) for SHMT in *Msp. hungatei*, while SHMT activity in *Msph. stadtmanae* could be detected by using the same BCE as source of the C_1 carrier.

Investigation of SHMT activities in some methanogens using purified H_4S as the potential $_1C$ carrier was also carried out (table III.1). In *Msp. hungatei*, *Ms. barkeri*, *Mst. concilii* and *Ml. tindarius*, no H_4S dependent activity was detected. In *Msph. stadtmanae*, a low level of H_4S dependent activity was observed.

III.3.2 Methane formation from L-serine using intact cells of *Msph. stadtmanae*, *Ms. barkeri* and *Msp. hungatei*

Cells of *Msph. stadtmanae* were able to produce methane from serine under hydrogen (see section II.3 of chapter II), which is consistent with the presence of a H_4M-dependent SHMT activity. The aforementioned SHMT activities from *Ms. barkeri* and *Msp.hungatei* indicated that the enzyme used H_4F instead of the major pterins (functioning in the methanogenic pathway) in *Ms. barkeri* and *Msp. hungatei* (Keltjens and Vogels 1988) under the *in vitro* conditions described. However, the situation *in vivo* might be different because the concentrations of the major pterins could be much higher than both the concentration of H_4F *in vivo* (if present) and the concentration of H_4S used *in vitro*. To determine if the major pterins used for methanogenesis in *Ms. bakeri* and *Msp. hungatei* could be used as the hydroxymethyl acceptors from L-serine *in vivo*,

experiments using whole cells to detect formation of methane from L-serine were carried out. Cells of *Msph. stadtmanae* produced methane from L-serine under a hydrogen gas phase while cells from *Ms. barkeri* and *Msp. hungatei* could not do so even after 20 min (figure III.1). These latter cells form methane when the gas phase was changed to hydrogen/carbon dioxide (80:20, v/v). Further experiments were performed to verify whether or not L-serine had indeed entered the cells. It was shown that in all three methanogens tested, L-serine had entered the cells under the aforementioned buffer conditions (table III.2) as indicated by the release of counts from the cells when a 5% TCA was used instead of buffer. The actual mechanisms for entry of serine into the cells was not determined.

III.3.3 Further investigations of SHMT activity in *Msp. hungatei* and *Ms. barkeri*

a) Air stability (fig III.2). The SHMT in the CFE of both organisms displayed no air sensitivity for the whole period of the experiment when the enzyme was stored at 4 °C. The slight decrease of activity observed might be caused by other factors.

b) Effects of pyridoxol-5-phosphate (PLP) and Mg^{2+} on the SHMT activity in *Ms. barkeri* and *Msp. hungatei* (table III.3). Other than the C_1 carrier (H_4F), pyridoxal-5-phosphate and Mg^{2+} are

Figure III.1. Methane formation from L-serine plus H_2 in methanogens. (■), *Msph. stadtmanae;* (▲),*Ms. barkeri;* and (●), *Msp. hungatei*. Arrows indicate the addition of serine or the change of gas phase from H_2 to H_2/CO_2.

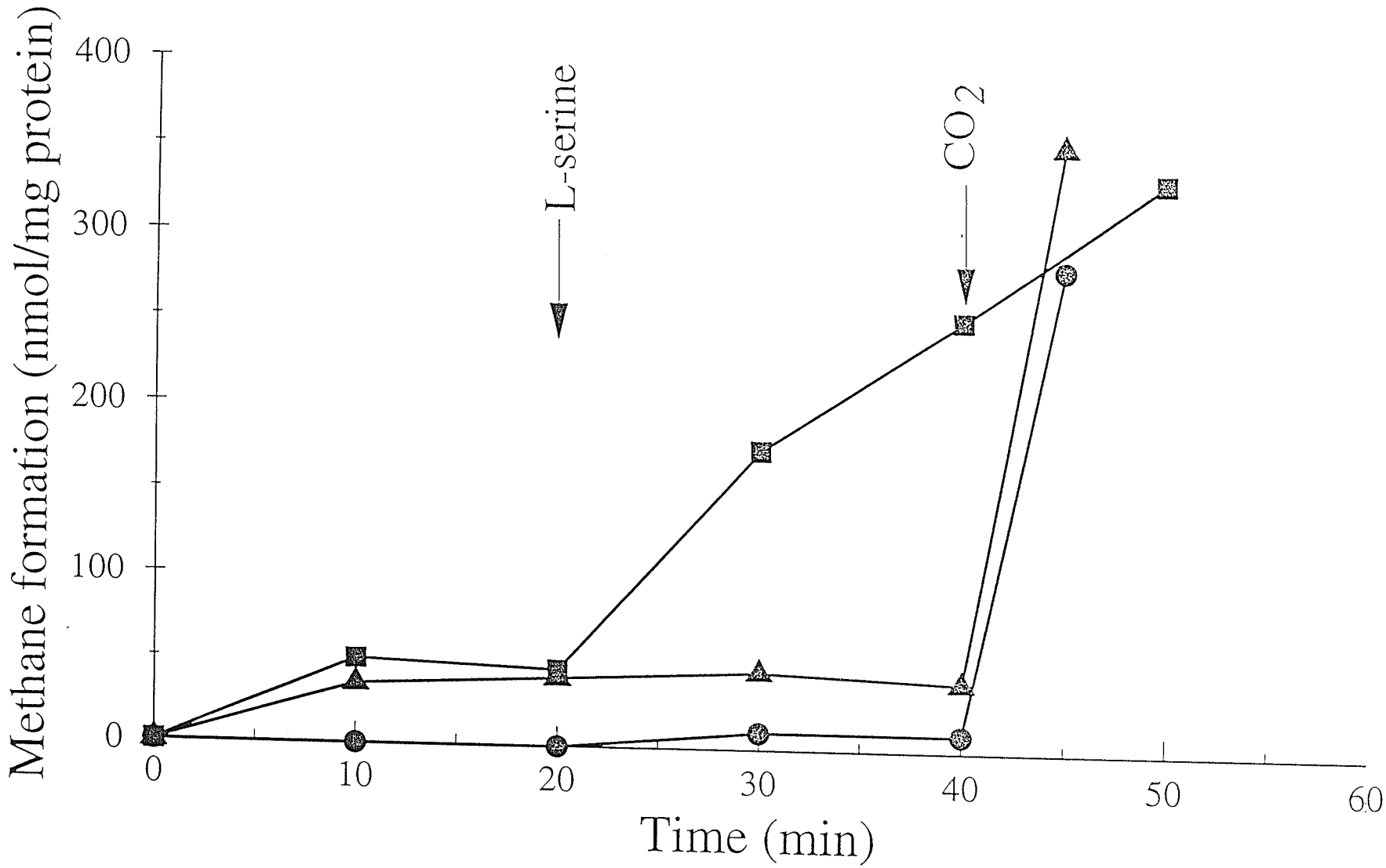

Methane formation (nmol/mg protein)
400
300
200
100
0
L-serine
CO_2
0
10
20
30
40
50
60
Time (min)

Table III.2 Entry of [^{14}C] serine into cells in some methanogens[a]

Washing solution	μmol/mg protein		
	Msp. hungatei	*Ms. barkeri*	*Msph. stadtmanae*
5% TCA	0.16±0.017	0.15±0.014	0.14±0.019
phosphate buffer	0.31±0.013	0.22±0.027	0.24±0.031

a. The specific radioactivity was 4400 cpm/μmol. All data listed were average of duplicates.

Figure III.2. Stability of SHMT activity under aerobic and anaerobic conditions. CFEs were stored at 4 °C. Symbols: (____), aerobic storage; (.....), anaerobic storage; (■), *Msp. hungatei;* (▲), *Ms. barkeri.*

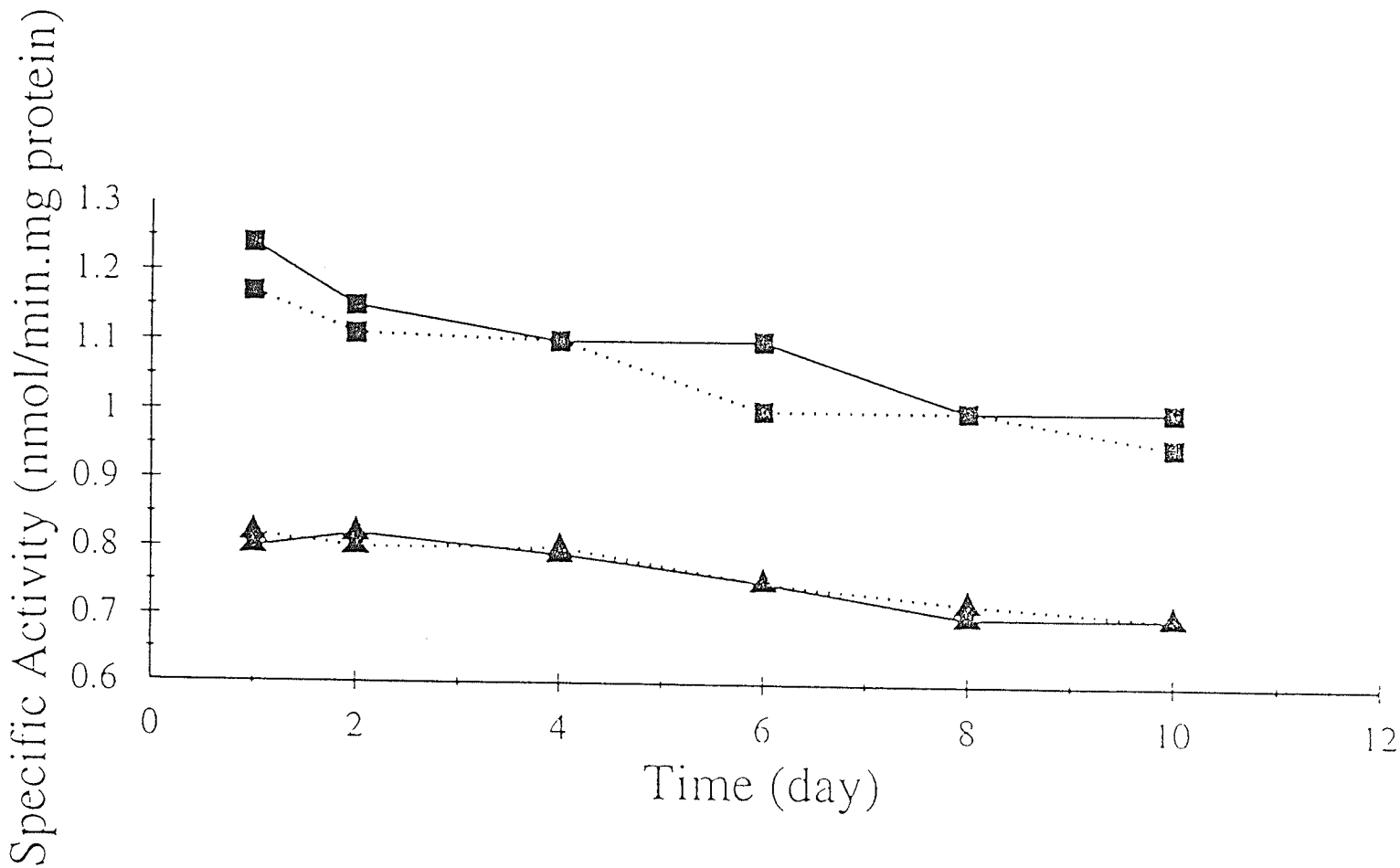

Specific Activity (nmol/min.mg protein)
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0
2
4
6
8
10
12
Time (day)

Table III.3 Effect of pyridoxal-5-phosphate (PLP) and Mg^{2+} on the activity of SHMT[a] (nmol/min.mg protein) in CFE

additions	*Msp. hungatei*	*Ms. barkeri*
none	1.0	0.4
0.25 mM PLP	1.5	0.8
20 mM magnesium acetate	0.9	0.5
0.25 mM PLP + 20 mM magnesium acetate	1.5	1.0

a. Enzyme assay was carried out as described in the Materials and Methods. 0.1 mg protein (10 - 20 μl CFE) was added in each assay tube.

both required for maximum activity of SHMT in eubacteria and most of the eucaryotic cells (Schirch and Mason 1963, Schirch et al. 1985). The effects of these compounds on the H_4F-dependent SHMT from both *Ms. barkeri* and *Msp. hungatei* were investigated. Added PLP (0.25 mM) was required for maximum activity in both organisms with about 50% and 33% activity stimulated respectively. Mg^{2+} had little effect on the enzyme activity of both organisms.

c) Other properties of the H_4F-dependent SHMT in *Ms. barkeri*. The optimum pH for the enzyme activity in CFE was found to be around 8.1 (figure III.3). The activity was dependent on the concentrations of H_4F with an apparent Km value of 0.086 mM (figure III.4a). The enzyme activity was also dependent on the concentrations of L-serine with an apparent Km of 0.29 mM (figure III.5a).

d) Other properties of the H_4F-dependent SHMT in *Msp. hungatei*. To prepare for further purification and investigation of the enzyme, assays of enzyme activity in the CFE from cultures harvested at various stages of the growth phases were performed. The enzyme activity appeared to be continuously increasing throughout the whole growth cycle. A total increase of about 40% activity was observed from early exponential phase till late stationary phase (Figure III.6). The optimum pH was determined to be 8.1 (figure III.7). Enzyme activity increased with increasing concentrations of H_4F, and the apparent Km for H_4F was determined to be 0.065 mM (figure III.8a).

Figure III.3. Effect of pH on the activity of SHMT in *Ms. barkeri*. Each assay tube contained 0.1 mg of 70% ammonium sulfate treated CFE. Various pHs were obtained by adjusting the regular assay buffer with HCl and KOH. Other assay conditions are the same as regular enzyme assays.

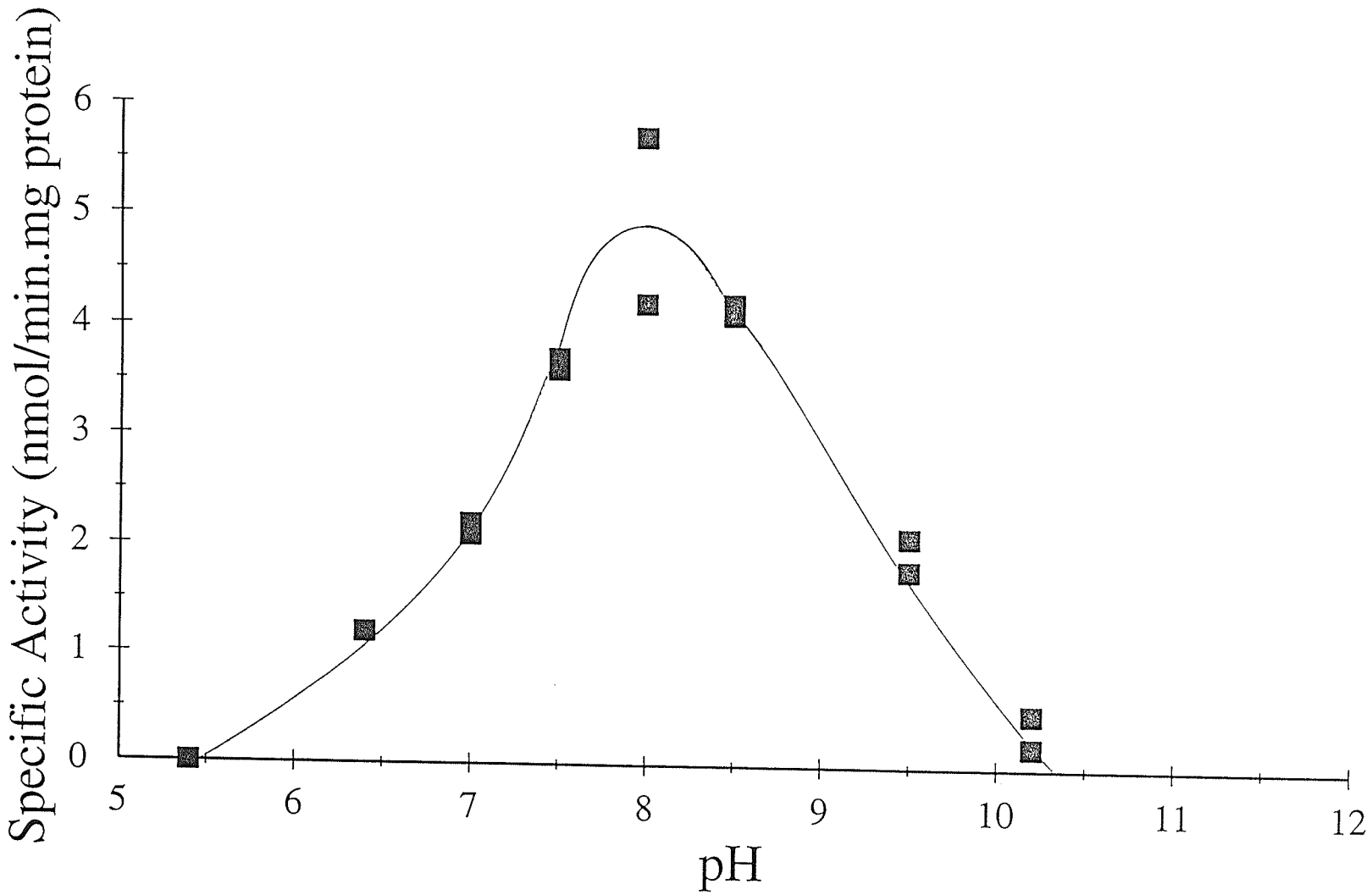
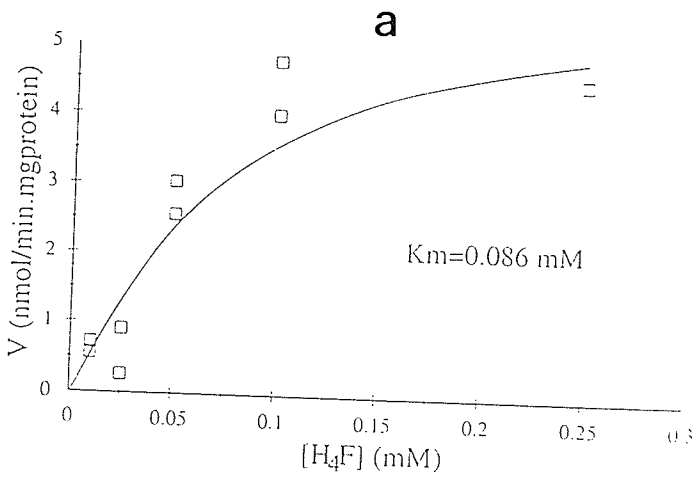

Specific Activity (nmol/min.mg protein)
0
1
2
3
4
5
6
5
6
7
8
9
10
11
12
pH

Figure III.4. Dependence of SHMT activity on the concentration of H_4F in *Ms. barkeri*. a. The first-order rate plot by Sigma Plot's Curve Fit (standard err for Km was 0.0034); b. Lineweaver-Burk plot. The correlation coefficient of the Lineweaver-Burk plot was 0.85.


a
V (nmol/min.mgprotein)
[H4F] (mM)
Km=0.086 mM
0
1
2
3
4
5
0.05
0.1
0.15
0.2
0.25

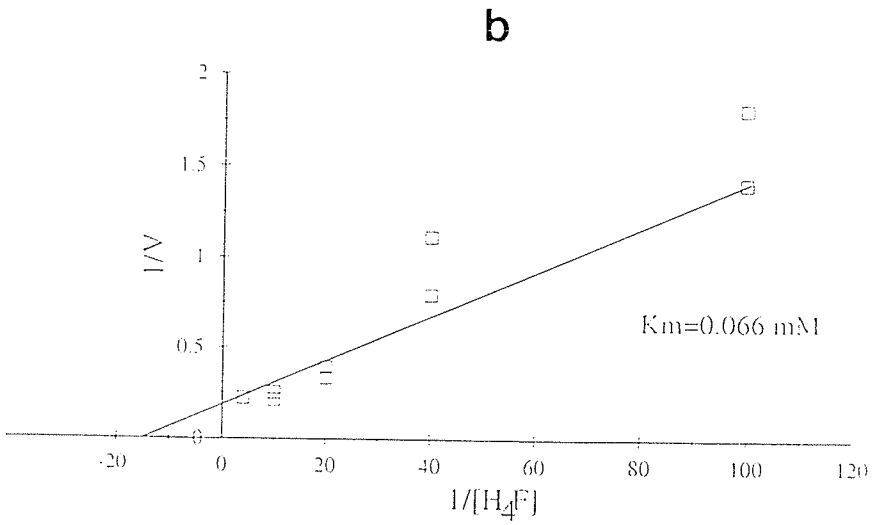
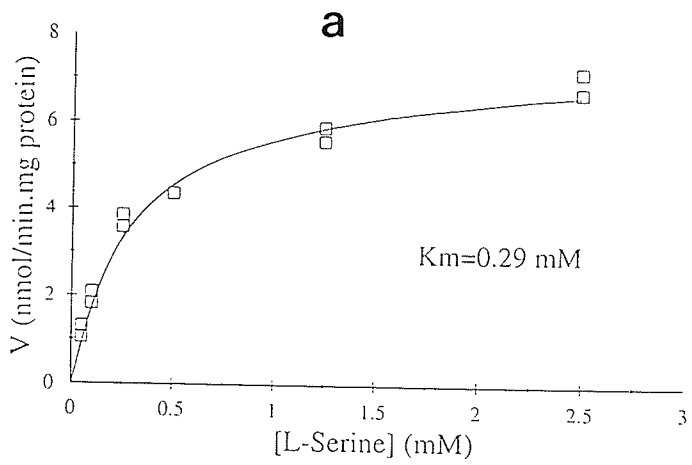

b
1/V
1/[H4F]
Km=0.066 mM
0
0.5
1
1.5
2
-20
0
20
40
60
80
100
120

Figure III.5. Dependence of SHMT activity on the concentration of L-serine in *Ms. barkeri*. a. The first-order rate plot by Sigma Plot's Curve Fit (standard err for Km was 0.033); b. Lineweaver-Burk plot. The correlation coefficient of the Lineweaver-Burk plot was 0.93.


a
V (nmol/min.mg protein)
[L-Serine] (mM)
Km=0.29 mM
0
2
4
6
8
0
0.5
1
1.5
2
2.5
3

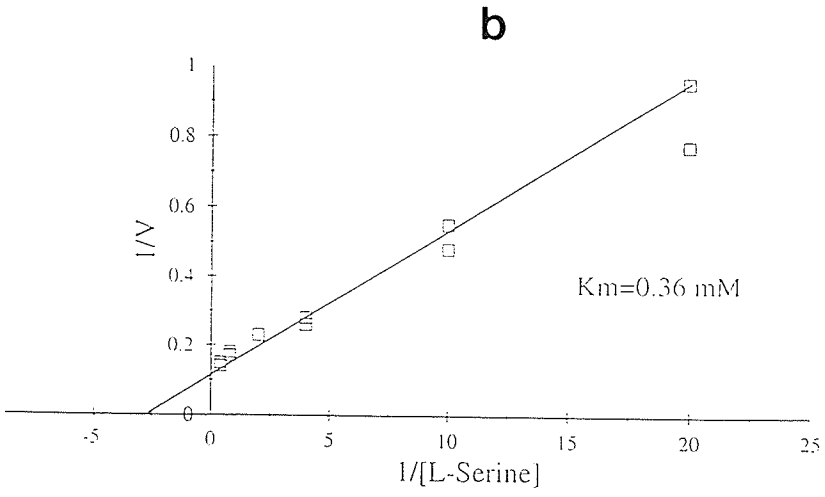

b
1/V
1/[L-Serine]
Km=0.36 mM
0
0.2
0.4
0.6
0.8
1
-5
0
5
10
15
20
25

Figure III.6. SHMT activity of *Msp. hungatei* in different growth phases. Arrows indicate the additions of H_2/CO_2.

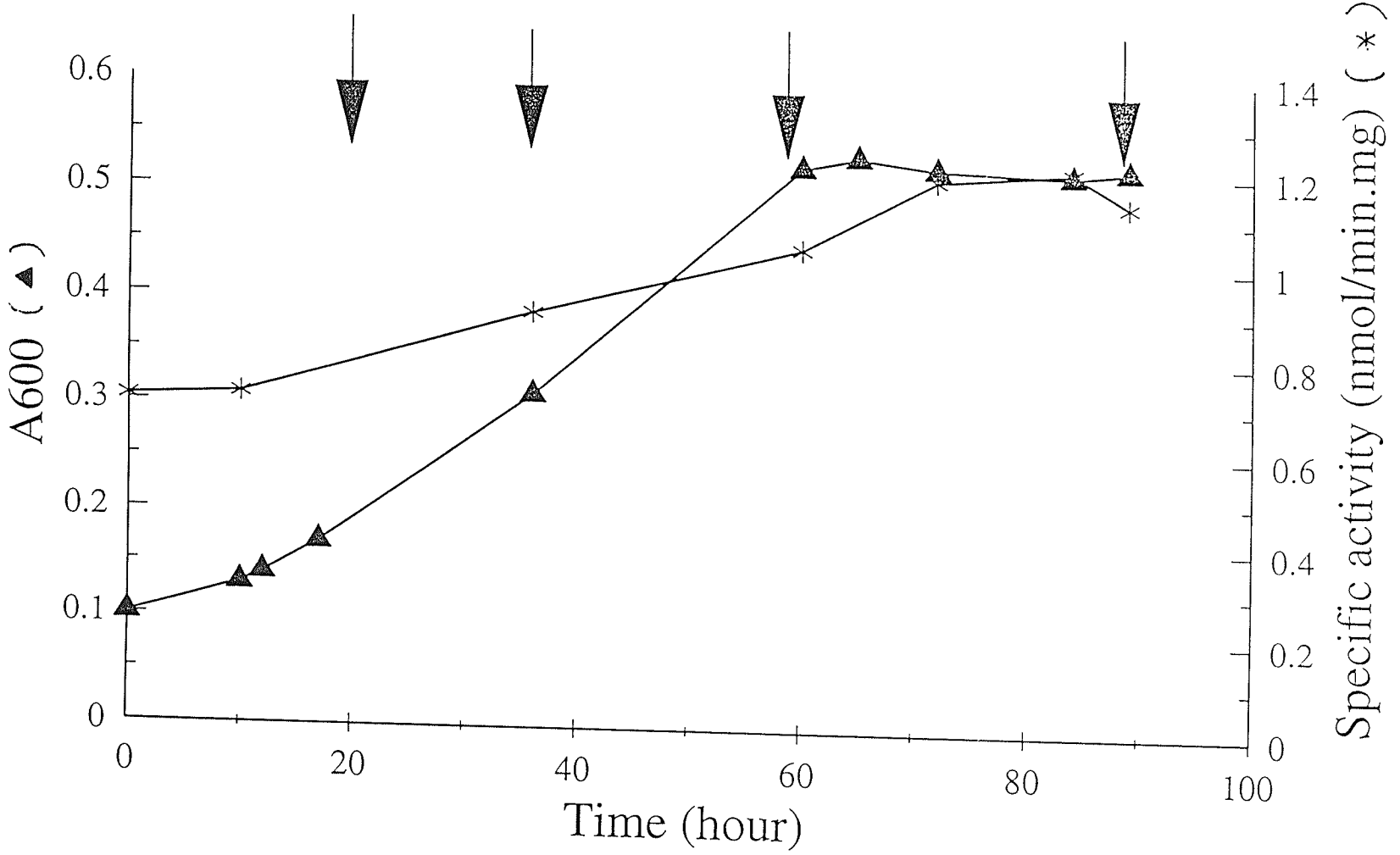
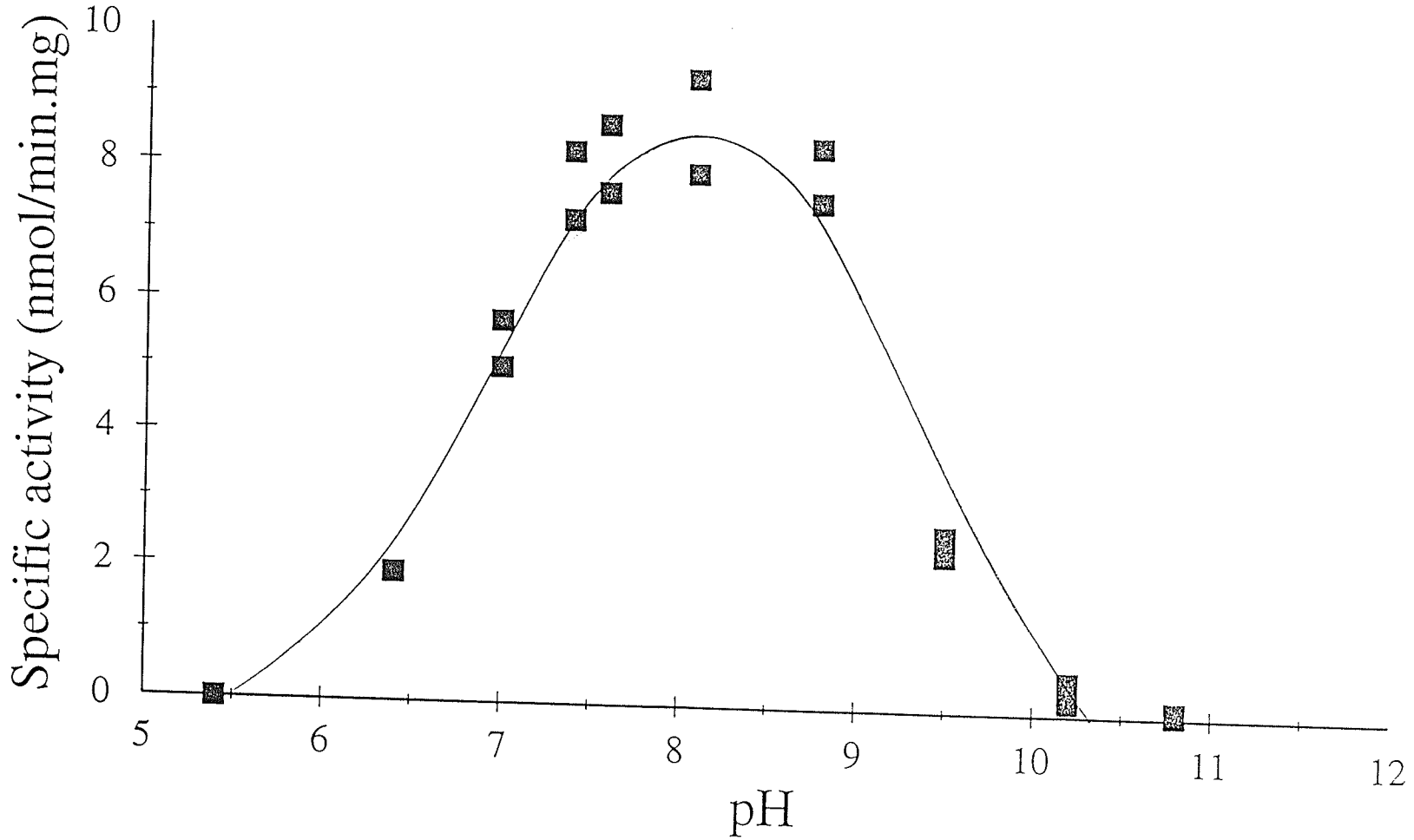

A600 (▲)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
Time (hour)
Specific activity (nmol/min.mg) (*)
1.4
1.2
1
0.8
0.6
0.4
0.2
0

Figure III.7. Effect of pH on the activity of SHMT in *Msp. hungatei*. Assay conditions were the same as described in figure III.3.


Specific activity (nmol/min.mg)
10
8
6
4
2
0
5
6
7
8
9
10
11
12
pH

The SHMT activity was also dependent on the concentrations of L-serine with an apparent Km of 0.31 mM (figure III.9a).

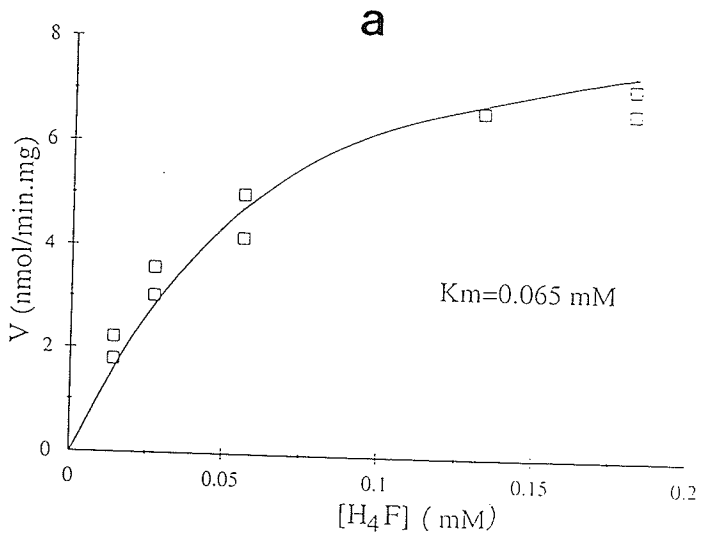
All the Km values were estimated based on the first-order rate plots instead of the Lineweaver-Burk plots. The former plot method relies more on the higher concentration points which are less fluctuable than the lower concentration points, while the latter one relies more on the lower concentration points which are normally more fluctuable.

III.3.4 Growth requirement test of folate in *Msph. stadtmanae* and *Msp. hungatei*

To determine if folate is required for cell growth, folate-free media were prepared. The folate-free media contained everything in the regular media except folate as described in the Materials and Methods. By using an air-tight syringe, 10% (v/v) of inoculum was taken out of a regular culture, filtered through a 0.45 μm sterile filter (Nalgene), then flushed with folate-free medium twice before injecting the cells into stoppered-bottles that contained folate-free medium. No growth requirement of folate was displayed in either *Msph. stadtmanae* or *Msp. hungatei* within 4 transfers of culture into folate-free media. The result of *Msph. stadtmanae* is consistent with the data of Miller and Wolin (1985).

Attempts to investigate the levels of folate in methanogens were not successful, using a folate assay kit from ICN. This was mainly because the folate-binding protein used in the assay kit

Figure III.8. Dependence of SHMT activity on the concentration of H_4F in *Msp. hungatei*. a. The first-order rate plot by Sigma Plot's Curve Fit (the standard err for Km was 0.0029); b. Lineweaver-Burk plot. The correlation coefficient of the Lineweaver-Burk plot was 0.92.


a
8
6
4
2
0
V (nmol/min.mg)
0
0.05
0.1
0.15
0.2
[H4F] (mM)
Km=0.065 mM

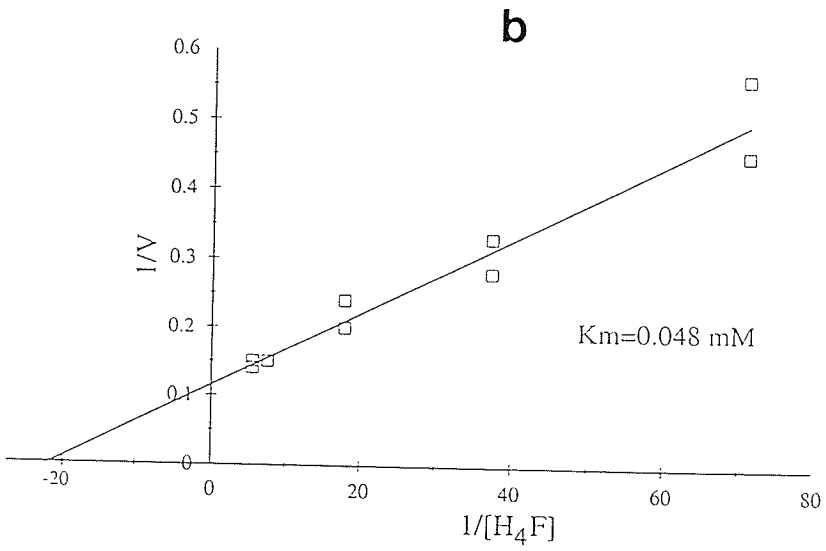
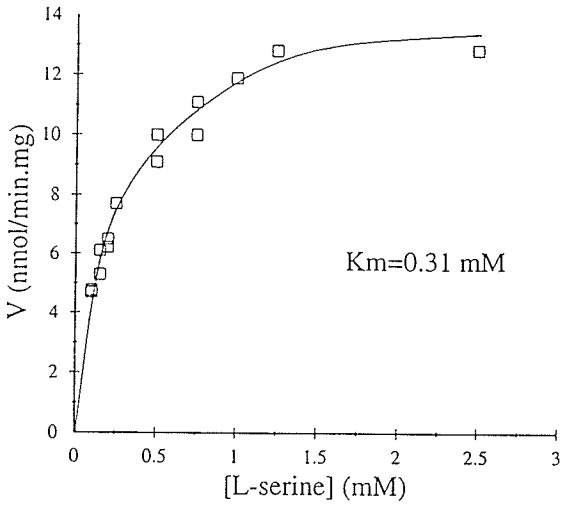
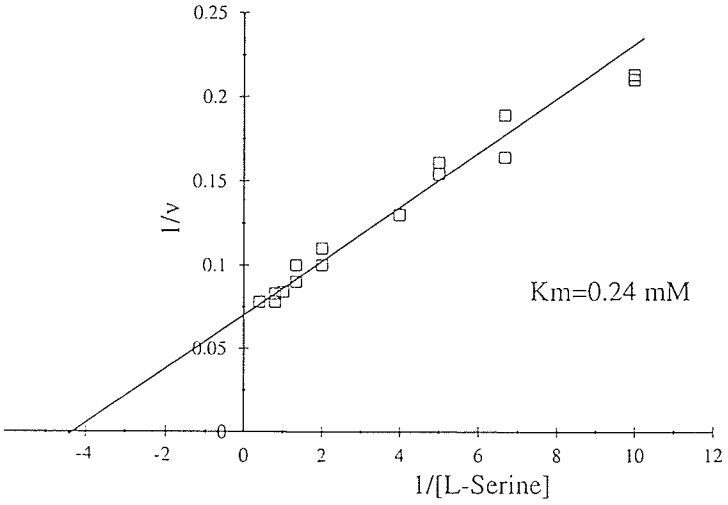

b
0.6
0.5
0.4
0.3
0.2
0.1
0
1/V
-20
0
20
40
60
80
1/[H4F]
Km=0.048 mM

Figure III.9. Dependence of SHMT activity on the concentration of L-serine in *Msp. hungatei*. a. The first-order rate plot by Sigma Plot's Curve Fit (the standard err for Km was 0.025); b. Lineweaver-Burk plot. The correlation coefficient of the Lineweaver-Burk plot was 0.97.

a


V (nmol/min.mg)
[L-serine] (mM)
Km=0.31 mM
0
2
4
6
8
10
12
14
0
0.5
1
1.5
2
2.5
3

b


1/v
1/[L-Serine]
Km=0.24 mM
0
0.05
0.1
0.15
0.2
0.25
-4
-2
0
2
4
6
8
10
12

failed to differentiate between folate and methanopterin or sarcinapterin, although the folate-binding protein was able to bind to folate with about 40 to 100 times higher affinity than to methanopterin or sarcinapterin. This method could detect folate levels between 2 to 45 nM. When purified methanopterin and sarcinapterin were used, 2 μM methanopterin and 5 μM sarcinapterin had the about the same reading as 45 nM folate. Nanomolar levels of methanopterin and sarcinapterin could not be detected using this method.

III.4 Discussion

Pterin derivatives distinct from H_4F, such as methanopterin, sarcinapterin and others have been shown to be responsible for the C_1 transfering reactions in the methanogenic pathway (Gorris and van der Drift 1986). Their functions in biosynthesis have never been directly assayed until now except for SHMT in *Mb. thermoautotrophicum* (Hoyt et al. 1986). Our enzymatic assays indicated that the acceptors for the serine hydroxymethyl transfer reaction were different among the methanogens. In *Msph. stadtmanae*, a member of the family *Methanobacteriaceae*, SHMT was dependent on H_4M, although the level of H_4M was reported as extremely low (van der Wijngaard et al. 1991) or not detectable (Gorris and van der Drift 1986). Indeed, a role for H_4M in biosynthetic reactions of C_1 transfer involving SHMT was demonstrated by ^{14}C labelling (chapter II of this thesis) and ^{13}C NMR (Choquet et al. 1994, Miller et al.

1995). In the family *Methanococcaceae*, SHMT of *Mc. thermolithotrophicus* was also found to be H_4M-dependent, while in *Mc. voltae*, no activity was detected. One explanation for the result in *Mc. voltae* is that the organism can take up amino acids from the medium (Whitman et al. 1982), which would contain amino acids from both yeast extract and trypticase. The ability of the organism to take up amino acids including glycine and serine might cause the loss or reduction of the enzyme being synthesized. ^{13}C-NMR results of *Mc. voltae* indicated that CO_2 instead of C-2 of acetate was incorporated into the methyl group of methionine, and the C-2 and C-8 of purine (Choquet et al. 1994). These data are consistent with either the presence of a H_4M dependent SHMT or the absence of SHMT activity.

In *Msp. hungatei*, *Ms. barkeri*, *Ml. tindarius* and *Mst. concilii*, members of the families *Methanomicrobiaceae* and *Methanosarcinaceae* (both belonging to order *Methanomicrobiales*), SHMT was found to be H_4F dependent but not H_4S or H_4M dependent. Attempts to detect enzyme activity in *Msp. hungatei* using boiled-cell extracts (BCE) of *Msp. hungatei* as the cofactor source were not successful, although the BCE contained a sufficient amount of a pterin derivative (Gorris and van der Drift 1986) which could be used as the hydroxymethyl acceptor by the enzyme in *Msph. stadtmanae*. Furthermore, it was shown that whole cells of *Msp. hungatei* and *Ms. barkeri* could not produce methane from L-serine under hydrogen, while cells of *Msph. stadtmanae* were able to form methane from L-serine. This implied that sarcinapterin in *Ms.*

barkeri or the pterin derivative in *Msp. hungatei* involved in C_1 transfer for methanogenesis was not responsible for serine hydroxymethyl transfer reaction *in vivo*. It could also be implied that the acceptor of the hydroxymethyl group in the serine hydroxymethyl transfer reaction in *Msp. hungatei* or *Ms. barkeri*, either H_4F or its derivatives but not the major pterin used in the methanogenic pathway, might be present only at a very low level in the cells. This would be similar to the case of *Msph. stadtmanae* where H_4M was present only at an extremely low level (van der Wijngaard et al. 1991), yet it did function as C_1 acceptor for both methanogenic and biosynthetic reactions (chapter II of this thesis; Choquet et al. 1994).

The results in *Msp. hungatei* and in *Ms. barkeri* were also consistent with the data that C-2 of acetate, which is the precursor of C-3 of serine in methanogens (Ekiel et al. 1983, Jones et al. 1987), but not CO_2 was incorporated into C-2 and C-8 of purine while the methyl group of methionine was derived from CO_2 (Ekiel et al. 1983, Choquet et al. 1994).

Our results also indicated that *Msp. hungatei* did not require folic acid to grow; this could be because of the ability of cells to synthesize folate. Unfortunately, attempts to investigate the levels of folate acid in methanogens were not successful since the folate-binding protein used in the assay failed to differentiate folate from methanopterin. Very low levels of folate were detected in some methanogens by using folate-requiring bacteria as the folate detectors (Worrell and Nagel 1988; Leigh 1983). Such results

may have been an artifact due to the reaction of methanopterin in the bioassay (Worrell and Nagel 1988).

The involvement of folate in SHMT of members of *Methanomicrobiales* but not in members of the other two orders of methanogenic bacteria is consistent with the phylogenetic divergence among the methanogens. It has been known that members of *Methanomicrobiales* were phylogenetically closer to the *Halobacteriales* than other methanogens (Boone et al. 1993, Woese et al. 1990). Non-methanogen archaea are known to use H_4F as C_1 carrier for C_1 transfer reactions (White 1988) with one exception, *Archaeoglobus fulgidus*. This organism, which was phylogenetically closer to methanogens than most other non-methanogen archaea (Boone et al. 1993), was found to use H_4M as the C_1 carrier in the lactate oxidation pathway (Moller-Zinkhan et al. 1989).

The SHMT from *Msp. hungatei* and *Ms. barkeri* required pyridoxal 5'-phosphate but not magnesium ions for maximum activity. This was different from its counterparts in *Mb. thermoautotrophicum* strain ΔH, eubacteria and eukaryotic cells (see chapter I of this thesis). Both pyridoxal 5'-phosphate and magnesium ions were required in these cells (Hoyt et al. 1986; Schirch et al. 1985; Schirch and Mason 1963). The enzyme displayed no air sensitivity in either *Ms. barkeri* or *Msp. hungatei* , similar to what was found in SHMT from another strict anaerobic organism, *Mb. thermoautotrophicum* ΔH (Hoyt et al. 1986). The optimum pH for SHMT in both organisms was found to be 8.1, similar to that of the enzyme in *Mb. thermoautotrophicum* (Hoyt et al. 1986). In *Msp. hungatei*, the Km of the enzyme for L-

serine was 0.31 mM, and the Km for H_4F was 0.065 mM, both much lower than the Kms of the enzyme for L-serine and H_4M in *Mb. thermoautotrophicum* (Hoyt et al. 1986), which were 2.8 and 1.8 mM respectively. On the other hand, they were similar to the Kms of the enzyme for L-serine and H_4F in *E. coli* (Schirch et al. 1985) 0.8 and 0.08 mM respectively. In *Ms. barkeri*, the Km of SHMT for L-serine was 0.29 mM , and the Km for H_4F was 0.086 mM, both similar to those of SHMT in *Msp. hungatei*.

The characterizations of H_4F dependent SHMT reported in this chapter were all based on the non-purified enzyme preparations. It would be interesting to study this enzyme further in a purified form, which would provide further information and understanding on this enzyme.

Chapter IV

Serine hydroxymethyltransferase in *Methanospirillum hungatei* GP1: purification and characterization

IV.1 Introduction

H_4M is well known as a C_1 carrier in the methanogenic pathway (Jones et al. 1987) and one biosynthetic reaction (Hoyt et al. 1985). In this reaction, SHMT catalyzes the reversible interconversion of serine and glycine, with H_4M in methanogens (Hoyt et al. 1986). In other parts of the biological world H_4F serves as the C_1 acceptors (Schirch et al. 1985; Schirch 1971). In the previous chapter, SHMT was determined to be H_4F dependent in four members of *Methanomicrobiales*, including *Msp. hungatei*, *Ms. barkeri*, *Ml. tindarius* and *Mst. concilii*, using H_4M purified from *Mb. thermoautotrophicum* Marburg and H_4S purified from *Ms. barkeri* Fusaro to test the efficiency of other potential cofactors. This is so far the first report of H_4F dependent SHMT in methanogens. In 1965, a H_4F dependent SHMT was purified and characterized from the culture known as *Methanobacillus omelianskii* (Wood et al. 1965). However, it was later demonstrated that the culture was a mixture of two organisms: a methanogen, *Methanobacterium bryantii*, formerly named as *Methanobacterium* M. o. H. and a non-methanogen bacterium, the "S" organism which oxidizes ethanol and produces hydrogen (Bryant et al. 1967).

Although the enzymes involved in the methanogenic pathway in various methanogens have been intensively studied (Thauer et al. 1993), those that are involved in C_1-carrier-requiring biosynthetic steps have received little attention. It has been stated that H_4M would function in methanogens as H_4F does in eubacteria and eukaryotic cells, as the major C_1 carrier for several biosynthetic reactions as well as for methanogenesis (Keltjens and Vogels 1988). Indeed, this was demonstrated when a H_4M-dependent SHMT was purified and characterized in *Mb. thermoautotrophicum* strain ΔH (Hoyt et al. 1986). However, our preliminary studies indicated that SHMT was H_4F dependent in some other methanogens (chapter III), specifically members of the *Methanomicrobiales*. In this chapter we describe the purification and characterization of the H_4F dependent SHMT from *Msp. hungatei* GP1.

IV.2 MATERIALS AND METHODS

IV.2.1 Chemicals

Formaldehyde, dimedone, glycine, sodium acetate, ammonium sulfate, DEAE-Sephadex A-50 and tetrahydrofolate were obtained from Sigma Chemical Co. [^{14}C] formaldehyde, 3-^{14}C-serine were purchased from Amersham. Hydroxylapatite was from BIORAD. Purified tetrahydrosarcinapterin was a gift from Dr. J. Krzycki.

IV.2.2 Microorganisms and culture conditions

Msp. hungatei GP1(DSM) was grown at 37°C under H_2/CO_2 (80/20, v/v) as described in chapter III.

IV.2.3 SHMT assay and calculation of activity

Unless otherwise specified, SHMT was assayed aerobically for the formation of methylene-H_4F from L-serine (Taylor and Weissbach 1965) as described in chapter III.

For the synthesis of serine from glycine, the enzyme was assayed using the following method: The complete assay system contained 0.2 μmole ^{14}C-formaldehyde (5.5 x 10^5 cpm/μmole), 1.0 μmole glycine, 0.1 μmole pyridoxal phosphate, 0.1 μmole tetrahydrofolate, 4.0 μmoles 2-mercaptoethanol, 30 μmoles potassium phosphate and enzyme in a total volume of 0.4 ml, pH 7.5. All components except enzyme were incubated 5 min at 37°C. Reaction was initiated by the addition of enzyme and terminated 15 to 30 min later with 0.3 ml 0.4 M sodium acetate, pH 4.5. Formaldehyde-dimedone complex was then extracted and counted for radioactivity as described earlier. For each set of experiments, controls with every component except the enzyme were used. The amount of serine formed in the reaction was calculated as the difference between the ^{14}C-formaldehyde of controls and the ^{14}C-formaldehyde of the experimental tubes.

The enzyme activity catalyzing the formation of methylene-H_4F

was calibrated as described in chapter III.

IV.2.4 Purification of Serine hydroxymethyl transferase in *Msp. hungatei* GP1

The following purification procedures were all performed aerobically at 4 °C.

a). Ammonium sulfate precipitation. Solid ammonium sulfate was added slowly to the cell free extracts to 50% of saturation (29.1 g/100 ml). After stirring for 10 min, the solution was centrifuged at 25,000 x g for 15 min, and the precipitate was discarded. Additional ammonium sulfate was added to the supernatant to 80% of saturation (19.4 g/100 ml). The solution was then stirred again for 15 min, centrifuged at 25,000 x g for 15 min. The pellet was dissolved in 20 mM potassium phosphate buffer, pH 7.2, containing 0.1 mM pyridoxal phosphate and 1 mM EDTA. The remaining ammonium sulfate was removed through ultrafiltration at 60 psi (both the membrane holder and the YM-10 membrane were from Amicon). The ultrafiltration process was repeated at least twice using the same buffer.

b). 1st DEAE-Sephadex A-50 column. The enzyme solution from the previous step (ammonium sulfate precipitate and ultrafiltration) was loaded to a DEAE-Sephadex A-50 column (18 x 2.5 cm) which was preequilibrated with 20 mM potassium phosphate

buffer, pH 7.2 containing 1.0 mM EDTA. After the enzyme solution was loaded, the column was then washed with 1 bed volume of the equilibrating buffer, containing 150 mM NaCl. The enzyme was eluted with a linear NaCl gradient of 150-400 mM and a pH gradient of 7.2-6.4. The mixing chamber contained 2.5 bed volumes of 20 mM potassium phosphate buffer, pH 7.2, containing 150 mM NaCl and 1.0 mM EDTA. The reservoir contained 2.5 bed volumes of 20 mM potassium phosphate buffer, pH 6.4 with 400 mM NaCl and 1.0 mM EDTA. The column was eluted with a flow rate of 1.0 ml per min, each fraction containing 6.0 ml. The fractions with activity higher than the starting enzyme solution were pooled and concentrated by using ultrafiltration as described earlier. The ultrafiltration step was repeated twice more with 20 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM of pyridoxal phosphate and 1.0 mM of EDTA, to significantly reduce the amount of salt remained in the enzyme solution.

c). **Hydroxylapatite column.** Enzyme solution from the first DEAE-Sephadex column was loaded onto a hydroxylapatite column (10 x 2.5 cm) which had been preequilibrated with 20 mM potassium phosphate buffer, pH 7.0, containing 1.0 mM EDTA. After loading, the column was washed with 1 bed volume of 100 mM potassium phosphate buffer, pH 7.0 containing 1.0 mM EDTA. The enzyme was eluted with a linear potassium phosphate gradient. The mixing chamber contained two bed volumes of the washing buffer, and the reservoir contained two bed volumes of 300 mM potassium phosphate

buffer, pH 7.0 containing 1.0 mM EDTA. The column was run with a flow rate of 1.0 ml/min, and each fraction contained 4 ml. Collected fractions with SHMT activity were concentrated and the amount of potassium phosphate was reduced by the use of ultrafiltration as described earlier.

d). 2nd DEAE-Sephadex A-50 column. The concentrated enzyme collection was added to another DEAE-Sephadex A-50 column (18 x 1.6 cm) which was preequilibrated with 20 mM potassium phosphate buffer, pH 6.4, containing 1.0 mM EDTA. The column was then washed with one bed volume of equilibrating buffer containing 150 mM NaCl, eluted with a linear NaCl gradient. The mixing chamber contained 2.5 bed volumes of washing buffer and the reservoir contained 2.5 bed volumes of 20 mM potassium phosphate buffer, pH 6.4, containing 300 mM NaCl and 1.0 mM EDTA. The enzyme was eluted with a flow rate of 0.5 ml per min, and each fraction contained 2.5 ml.

IV.2.5 Electrophoresis, criteria for the purity of SHMT in *Msp. hungatei GP1*, and the estimation of subunit mass

SDS-denaturing polyacrylamide gel electrophoresis was performed using the Tris/glycine buffer system as described by Laemmli (1970). Several stock solutions were made for the preparation of gels. Solution A: Acrylamide concentrate containing 29.2 g acrylamide and 0.8 g methylene-bisacrylamide and water in a total volume of 100 ml; Solution B: 1.5 M Tris-Cl, pH 8.8;

Solution C: 0.5 M Tris-Cl, pH 6.8; Solution D: 10% (w/v) SDS. The stacking gel was prepared by mixing 1.3 ml of A, 2.5 ml of C, 0.2 ml of D and 6.1 ml water. The separating gel was prepared by mixing 5 ml of A, 5 ml of B, 0.2 ml of D and 9.7 ml water. Both gel solutions were deaerated for >15 min before adding TEMED (0.05%) and ammonium persulfate (0.05%). Protein samples were dissolved in the sample buffer containing 60 mM tris-Cl, pH 6.8, 0.5% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.025% Bromophenol Blue. Samples were heated in a hot water bath for 5 min at 95 °C before loading into the gel wells. The running buffer contains 192 mM glycine, 25 mM tris-base and 0.1% (w/v) SDS (pH was about 8.3). The following protein markers were used as references: myosin of rabbit muscle (205 KDa), β-galactosidase of *E. coli* (116 KDa), phosphorylase B of rabbit muscle (97KDa), bovine plasma albumin (66 KDa), egg albumin (45 KDa) and carbonic anhydrase (29 KDa). After running at a constant current of 36 mA per gel for about 4 hours, the gel was fixed with a fixative solution containing methanol (40%) and trichloroacetic acid (10%). Protein bands were visualized by using the Sigma silver stain kit developed from the method of Merrill et al. (1981). The protein was considered pure when only one band was developed in the denaturing gel by using this staining method.

Protein staining was also carried out with 0.1 % Coomassie Brilliant Blue R250 in 40% methanol and 10% acetic acid for 30 min. The-dye-stained gel was destained using a destained solution containing 10% acetic acid and 40% methanol (Fairbanks et al. 1971).

IV.2.6 Molecular mass determination.

The molecular mass was determined on a Sephacryl HR 200 column (60 x 1.6 cm) previously equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 100 mM NaCl and 1.0 mM EDTA. Half to 1.0 ml of sample was loaded onto the column. A constant flow rate of 0.2 ml per min was maintained with 2.0 ml of eluted buffer collected in each fraction. The standard molecular weight markers used for calibration were all purchased from Sigma: blue dextran (2,000 kDa), amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa). The molecular mass of the enzyme was estimated according to Andrews (1965).

IV.2.7 Amino-terminal sequence and amino acid composition of SHMT in *Msp. hungatei*.

About 0.1 μg of pure protein was loaded into each well of a SDS-denaturing polyacrylamide gel (7.5%) and run as described in section IV.2.5 (Laemmli 1970). The protein band was then transferred onto a PVDF membrane by electroblotting (Matsudaira 1987) through the following procedures: gel was removed from the electrophoresis cell and soaked in the electroblotting buffer (10 mM CAPS buffer, pH 11, containing 10% methanol) for 5 min; electroblotting was performed at room temperature at 90 volt for 30 min; the blotted PVDF membrane was stained with 0.1% Coomassie

Brilliant Blue R-250 in 50% methanol for 5 min, and destained in 50% methanol, 10% acetic acid for 5 min three times at room temperature; the membrane was finally rinsed in H_2O for 10 min three times, air dried and stored at -20°C; the protein was sent to the Department of Biochemistry, University of Victoria, B. C. for the determination of N-terminal sequence and amino acid composition, where it was analysed using the following method (Yuen et al. 1988): a). For N-terminal sequencing, the protein band was hydrolysed in a clean hydrolysis tube containing 500 μl of 6N HCl at 165 °C for 45 min; automatic derivatization and online PTC-amino acid analysis were started by placing the membrane band on a derivatizer sample slide; b). For amino acid composition, the protein band was hydrolysed as described above, and amino acids were identified and quantitated through HPLC using an ion exchange column.

IV.2.8 Protein determination

The protein concentration was determined by measuring the absorbance at 280 nm or by the method of Bradford (1976).

IV.2.9 Radioactivity measurements

Radioactivity was quantitated as described in chapter III. The counting efficiency of the radioactive SHMT assay was determined to be 90% using the external standard method.

IV.2.10 Storage of the enzyme

When the enzyme was not to be used soon, it was stored under -60 °C. In early purification steps, this did not cause appreciable loss of activity. When the enzyme was at a highly purified stage, repeated thawing and freezing caused significant loss of activity.

IV.3 Results

IV.3.1 Purification of SHMT

The H_4F-dependent SHMT in *Msp. hungatei* was purified to apparent homogeneity as follows. The 50-80% ammonium sulfate precipitation step successfully removed a large portion of proteins in the CFE prepared from the culture and gave a purification factor of 3.7 with a 99% yield of the enzyme (Table IV.1). Following the ammonium sulfate precipitation, a DEAE-Sephadex A-50 column was run with a concurrent NaCl gradient-(150-400 mM) and a pH gradient (pH 7.2-6.4). Using a NaCl gradient combined with a pH gradient in this early purification step was found to have better resolution (about twice higher purification factor) than using a single NaCl gradient tested previously. The elution profile of this first DEAE-Sephadex column showed a major peak, starting at the washing step with equilibration buffer containing 150 mM NaCl. The protein peak tailed to the end of

Table IV.1. Purification of the H_4F-dependent serine hydroxymethyl transferase in *Msp. hungatei*

purification step	total protein (mg)	specific activity (nmol/min.mg protein)	purification factor	yield (%)
CFE	1080	1.2	1	100
50-80% $(NH_4)_2SO_4$	285	4.5	3.7	99
1st DEAE-Sephadex A-50	27.3	44.6	37.2	94
Hydroxyapatite	2.86	250	208	59
2nd DEAE-Sephadex A-50	0.091	1200	1000	15

gradient. SHMT was eluted at 220 mM NaCl, pH 6.8, separated from the major protein peak (Figure IV.1). A hydroxylapatite column was applied as the 2nd column. One major non-SHMT protein peak appeared at the washing step, while the SHMT was eluted early at the potassium phosphate gradient (120 mM) (Figure IV.2). The 2nd DEAE-Sephadex A-50 column was run with a NaCl gradient and a single pH of 6.4. The SHMT was eluted at the mid-gradient at about 220 mM NaCl, away from the major protein peak eluted earlier in the gradient (Figure IV.3). The SHMT peak was collected as three parts, pool I (p I) contained protein recovered from earlier fractions of the activity peak (about 1/3 of the peak in volume); pool II (p II) was the enzyme recovered from fractions with the highest activity at the middle of the activity peak and pool III (p III) contained protein from later fractions (also about 1/3 of the peak in volume). The protein in p II exhibited one band on electrophoresis gels with silver stain applied (Figure IV.4), the activity yield from p II was about 15% of the total activity gained in CFE. Protein from p I and p III contained a couple of minor bands and a major enzyme protein band. Table IV.1 summarizes the whole purification procedures and results of the enzyme. The overall purification factor was 1000 with 15% yield.

IV.3.2 Determination of molecular mass and structure of the subunit

a. Sephacryl GR-200 gel filtration. The Sephacryl GR-200

Figure IV.1. Elution profile of the 1st DEAE-Sephadex A-50 column. Symbols: (■)A280; (▲) Activity (nmol/min . fraction); (●) pH gradient and (....) [NaCl] gradient (mM).

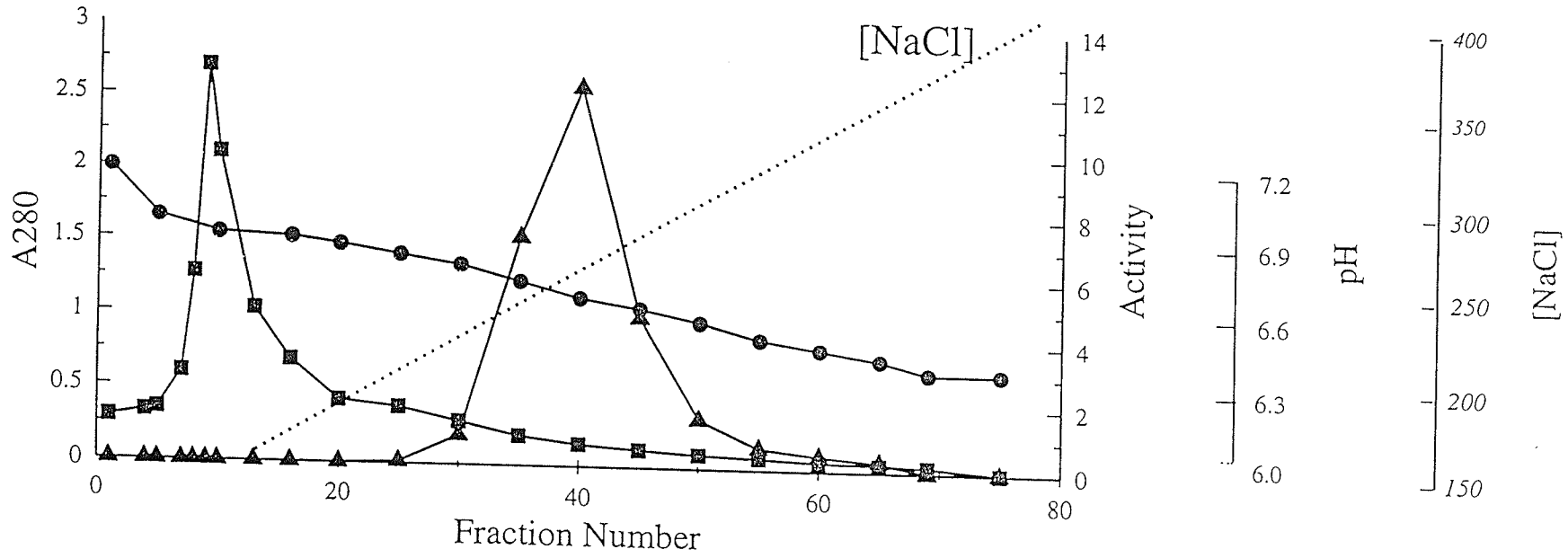
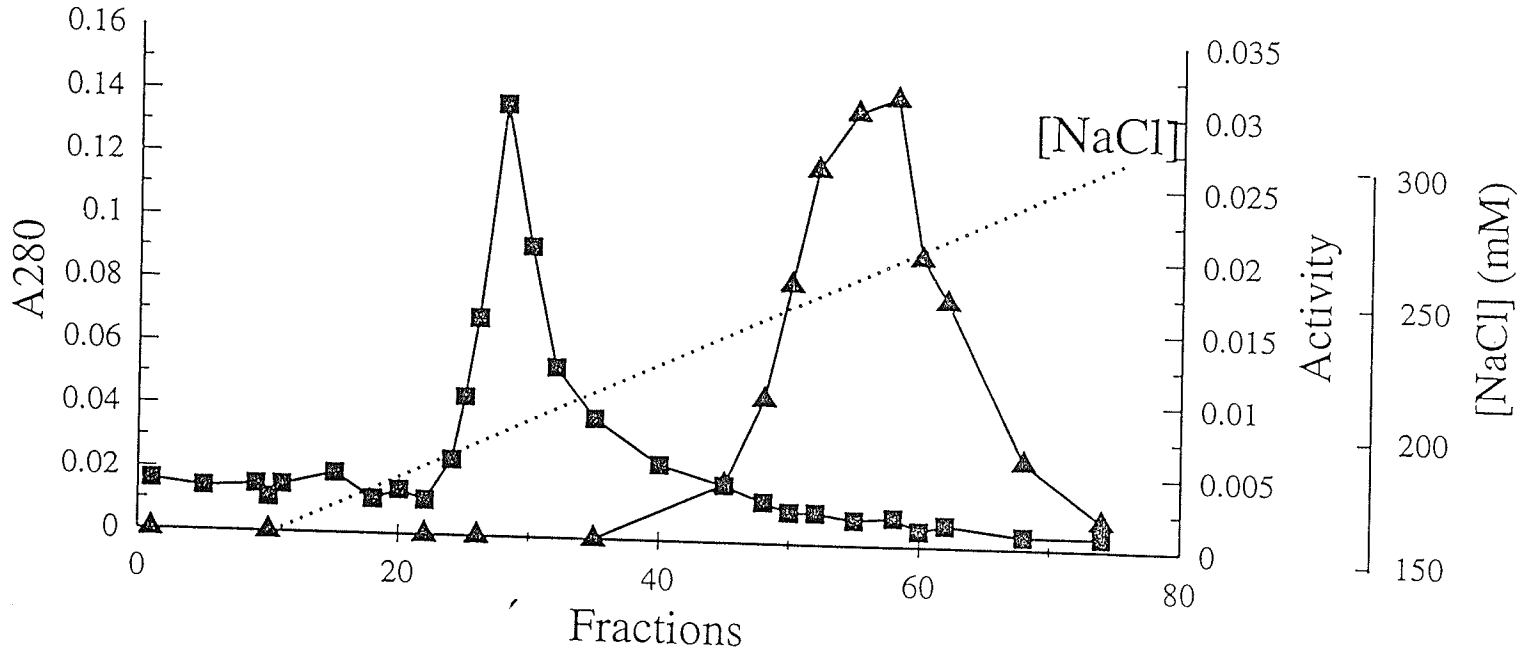

[NaCl]
A280
Activity
pH
[NaCl]
Fraction Number
0
20
40
60
80
0
0.5
1
1.5
2
2.5
3
0
2
4
6
8
10
12
14
6.0
6.3
6.6
6.9
7.2
150
200
250
300
350
400

Figure IV.2. Elution profile of hydroxylapatite column. Symbols: (■) A280; (▲) Activity (μmol/min. fraction) and (....) potassium phosphate gradient (mM).

A280
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
70
Fractions
0.12
0.1
0.08
0.06
0.04
0.02
0
Activity
300
250
200
150
KH_2PO_4 Concentration (mM)

Figure IV.3. Elution profile of 2nd DEAE-Sephadex A-50 column. Symbols: (■)A280; (▲) Activity (μmol/min. fraction) and (....) [NaCl] gradient.


A280
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
20
40
60
80
Fractions
[NaCl]
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Activity
300
250
200
150
[NaCl] (mM)

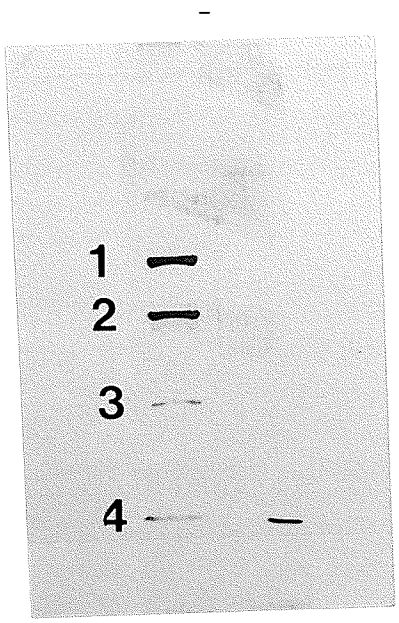


Figure IV.4. The purified SHMT on a silver-stained SDS gel. protein markers (from the cathode (top) to anode): (1), 116 KDa; (2), 97 KDa; (3), 66 KDa; and (4), 48.5 KDa.

chromatography was performed aerobically at 4 °C to determine the molecular mass of the SHMT. Based on its mobility relative to that of the various standards, a molecular weight of 93 KDa was calculated for SHMT (Figure IV.5a).

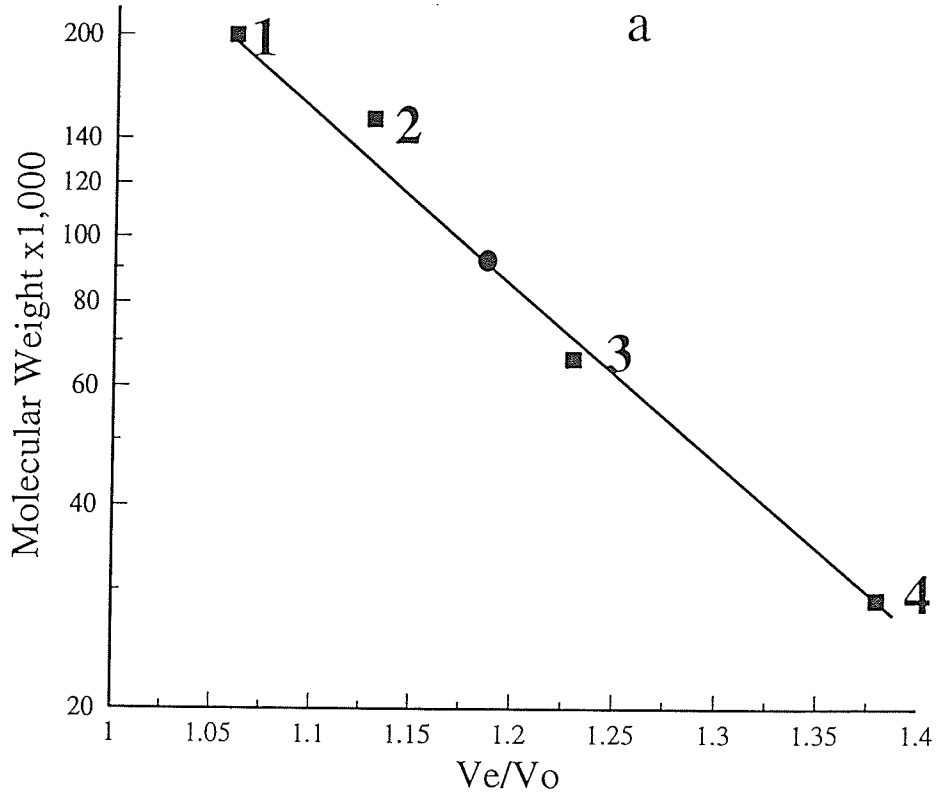
b. SDS-PAGE. SDS-PAGE of the purified SHMT was carried out at 4°C with a constant current of 36 mA per gel. The molecular mass of the SDS-treated SHMT was determined to be 47 KDa by comparing the R_f value of the SHMT band with various molecular standards (Figure IV.5b).

The combined results of the above experiments indicated that SHMT in *Msp. hungatei* was a homodimer protein with a total molecule weight of 94 kDa.

IV.3.3 N-terminal sequence analysis

The N-terminal sequence of SHMT was determined at the University of Victoria. We compare the N-terminal sequence of SHMT in *Msp. hungatei* with the sequences from other sources. The N-terminal sequence was aligned with the SHMT sequence of *Solanum tuberosum* (from GenomeNet, accession #S40218), and several other sequences were aligned with the nearest highly conserved region of SHMT sequence in *S. tuberosum* (table IV.2). Although the N-terminal sequence of SHMT is not typically a region containing a highly conserved domain of the enzyme, similarity was shown among sequences of SHMT from various sources. Several highly conserved

Figure IV.5. Determination of the molecular mass of SHMT. (a). by Sephacryl GR-200 gel filtration. (■), protein markers: (1), 200 KDa; (2), 150 KDa; (3), 66 KDa; and (4),29 KDa. (●), SHMT; (b) by SDS-PAGE. (■), protein markers: (1), 205 KDa; (2), 116 KDa; (3), 97 KDa; (4),66 KDa; (5), 45 KDa; and (6), 29 KDa. (●), SHMT.


a
Molecular Weight x1,000
200
140
120
100
80
60
40
20
1
2
3
4
1
1.05
1.1
1.15
1.2
1.25
1.3
1.35
1.4
Ve/Vo

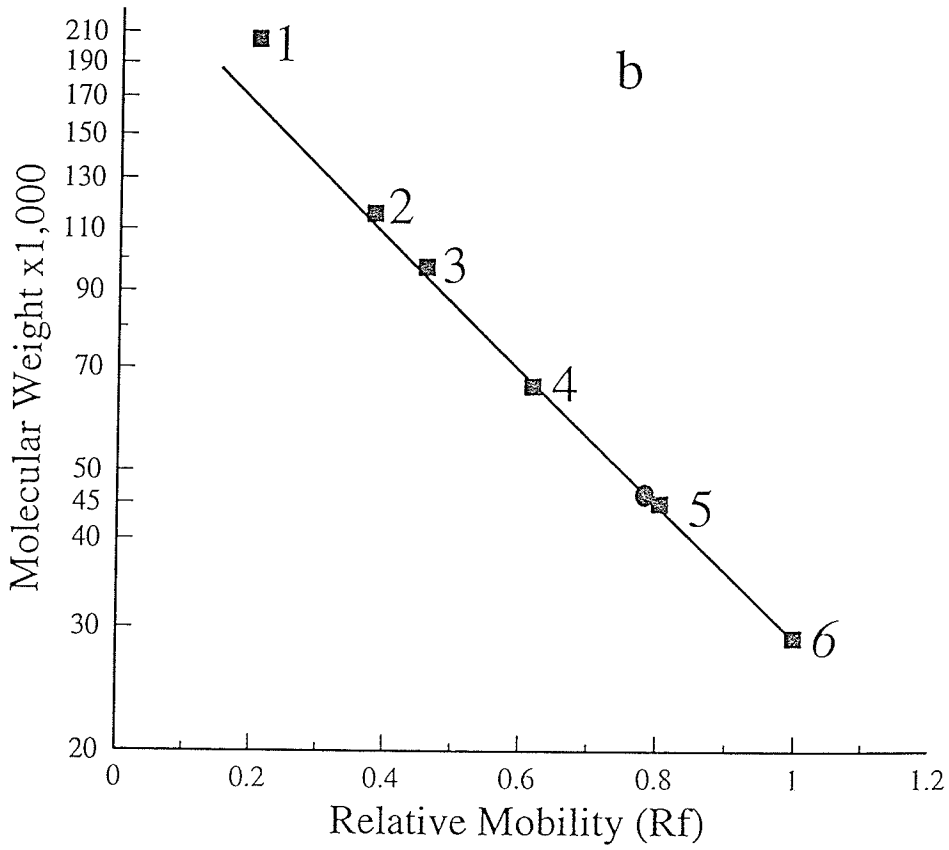

b
Molecular Weight x1,000
210
190
170
150
130
110
90
70
50
45
40
30
20
1
2
3
4
5
6
0
0.2
0.4
0.6
0.8
1
1.2
Relative Mobility (Rf)

Table IV.2 Comparison of N-terminal amino acid sequences of SHMT from *Msp. hungatei* to the relevant sequences of SHMT from various sources[a]

Sources	Start Position	A.A. Sequences	mw of subunit	A.A. number
Msp.hungatei	1	SYLETIDAEI AAIIEKEING G	47k	430
Solanum tuberosum (potato)	54	APLEVVDPEI ADIIELEKAR Q	57k	518
Pisum sativum (garden pea)	54	SPLEVIDPEI ADIIELEKAR Q	57k	518
E.coli	6	MNIADYDAEL WQAMEQEKVR Q	45k	417
Actinobacillus actinomyce-temcomitans	6	MNIADYDPVL WQAIENEMRR Q	46k	420
Bacillus subtilis	2	KHLPAQDEQV FNAIKNERER Q	45k	415
Hyphomicrobium methylovorum	18	SHVSETDPDI FSAIQKEFGR Q	46k	434
Human cytosol	24	QPLKDSDVEV YNIIKKESNR Q	53k	483
Human mitochondria	17	ESLSDSDPEM WELLQREKDR Q	52k	474
Saccharomyces cerevisiae	15	SHLVDTDPEV DSIIKDEIER Q	52k	469
Neurospora crassa	15	HSLVESDP-Q AEIMKKEVQR Q	52k	479
Mb. thermoautotrophicum Marburg	2	VSNQDYTERI RDLMKDHNSW M	47k	424

a. The sequence in *Mb. thermoautotrophicum* Marburg was from personal communication with Dr M. Vaupel which was based on the homology of an open reading frame sequence to the DNA sequence of gly A of *E. coli*. Other data were obtained from GenomeNet: Pisum sativum (garden pea), accession #p34899; Solanum tuberosum, accession #s40218; *H. methylovorum*, accession #p34895 ; *E. coli*, accession #p00477 ; *Actinobacillus actinomycetemcomitans* accession #p34897; *Bacillus subtilis*, accession #p39148; *Hyphomicrobium methylovorum*, #34895; human cytosol, #p34896; human mitochondria, #34897; *Saccharomyces cerevisiae*, #p37291; *Neurospora crassa*, #p34898. Conserved residues are shadowed. Homologous substitutions are bold.

amino acids were observed within the 21 amino acid sequences. This result further confirmed the identity of the enzyme.

IV.3.4 Analysis of amino acid compositions

The amino acid composition was calculated based on the data of analysis provided by the University of Victoria with minor adjustments, and on the subunit molecular mass of 47000 Da. The raw data provided were pmol of each amino acid, with extremely high level of glycine detected (table IV.3). Since the higher level of glycine might be contributed by the procedures of electrophoresis which involved a high concentration of glycine, an estimated number of 7.84% of the total number of amino acids, which was the average of glycine in several other SHMT, was used to calculate the composition of glycine. Tryptophan and cysteine were not detected as well, due to the acid hydrolysis process required for the amino acid composition determination. The pmole numbers of these two amino acids in table 3 were made up based on the numbers from other SHMT. The amino acid composition of SHMT from *Msp. hungatei* is listed in Table IV.4 together with those of several other organisms.

Table IV.3 Calculation of amino acid compositions of SHMT in *Msp. hungatei*[a]

AA	AAmw	Pmoles	Pgrams	AA comp	WtAA
Asx	115	171	19665	50	5750
Glx	129	113	14577	33	4257
Ser	87	159	13833	46	4002
Gly	57	114	6498	33	1881
His	137	33	4521	10	1370
Arg	156	90	14040	26	4056
Thr	101	81	8181	24	2424
Ala	71	147	10437	43	3053
Pro	97	52	5044	15	1455
Tyr	163	63	10269	18	2934
Trp	180	10	1862	3	558
Val	99	76	7524	22	2179
Met	131	27	3537	8	1048
Ile	113	66	7458	19	2147
Leu	113	127	14351	37	4181
Phe	147	51	7497	15	2205
Lys	128	84	10752	25	3200
Cys	103	10	1031	3	309
Total			161077	430	47007

a. The calculation of amino acid composition was based on the estimation that subunit molecular weight is 47,000. Number of glycine used was an estimated number calculated from 114 pmoles. The actual number from analytic result was about twice higher, which might be partly contributed from high glycine concentration of gel running buffer (192 mM). Neither Trp nor Cys was detected, because that the amino acid was degraded during the acid hydrolysis process. The numbers of Trp and Cys were made up based on the numbers from other SHMT.

Table IV.4 Amino acid composition of SHMT in *Msp. hungatei* and several other organisms[a]

	Composition (Number of amino acid residues per subunit)				
Amino acid	*Msp. hungatei*	*Mb. thermo-autotrophicum*	*H. methy-lovorum*	*E. coli*	S. tuber-osum (potato)
Asx	50	43	40	39	45
Glx	33	49	37	43	52
Ser	46	31	22	17	32
Gly	33	32	45	41	39
His	10	15	13	13	12
Arg	26	23	18	14	20
Thr	24	18	20	18	27
Ala	43	35	56	47	52
Pro	15	16	22	19	25
Tyr	18	11	9	20	26
Trp	3	3	1	3	3
Val	22	28	32	36	42
Met	8	15	8	11	14
Ile	19	30	31	21	25
Leu	37	34	30	32	41
Phe	15	16	21	11	18
Lys	25	20	27	29	43
Cys	3	5	4	3	2
Total	430	424	434	417	518

a. Data of *Mb. thermoautotrophicum* was provided by Dr. M. Vaupel. Amino acid composition of SHMT from other sources was obtained from GenomeNet as described in table IV.2.

IV.3.5 Effect of pH and temperature on the activity and stability of SHMT

The optimal pH for both degradation and synthesis of L-serine was 8.1 (Figure III.2 and IV.6). The effect of pH on the stability of SHMT was examined by monitoring activity of L-serine degradation (formation of methylene-H_4F). The enzyme was most stable between pH 7.0 and 7.6. When the enzyme was stored at pH 6.4 or lower and pH 8.7 or higher, appreciable loss of activity was observed (Figure IV.7). The optimal temperature for the SHMT activity was determined. Highest activity was obtained when the enzyme assay was carried out at 27°C (Figure IV.8). The effect of temperature on the enzyme stability was also examined. Appreciable loss of activity was observed with enzyme preincubated above 55 °C and then assayed at 37 °C (Figure IV.9).

IV.3.6 Determination of kinetic constants

The apparent Michaelis constants (Km) and the maximum reaction velocities (Vmax) of SHMT for H_4F, L-serine and glycine were determined based on the first-order rate plot generated by Sigma Plot's Curve Fit. The apparent Km for H_4F, L-serine and glycine were determined to be 0.06 mM, 0.29 mM and 0.62 mM respectively (Figure IV.10, 11 and 12). The Vmax for the degradation (Figure IV.11) and synthesis (Figure IV.12) of L-serine was determined to be 0.9 and 1.0 μmol/min. mg protein respectively when enzyme

Figure IV.6. Effect of pH on the SHMT activity for the synthesis of serine from glycine. 1.1 μg of pIII protein from the 2nd DEAE column was used for each assay. pH was adjusted by using HCl or KOH.

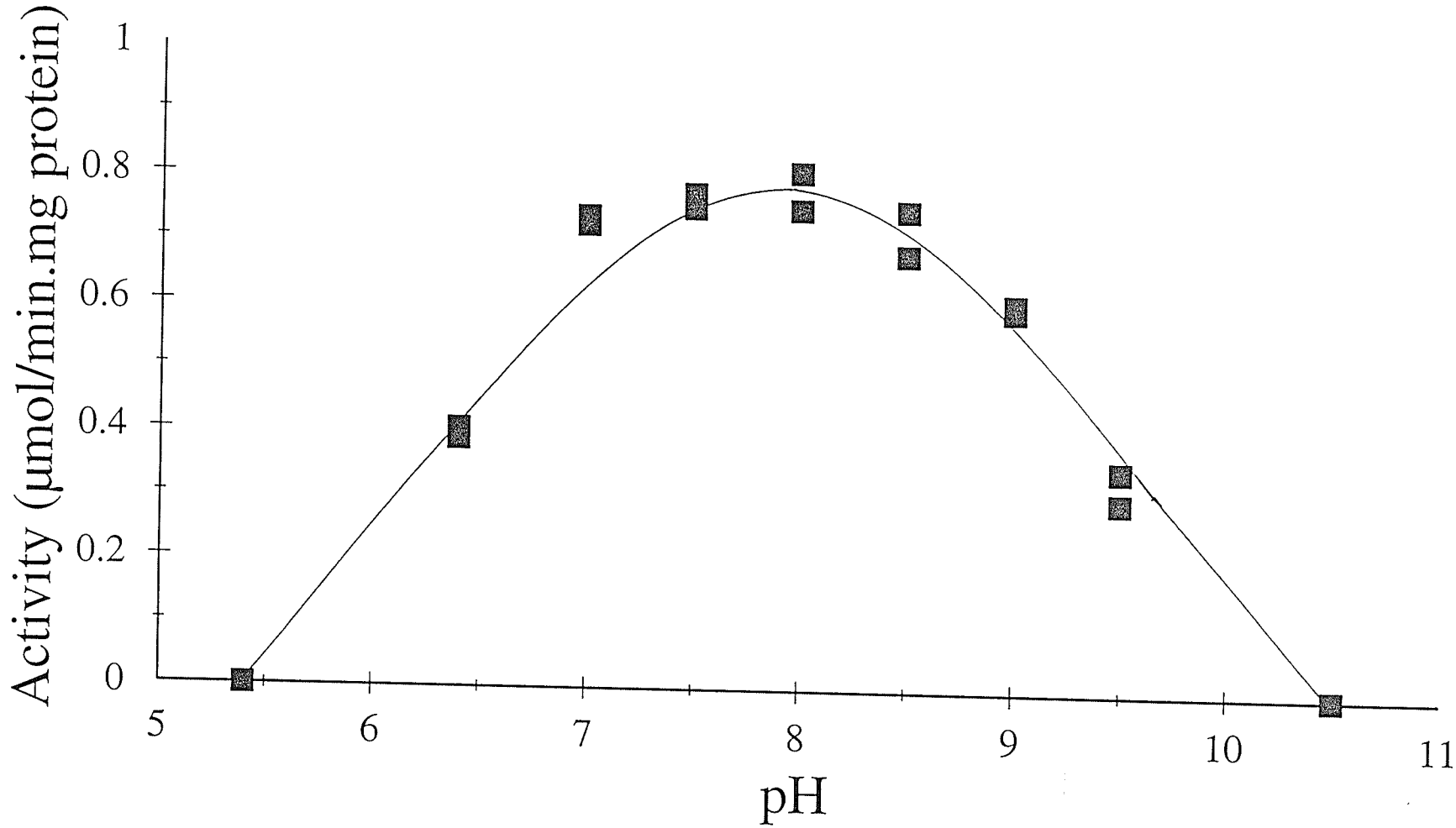

Activity (µmol/min.mg protein)
1
0.8
0.6
0.4
0.2
0
5
6
7
8
9
10
11
pH

Figure IV.7. Effect of pH on the stability of SHMT. 1.0 μg of enzyme (pII of 2nd DEAE-Sephadex column) was added into each assay tube containing assay buffer of various pH. pH was adjusted as in Figure IV.6. The enzyme-buffer mixture was incubated at 4 °C for 48 hrs, pHs were then adjusted back to 7.5, and enzyme assay was carried out under standard assay conditions.

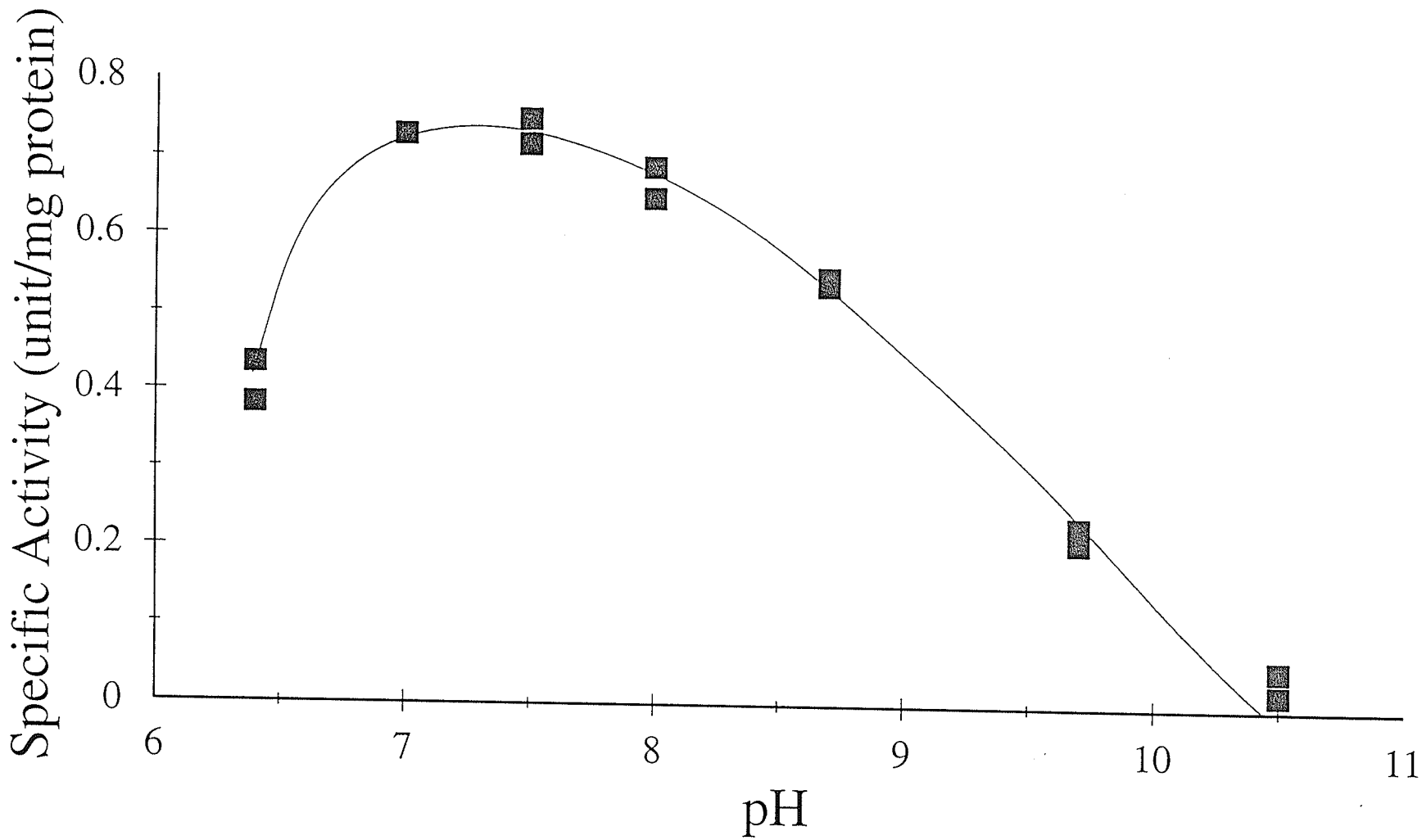

Specific Activity (unit/mg protein)
0.8
0.6
0.4
0.2
0
6
7
8
9
10
11
pH

Figure IV.8. Effect of temperature on the activity of SHMT. 1.1 μg of enzyme (pIII of the 2nd DEAE-sephadex column) was added into each assay tube.

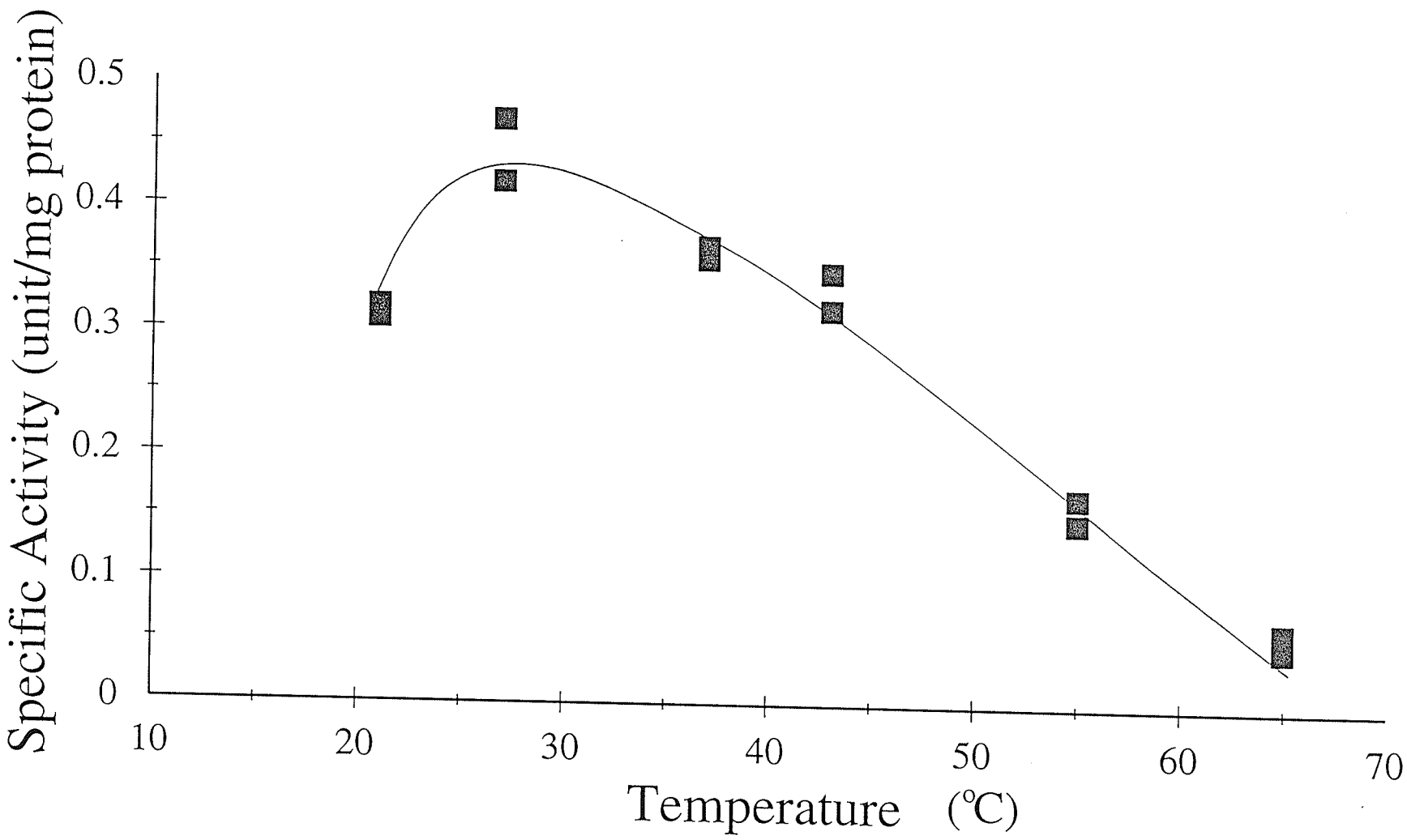

Specific Activity (unit/mg protein)
0.5
0.4
0.3
0.2
0.1
0
10
20
30
40
50
60
70
Temperature (°C)

Figure IV.9. Effect of temperature on the stability of SHMT. Assay buffer containing 1.0 μg of enzyme (pII of 2nd DEAE-Sephadex column) was incubated under various temperature for 45 min. The residual activity was measured under standard assay conditions at 37 °C. 100% activity was 1.0 μmol/min. mg protein.

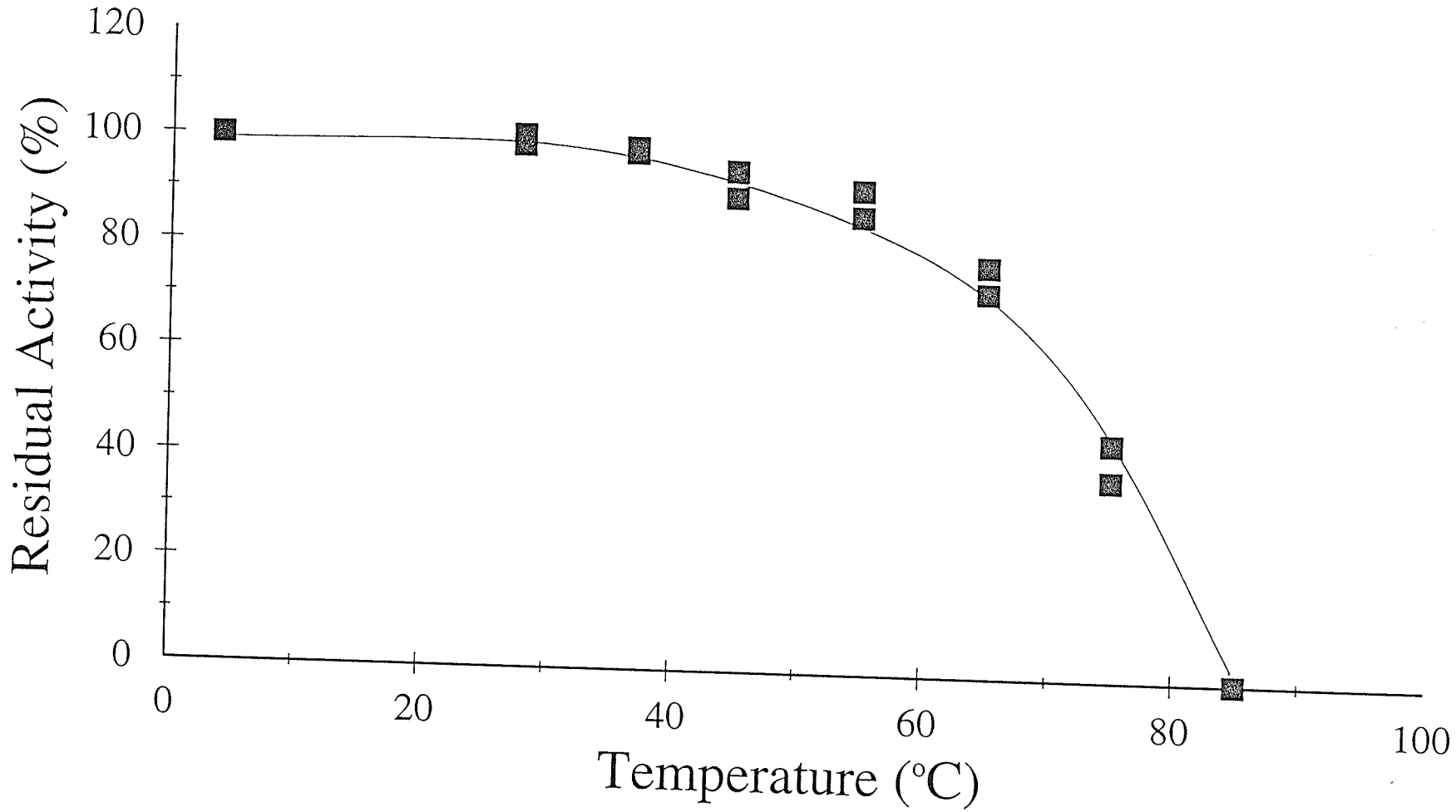

Residual Activity (%)
120
100
80
60
40
20
0
0
20
40
60
80
100
Temperature (°C)

Figure IV.10. Dependence of SHMT activity on the concentration of H_4F. Enzyme assay was carried out with various H_4F concentrations under standard assay conditions. 1.1 μg of enzyme (pIII of 2nd DEAE-Sephadex column) was added into each assay tube. The standard err for Km was 0.005.

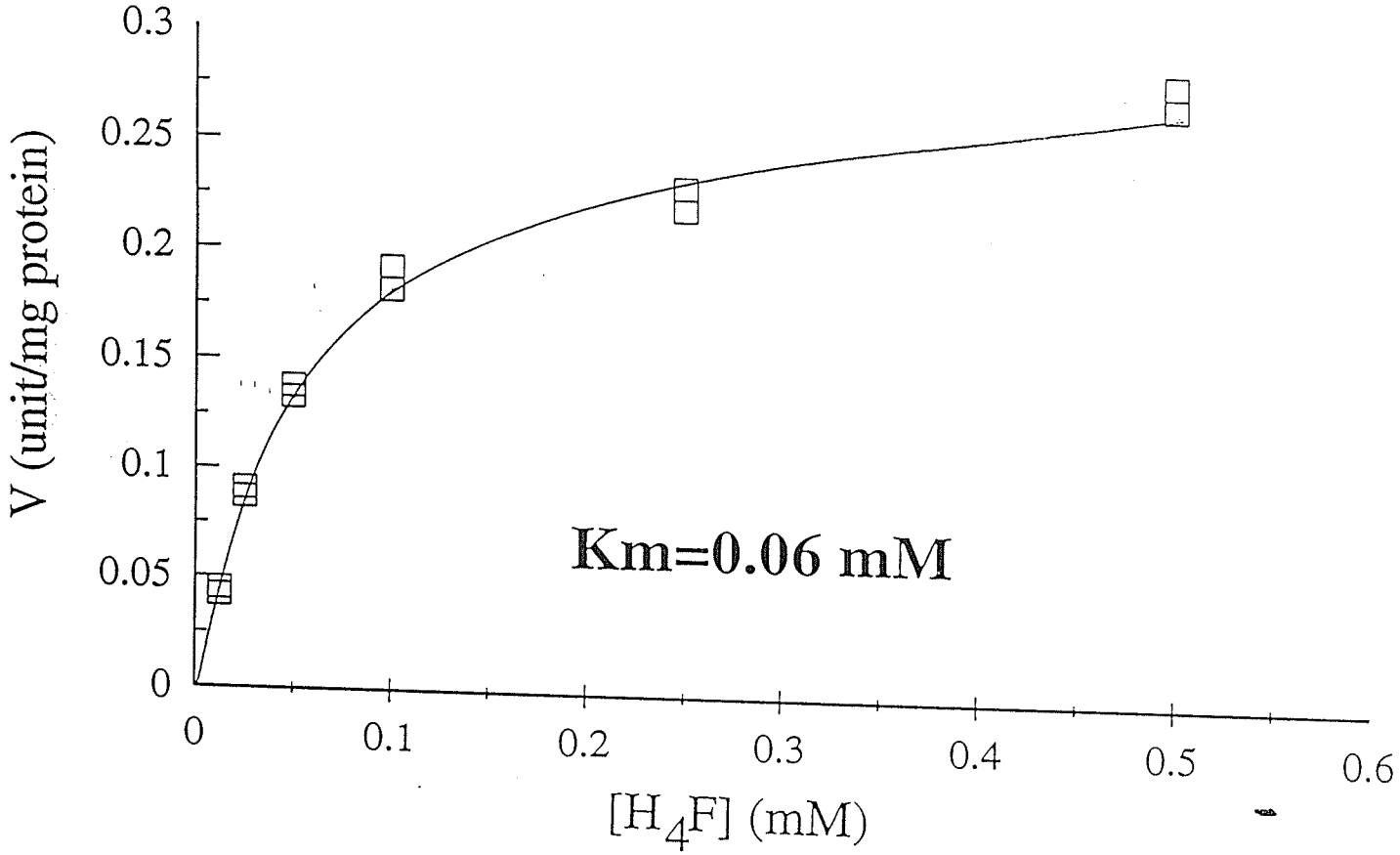

V (unit/mg protein)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.1
0.2
0.3
0.4
0.5
0.6
[H4F] (mM)
Km=0.06 mM

Figure IV.11. Dependence of SHMT activity on the concentration of L-serine. Enzyme assay was carried out with various L-serine concentrations under standard assay conditions. 1.1 μg of enzyme (pIII of 2nd DEAE-Sephadex column) was added into each assay tube. The standard err for Km was 0.037.

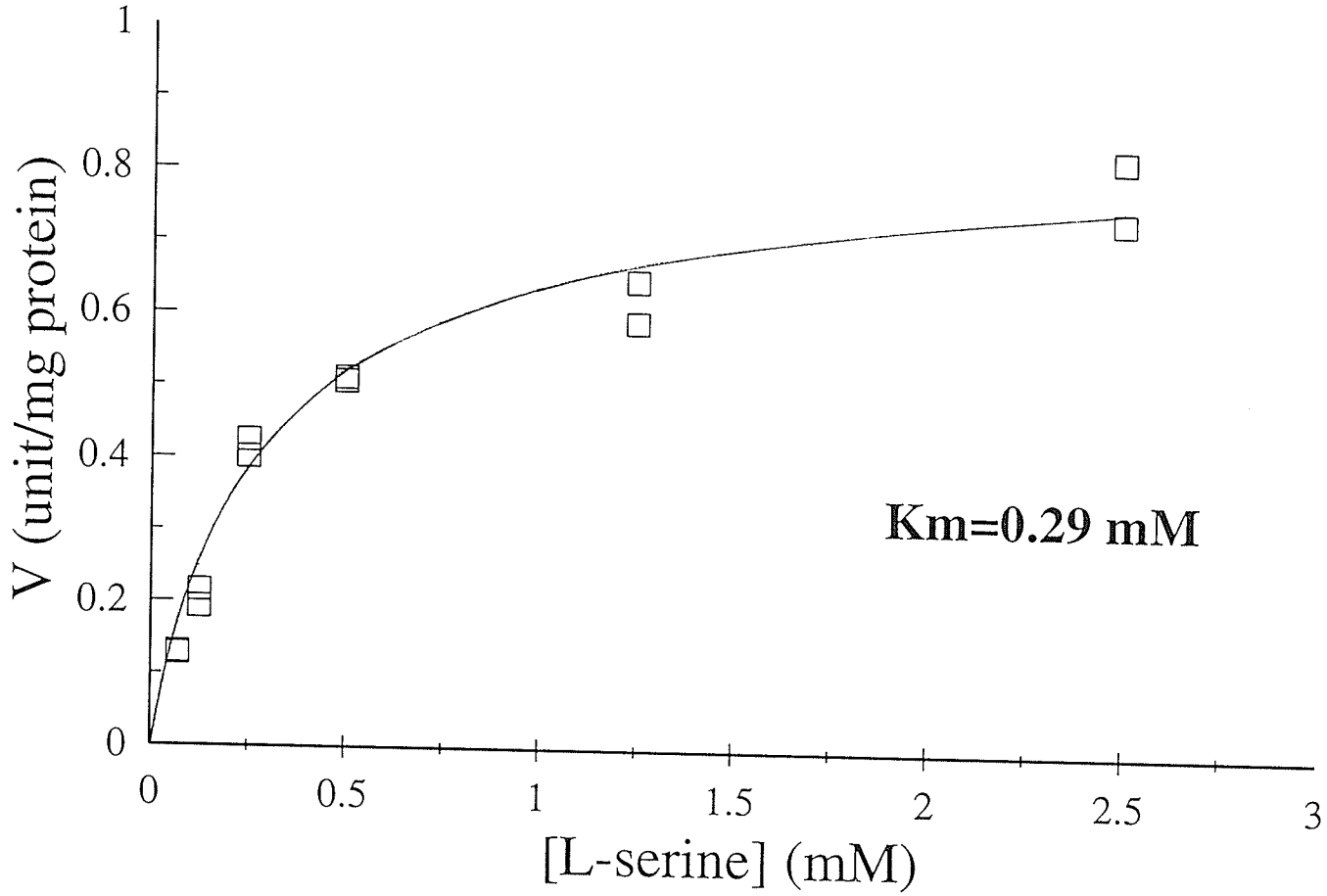
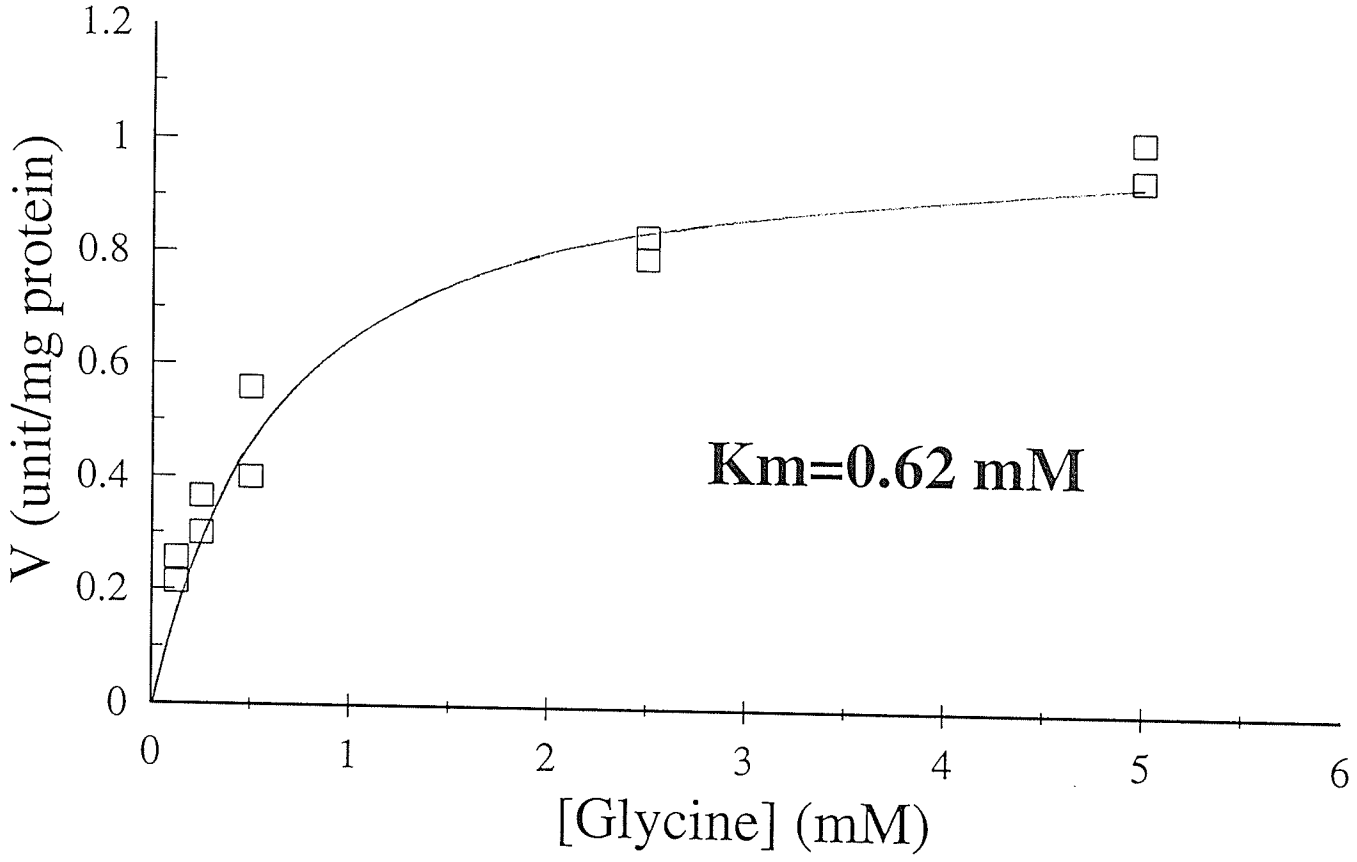

V (unit/mg protein)
1
0.8
0.6
0.4
0.2
0
0
0.5
1
1.5
2
2.5
3
[L-serine] (mM)
Km=0.29 mM

Figure IV.12. Dependence of SHMT activity for the synthesis of serine on the concentration of glycine. 1.1 μg of enzyme (pIII of 2nd DEAE-Sephadex column) was added into each assay tube. The standard err for Km was 0.081.


1.2
1
0.8
0.6
0.4
0.2
0
V (unit/mg protein)
0
1
2
3
4
5
6
[Glycine] (mM)
Km=0.62 mM

from pool III of the 2nd DEAE-Sephadex column was used.

IV.3.7 Effect of PLP inhibitors and sulfhydryl inhibitors on SHMT activity

Pyridoxal 5'-phosphate was found to increase the SHMT activity in CFE by about 50% (table III.3, chapter III). However, attempts to determine the effect of pyridoxal 5'-phosphate on activity of purified enzyme were not successful, possibly because the enzyme had become covalently bound with pyridoxal 5'-phosphate during the purification steps, in which 0.1 mM PLP was present in the enzyme storage buffer. To clarify if pyridoxal 5'-phosphate was required for the SHMT activity, penicillamine, an inhibitor of pyridoxal 5'-phosphate enzymes (Sukanya et al. 1991) was used. The enzyme activity was inhibited by 32%. when the enzyme was preincubated with the inhibitor for 30 min (Figure IV.13). Further experiments were carried out to assay enzyme activity in buffer containing 5 mM cysteine, a compound known to recover apo-SHMT by chemically reacting with PLP in *E. coli* (Schirch et al. 1985) and eucaryotes (Schirch and Mason 1965). It was shown that only about 40% of the original activity was detected when cysteine was present in the assay buffer (table IV.5). These results indicated that pyridoxal 5'-phosphate was required for optimal enzyme activity.

SHMT has been reported to contain sulfhydryl groups which were important for maintaining enzyme activity in both eubacteria and eukaryotes (Miyazaki et al. 1987; Schirch et al. 1985). Preliminary investigations were performed using common sulfhydryl

Figure IV.13. Effect of pencillamine on the activity of SHMT enzyme containing pyridoxal phosphate. 100 μl (5.5 μg) of enzyme (pIII of 2nd DEAE-Sephadex column) was incubated with pencillamine (1 mM) for 0 to 30 min. At various time points, a 20-μl aliquot was withdrawn for monitoring residual activity. Enzyme assay was performed under standard assay conditions. ■, no pencillamine; ▲, 1 mM pencillamine.

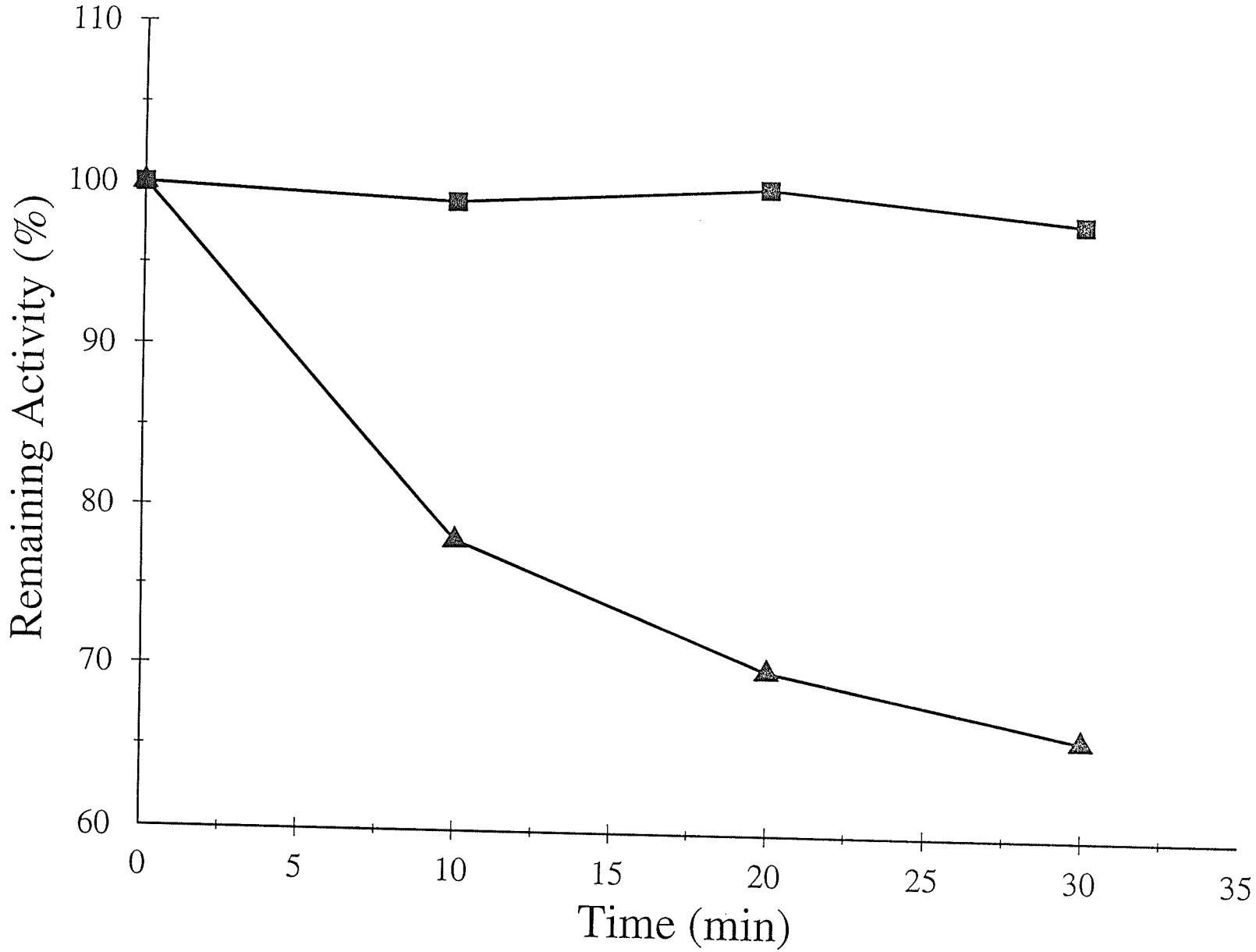

110
100
90
80
70
60
Remaining Activity (%)
0
5
10
15
20
25
30
35
Time (min)

Table IV.5 Effect of cysteine and sulfhydryl inhibitors on SHMT activity[a]

Agent	Concn. mM	Relative activity %
None	-	100
cysteine	5	40
DTNB	0.2	85
$HgCl_2$	0.1	80

a. 0.2 μg of SHMT from pIII of 2nd DEAE-Sephadex column was added into the assay buffer and preincubated with agent for 20 min. Assay method was described under the section of Materials and Methods. 100% activity was 0.9 μmol/min. mg protein.

group reagents, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and $HgCl_2$ to determine the effect of these sulfhydryl group inhibitors on SHMT activity. Experiments were carried out as described previously (Schirch et al. 1985): the purified enzyme (0.2 Fg) was incubated with 0.2 mM DTNB or 0.1 mM $HgCl_2$ at room temperature for 20 min, enzyme activity was assayed under standard conditions. Only less than 20% decrease of activity was observed with DTNB or $HgCl_2$ added (table IV.4). This would indicate that no sulfhydryl group was involved in the active site.

IV.4 Discussion

The present work involved the purification of a H_4F-dependent SHMT from *Msp. hungatei* GP1. This is the first report of the purification of a H_4F-dependent enzyme involved in C_1 biosynthesis from methanogens. Homogeneity of the enzyme was based on the result of SDS-PAGE with silver stain applied. The enzyme was determined to be a 93 KDa homodimer-protein. This was similar to SHMT of *E. coli* which is 95 KDa homodimer (Schirch et al. 1985) and SHMT of a methylotrophic bacterium, *Hyphomicrobium mrthylovorum* GM2, which is a 98 KDa homodimer (Miyazaki et el 1987). The molecular mass was also close to that of the other purified SHMT from *Mb. thermoautotrophicum* ^{a}H (Hoyt et al. 1986), which is a H_4M-dependent protein with a molecular weight of 102 KDa, determined by the non-denaturing PAGE technique.

Through N-terminal sequencing of the enzyme, the first 21

amino acid residues were identified. Comparison of this N-terminal sequence with relevant sequences of SHMT from other sources were carried out: various eubacteria including a methylotroph, a methanogen, and eucaryotes. Several highly conserved amino acids were observed. Amino acid composition analysis was also performed. However, no cysteine was found to be involved in the active site, since both DTNB and $HgCl_2$ did not inhibit SHMT activity of *Msp. hungatei*. In *E. coli* (Schirch et al. 1985) and *H. methylovorum* (Miyazaki et al. 1987), DTNB completely inhibited SHMT activity of both organisms. SHMT activity of *H. methylovorum* was also 100% inhibited by $HgCl_2$ (Miyazaki et al. 1987). SHMT of *E. coli* and *H. methylovorum* contains 3 and 4 cysteine residues respectively (from GenomeNet, accession #p00477 and p34895, respectively).

The temperature for maximal enzyme activity was determined to be 27 °C, being different from the organism's optimal growth temperature (35°C) (Patel et al. 1976). At 37°C, about 75% of the maximal enzyme activity was still present. The optimal pH was 8.1 for both biodegradation and biosynthesis of L-serine. This is similar to that of other SHMT reported (Hoyt et al. 1986; Schirch 1971; Miyazaki et al. 1987). The affinity of the enzyme for L-serine (Km = 0.29 mM) and H_4F (Km = 0.06 mM) was about 10- and-30-fold higher than that of the SHMT from *Mb. thermoautotrophicum* for L-serine and H_4M respectively (Kms were 2.8 mM and 1.8 mM) (Hoyt et al. 1985), but relatively similar to other H_4F dependent SHMT, such as in *E. coli* (Kms were 0.8 mM and 0.08 mM respectively) (Schirch et al. 1985) and in *Hyphomicrobium methylovorum* (Kms for

L-serine and glycine were 0.15 and 0.046 mM respectively) (Miyazaki et al. 1987). Km for glycine (0.62 mM) of SHMT from *Msp. hungatei* was about two fold higher than the Km for L-serine, this is consistent to the ^{13}C-NMR result that glycine was originated from serine (Ekiel et al. 1983) in this organism. It was therefore concluded that the function of SHMT in *Msp. hungatei* is to provide glycine and C_1 units for biosynthetic purposes.

Chapter V

A secondary alcohol dehydrogenase in *Methanosphaera stadtmanae*: preliminary characterization and its potential functions[1]

V.1 Introduction

Alcohol dehydrogenases (ADH) are widespread in nature. They have been found present in human (Branden et al. 1975) and animals (Eklund et al. 1990), eubacteria (Breicher and Winter 1991, Jones and Woods 1989, Ismaiel et al. 1993), yeast (Jornvall et al. 1988) and archaea (Zellner and Winter 1987, Zellner et al. 1990). In mammalian and yeast cells, the metabolism of ethanol is a recognized physiological role of ADH. In anaerobic eubacteria that produce secondary alcohols as major end products (Jones and Woods 1989), the major physiological role of the secondary ADH is the formation of secondary alcohols. Indeed, some *Clostridium* sp. possess high levels of secondary ADH. These organisms are able to produce significant amounts of 2-propanol and 2-butanol in natural environments (George et al. 1983, Kutzenok and Aschner 1952).

In methanogens (Zellner and Winter 1987, Widdel et al. 1988,Frimmer and Widdel 1989), secondary alcohols have been found to support both methanogenesis and growth as electron donors in

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[1]. The secondary ADH was accidentally found during studies of the C_1 transfer reactions for biosynthesis. This chapter has no direct connection with the rest of the thesis.

all methanogens that were found to possess secondary ADH. Some secondary ADHs were also able to use primary alcohols as electron donors for methanogenesis (Frimmer and Widdel 1989). The major physiological role of secondary ADH in methanogens might be the oxidation of secondary alcohols and/or primary alcohols which then provide electrons for methanogenesis and support growth, since these reactions are thermodynamically favourable.

Secondary ADH has been detected and studied in several methanogens during the past decade (Zellner and Winter 1987, Widdel 1986, Widdel et al. 1988, Lalitha and Krishnan 1991). Although primary ADH activity in methanogens was first reported as early as in 1940 (Barker 1939, Barker 1941) from *Methanobacillus omelianskii*, the culture was later proved to a syntrophic mixed culture of two organisms, a methanogen (*Mb. bryantii* M.O.H.) and a eubacterium (the "S" organism)(Bryant et al. 1967). The primary ADH activity found in the mixed culture belonged to the non-methanogen organism, however, a secondary ADH was found later in *Mb. bryantii* M.O.H. (Widdel et al. 1988). Studies of secondary ADH in methanogens have focused mainly on growth tests of known methanogens and the isolation of new methanogens that are able to grow on the expense of alcohols as the electron donor for methanogenesis (Lalitha and Krishnan 1991, Widdel et al. 1988,Zellner and Winter 1987, Widdel 1986). As well, the secondary ADH have been purified and characterized in *Methanogenium liminatans, Methanogenium thermophilum* TCI and *Methanobacterium palustre* (Bleicher and Winter 1991, Widdel and Wolfe 1989).

So far, there seems to be no clustering for the presence of secondary ADH in methanogens. Many methanogens have not been tested for ADH activity. It would not be surprising to see more methanogens containing ADH activity if tested.

V.1.1 Alcohols as electron donors for methanogenesis and growth

Several methanogens have been found to use secondary alcohols (Zellner and Winter 1987) as electron source for CO_2 reduction and growth of cells, *Methanogenium organophilum* CV and an unidentified isolate use also primary alcohols (Frimmer and Widdel 1989) (table V.1). Most of the methanogens tested that possessed secondary ADH are able to use secondary alcohols only but not primary alcohols. Although alcohols can be used as electron donors, the rate of methanogenesis with alcohols was very much slower than when molecular hydrogen was used. Growth with alcohols was also very poor compared with cultures supplied with H_2/CO_2 (Zellner and Winter 1987). When 2-propanol or 2-butanol was used, acetone or 2-butanone was produced during CO_2 reduction to methane. The addition of hydrogen gas reduced the acetone back to 2-propanol, which was formed during the oxidation of 2-propanol in the absence of molecular hydrogen (Zellner and Winter 1987). In *Mg. organophilum* (Frimmer and Widdel 1989, Widdel 1986), both secondary and primary alcohols can be used to support methanogenesis and cell growth. When 1-propanol or ethanol was utilized as the electron donor, propionate or acetate was formed as the oxidation product.

The reduction of 1 mol of CO_2 to 1 mol of methane required

the oxidation of 2 mol of primary alcohols or 4 mol of secondary alcohols into their corresponding carboxylic acids or ketones respectively (Widdel 1986). The stoichiometry and free energy changes would be as follows:

For secondary alcohols such as 2-propanol,

$$4CH_3CHOHCH_3 + HCO_3^- + H^+ \rightarrow 4CH_3COCH_3 + CH_4 + 3H_2O \quad (\Delta G^{\circ\prime} = -36.5 \text{ kJ})$$

For primary alcohols such as ethanol,

$$2CH_3CH_2OH + HCO_3^- \rightarrow 2CH_3COO^- + CH_4 + H_2O + H^+ \quad (\Delta G^{\circ\prime} = -116.3 \text{ kJ})$$

The net free energy gain of primary alcohol oxidation is larger than that of secondary alcohol. This may partly explain why methanogens which were able to use primary alcohols as electron donors grew faster on primary alcohols than on secondary alcohols (Widdel 1986).

V.1.2 Substrates and electron acceptors (table V.1)

Besides primary and secondary alcohols as well as their corresponding carboxylic acids and ketones, a NADP-dependent secondary ADH was also found to catalyze the reduction of acetaldehyde to ethanol or its reverse direction in *Mg. organophilum* (Frimmer and Widdel 1989). Acetaldehyde could be further oxidized to acetic acid, catalyzed by a NADP-dependent enzyme in the cell-free extracts. Whether or not the oxidation of both ethanol and acetaldehyde is catalyzed by the same enzyme is still not clear.

Although both NAD and NADP can be used as the electron acceptors for secondary ADH in eubacteria (Bryant et al. 1988), eucaryotes (Eklund et al. 1990) and non-methanogen archaea (Rella

Table V.1 Substrate and cofactor specificity of ADH in methanogens

Species and strain	substrates[a]	electron acceptors	References
Methanogenium			
marisnigri JR1	2-pro, 2-but	_[b]	Widdel et al. 1988
thermophilum TCI	2-pro, 2-but	F_{420}	Widdel and Wolfe 1989
organophilum CV	2-pro, 2-but Etoh, 1-pro	NADP	Frimmer and Widdel 1989
liminatans	2-pro, 2-but, cyclic alc	F_{420}	Bleicher and Winter 1991
Methanospirillum			
hungatei GP1	2-pro, 2-but	_	Widdel et al. 1988
hungatei SK	2-pro, 2-but	_	Widdel et al. 1988
Methanobacterium			
bryantii MoH	2-pro, 2-but	_	Widdel et al. 1988
palustre	2-pro, 2-but	NADP	Zellner et al. 1989
Methanobacterium -like strain	Etoh, 1-pro 2-pro	NADP	Frimmer and Widdel 1989
Isolate KL	2-pro	_	Lalitha and Krishnan 1991

a. 2-pro, 2-propanol; 2-but, 2-butanol; Etoh, ethanol; 1-pro, 1-propanol; cyclic alc, cyclic alcohols.
b. -, not determined. The presence of ADH was determined on the basis of growth and methanogenesis with various substrates acting as sole electron donors.

et al. 1987), of the two, only NADP but not NAD has been reported so far to serve as the electron acceptor for secondary ADH (Bleicher and Winter 1991, Frimmer and Widdel 1989, Zellner et al. 1989) in methanogens. In other methanogens tested, F_{420} rather than NADP is used as the electron acceptor (Bleicher and Winter 1991, Widdel and Wolfe 1989).

V.1.3 Kinetic studies and regulation of ADH

Very few kinetic studies have been done so far on secondary ADH from methanogens. In *Mg. thermophilum* TCI, the apparent Km of the enzyme for 2-propanol was about 10-fold higher than the Km for acetone. The Kms were determined to be 2.5 and 0.25 mM for 2-propanol and acetone respectively (Widdel and Wolfe 1989) with a pH optimum at about 4.2 (for 2-propanol oxidation). The pH optima for the two NADP-specific secondary ADH from *Mg. organophilum* and *Mb. palustre* were 10.0 and 8.0 respectively (Bleicher and Winter 1991,Frimmer and Widdel 1989). Unfortunately, no further experiments have been done to determined the toxicity of 2-propanol and acetone to the cells of *Mg. thermophilum*, where the affinity of the secondary ADH for acetone was very much higher than that for 2-propanol. A whole cell toxicity test of the two substrates may be helpful for understanding the physiological direction of the secondary ADH in this organism.

Expression of secondary ADH has been studied in several methanogens (Widdel and Wolfe 1989) including *Mg. thermophilum* TCI,

Mg. organophilum CV, *Msp. hungatei* SK and *Mb. bryantii* M.O.H. In all methanogens examined, it was found that secondary ADH expression could be enhanced by the addition of both secondary alcohols and structurally related compounds which can not be used as substrates of the enzyme (such as tertiary alcohols). Hydrogen limitation condition, primary alcohols and acetone were also found to stimulate the expression of ADH in some but not all methanogens tested.

In *Msph. stadtmanae*, a strict methylotrophic methanogen that requires both methanol and hydrogen for methanogenesis, but still requires CO_2 for growth (Miller and Wolin 1985), a secondary ADH was detected. In this chapter, we describe some preliminary characterizations and investigation of the physiological roles as well as factors that may control the expression of the secondary ADH in *Msph. stadtmanae*.

V.2 Materials and methods

V.2.1 Chemicals

1-propanol, 2-propanol, acetone,1-butanol, 2-butanol, Glycine, NADP and NADPH were purchased from Sigma Chemical Co..

V.2.2 Microorganism and culture conditions

Msph. stadtmanae (DSM 3091) was grown as previously described (Miller and Wolin 1985; Sparling et al. 1993) in the Materials and

Methods section of chapter II (section II.2.2).

For the growth test using 2-propanol to support cell growth, the medium was made as described in the section of Materials and Methods in chapter II but under a gas phase of N_2/CO_2. Various amounts of 2-propanol (sterilized through a 0.45 μm membrane filter) were added into the medium to give different final concentrations. The toxicity of 2-propanol and acetone to the cells was studied using the same medium and culture conditions with various concentrations of 2-propanol and acetone under a H_2/CO_2 gas phase.

For the determination of the effect of 2-propanol on the survival of cells under a hydrogen-deficient environment, no 2-propanol or 100 mM 2-propanol was added to the growth medium containing 0.4% methanol (v/v) under N_2/CO_2. A positive control was used with a gas phase of H_2/CO_2. About 20% (v/v) of inoculum was taken from a regular culture which had not been subjected to 2-propanol, and was washed twice with medium prepared under nitrogen using a syringe attached with a 0.45 μm membrane filter. This step is taken to remove hydrogen carried over from the inoculum into the culture. After inoculation, the cultures were incubated at 37 °C for 60 hrs. The N_2/CO_2 gas phase was then removed and replaced with H_2/CO_2 by venting and feeding the culture three times using pressurized H_2/CO_2 aseptically and anaerobically. The cultures were subsequently incubated in a shaker at 37 °C for several more days. Methane levels were measured by gas chromatography using a Chromosorb 102 column (Supelco, Oakville, Ont.) and a flame

ionization detector (Varian Aerograph series 2100). The absorbance (A660) of cultures was measured using a LKB Novaspec II spectrophotometer.

V.2.3 Preparation of cultures grown under hydrogen-limited conditions

Cultures exposed to hydrogen-limited conditions were prepared as following: the regular medium was inoculated and cells incubated as described in the Materials and Methods. When the cells reached late exponential growth phase (48 hrs incubation), 1 litre of culture (two bottles) was harvested and was referred to as control cultures; The gas phase of the the other cultures (two bottles) was changed to N_2/CO_2 through 3 cycles of evacuating (10 min) and gassing (3 min), then incubated (shaking) for 2 hours. Cultures were then harvested and were referred to as cultures exposed to hydrogen-limited conditions.

V.2.4 Preparation of cell suspensions and manipulation of whole cell methanogenesis experiments using secondary alcohols

Cells from 1 litre of culture were harvested in the exponential growth phase (O.D = 0.7 - 0.8), washed three times with 40 mM potassium phosphate pH 6.9, and suspended in 10 ml of the same buffer to form concentrated cell suspensions. All the

procedures were performed anaerobically in a Coy anaerobic chamber furnished with an oxygen-detector.

Before 2-propanol or 2-butanol was added, 0.5 ml of cells (2 to 5 mg protein) was first added to a 25 ml stoppered tube which contained 9.5 ml of the 40 mM potassium phosphate buffer, pH 6.9. To remove the residual methane from the cultures, the tubes were incubated in a reciprocal shaker (Haake) at 37°C for 10 min, and the gas phase replaced through 3 cycles of evacuating (5 min each) and flushing with pure nitrogen (1 min each). Methanol was added to the final concentration of 0.4% (v/v). Experiments were started with addition of 2-propanol or 2-butanol. Methane was measured as described earlier. Acetone was also measured by gas chromatography equipped with a flame ionization detector.

V.2.5 Preparation of Cell-free extracts (CFE) from cultures

Unless otherwise specified, cells in the exponential growth phase were harvested and lysed anaerobically as described in chapter III. CFE was obtained after centrifugation at 25,000 x g at 4 °C for 30 min.

V.2.6 CFE storage

CFE was stored at -20 °C or -60 °C for a long period of time without appreciable loss of activity.

V.2.7 Enzyme assay

The secondary ADH in *Msph. stadtmanae* was assayed photometrically in anaerobic rubber-sealed round cuvettes (Daniels and Wessels 1984) (diameter = 1 cm) under nitrogen at 37 °C in 40 mM glycine buffer, pH 9.2. Assays were performed on a LKB Novaspec II spectrophotometer at various wavelengths depending on the cofactors: 340 nm for NAD, NADP and NADPH (ϵ=6.22 / mM. cm), 560 nm for methyl viologen (ϵ=8.00 / mM. cm), 578 nm for benzyl viologen (ϵ=8.65 / mM. cm) and 401 nm for F_{420} (ϵ=25.0 / mM. cm). A regular assay included the following components (2.5 ml total volume): glycine, 40 mM; NADP or NADPH, 0.06 mM; 2-propanol or acetone, 0.4 mM; and CFE, 0.1-0.3 mg protein. When other potential electron acceptors were used for enzyme assays, various concentrations ranging from 0.01 to 0.5 mM were used. All assay components were made anaerobic and stored under a nitrogen gas phase except for the air stability test of the enzyme which was exposed to air before enzyme assays.

V.2.8 Calibration of enzyme activity

A background activity was present when the activity of NADP reduction was assayed in the CFE. All relevant data were presented after correction for background activity.

V.2.9 Determination of the pH optimum

For the determination of the optimal pH for enzyme activity, enzyme assays were carried out as described earlier except in 40 mM potassium phosphate buffer instead of glycine buffer. Different pH was obtained by adjusting the buffer with HCl or KOH.

V.2.10 Determination of kinetic constants

For the determination of kinetic constants, enzyme assays were performed in 40 mM glycine buffer, pH 9.2 for both NADP reduction and NADPH oxidation as described earlier with some modifications: for Km of 2-propanol or acetone, 0.06 mM NADP or NADPH was used; for Km of NADP or NADPH, 0.4 mM 2-propanol or acetone was used.

V.2.11 Protein determination

The protein concentration was determined by the method of Bradford (1976).

V.3 Results

V.3.1 Substrate and electron acceptor specificity

Several primary and secondary alcohols were used as potential substrates for the secondary ADH in *Msph. stadtmanae*, using NADP as the electron acceptor (table V.2). None of the primary alcohols tested, including ethanol, 1-propanol and 1-butanol, were used as

substrate by the enzyme, while both 2-propanol and 2-butanol could be oxidized to reduce NADP. Several common electron carriers were also tested for the possibility of accepting electron from 2-propanol (table V.2). Only NADP was able to accept electron from the secondary alcohol.

V.3.2 Air sensitivity of secondary ADH in *Msph. stadtmanae*

The air stability of the secondary ADH was tested for a period of 24 hours. The CFE was kept separately under air and nitrogen gas at 4 °C. When the CFE was exposed to air, the activity dropped quickly to less than 35% within the first 4 hours while the activity of the enzyme kept under nitrogen remained the same level (figure V.1). Attempts to recover the enzyme activity by making the CFE anaerobic again were not successful, indicating that the air inactivation of the enzyme was irreversible.

V.3.3 Determination of optimal pH

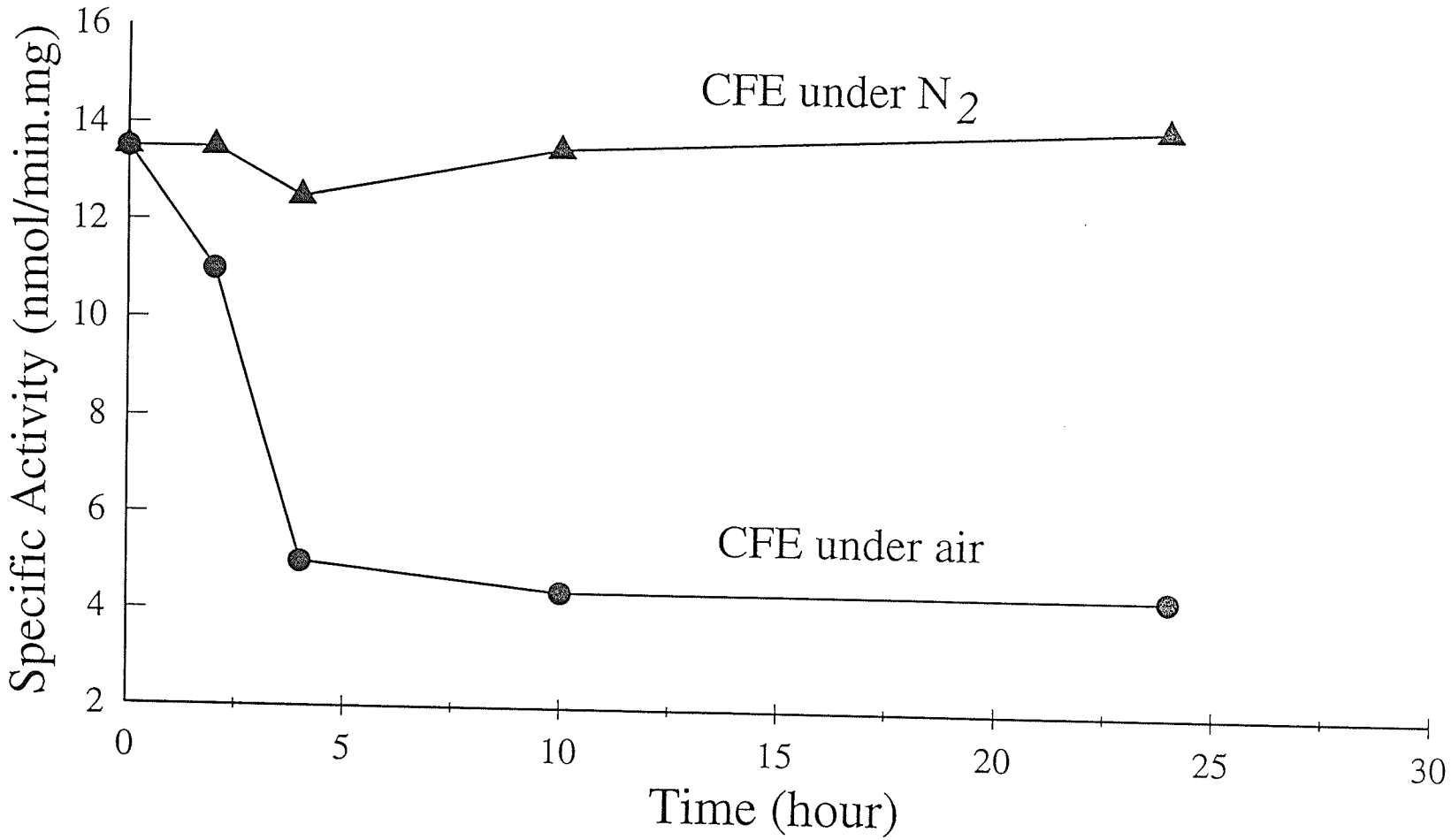
The optimal pHs for both the oxidation of 2-propanol and reduction of acetone were performed in 40 mM potassium phosphate

Table V.2 Substrate and electron acceptor specificity of secondary alcohol dehydrogenase activity

	Specific Activity (nmol/min. mg protein)
Substrate[a]	
2-Propanol	14.5
2-Butanol	13.8
Ethanol	<0.01
1-Propanol	<0.01
1-Butanol	<0.01
Electron acceptor[b]	
NADP	14.5
NAD	<0.01
MV	<0.01
BV	<0.01
F_{420}	<0.01

a. NADP was used as the electron acceptor.
b. 2-Propanol was used as the substrate.

Figure V.1. Air stability of secondary ADH in the CFE of *Msph. stadtmanae*. (▲), CFE stored under nitrogen gas; (●), CFE exposed to air.


Specific Activity (nmol/min.mg)
16
14
12
10
8
6
4
2
0
5
10
15
20
25
30
Time (hour)
CFE under N 2
CFE under air

buffer. The pH optimum for the oxidation of 2-propanol in the presence of NADP was determined to be 9.2, while the pH optimum for the reduction of acetone in the presence of NADPH was found to be 8.6 (figure V.2). At pH 7-8, the activity of acetone reduction was about twice as high as that of 2-propanol oxidation.

V.3.4 Determination of kinetic constants

The apparent Michaelis constants (Km) of secondary ADH in CFE for 2-propanol, acetone, NADP and NADPH were determined based on the results of the first-order rate plots. The apparent Km were determined to be, for 2-propanol, 0.053 mM; acetone, 0.032 mM; NADP, 0.013 mM, and NADPH, 0.0043 mM (figure V.3, V.4, V.5 and V.6).

V.3.5 The secondary ADH levels in different growth stages of cell culture

To investigate growth stage dependent fluctuations in the secondary ADH activities, two-week old cultures kept at 4 °C were used as inoculum. At the times indicated, samples were taken to measure the absorbance (A660) and secondary ADH activity. The results indicated that cells during the lag phase and early exponential phase had the highest level of secondary ADH (figure V.7). The specific activity of secondary ADH after mid-exponential growth phase dropped down to between one third and one half of the starting level.

Figure V.2. Effect of pH on the ADH activity: ■, 2-propanol oxidation; ▲, acetone reduction.

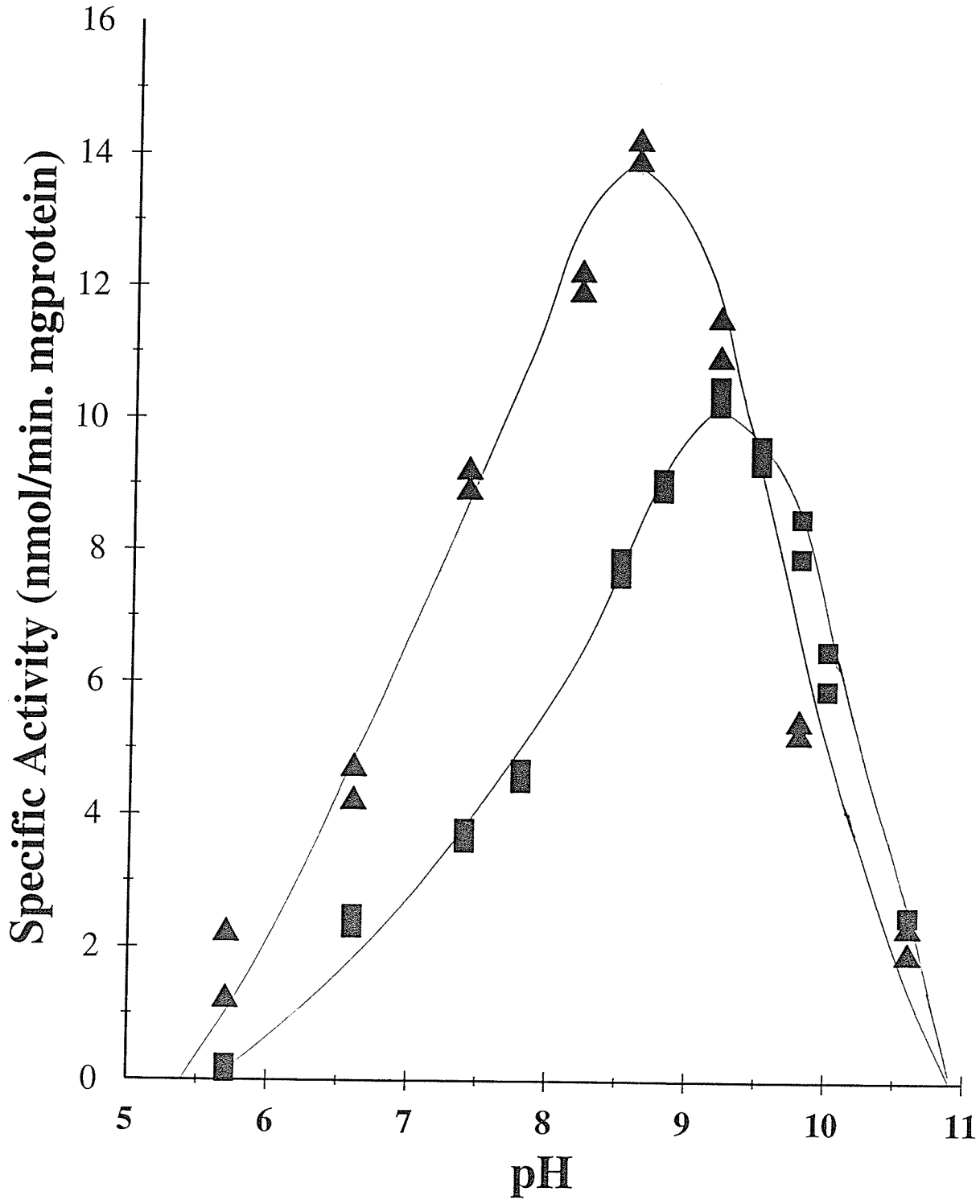

Specific Activity (nmol/min. mgprotein)
16
14
12
10
8
6
4
2
0
5
6
7
8
9
10
11
pH

Figure V.3. Dependence of ADH activity for NADP reduction on the concentration of 2-propanol. The standard err for Km was 0.0068.

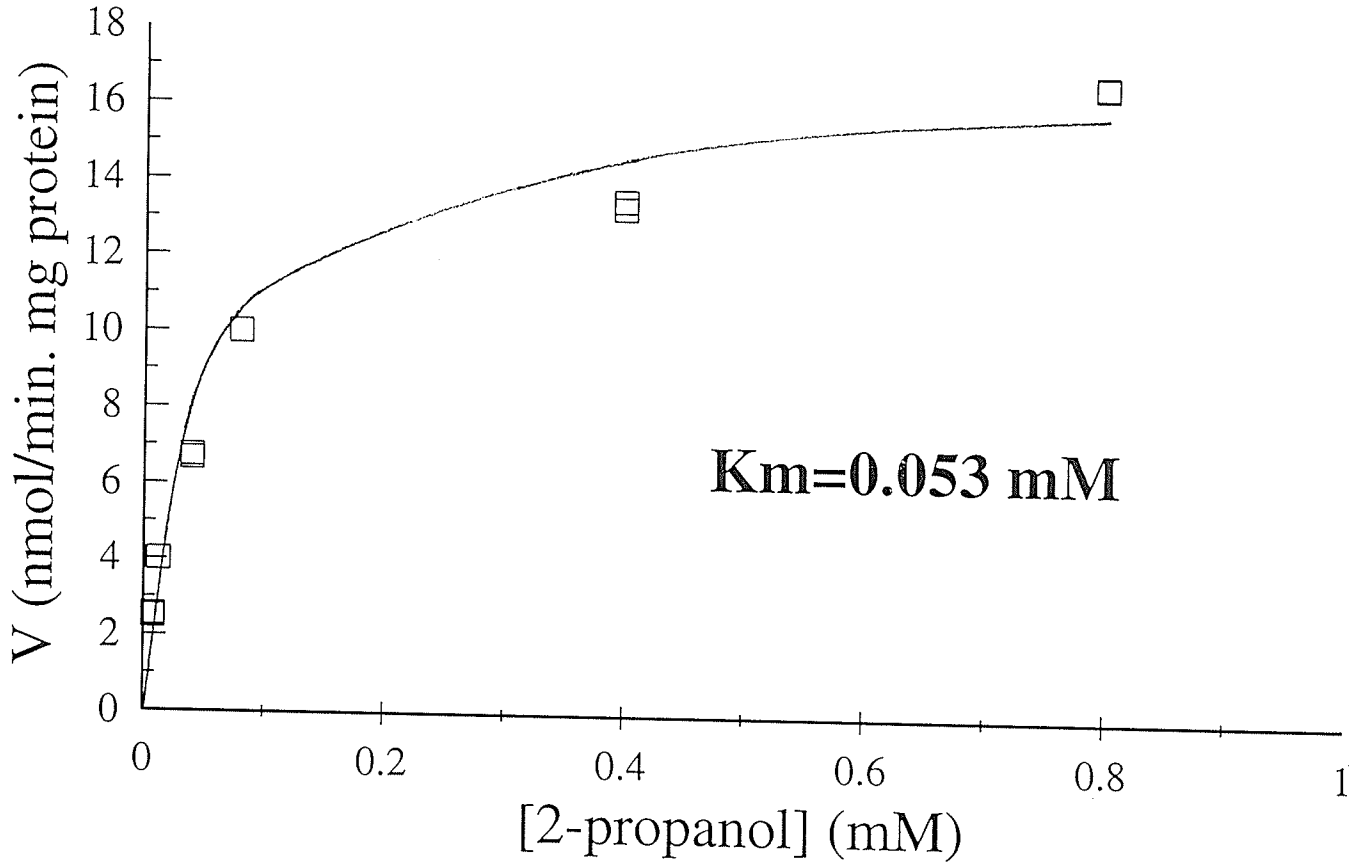

V (nmol/min. mg protein)
18
16
14
12
10
8
6
4
2
0
0
0.2
0.4
0.6
0.8
1
[2-propanol] (mM)
Km=0.053 mM

Figure V.4. Dependence of ADH activity for 2-propanol oxidation on the concentration of NADP. The standard err for Km was 0.0013.

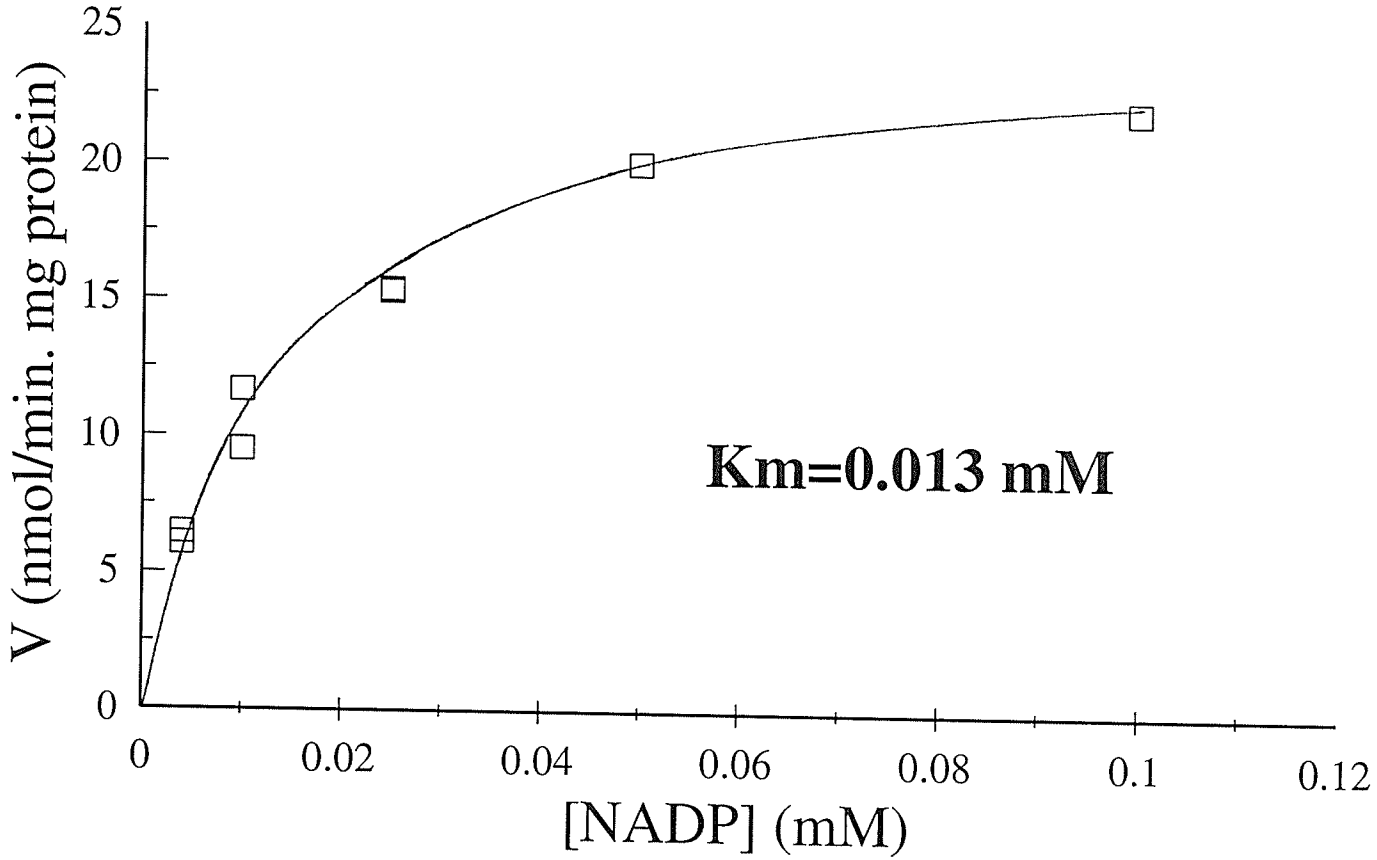

25
20
15
10
5
0
V (nmol/min. mg protein)
0
0.02
0.04
0.06
0.08
0.1
0.12
[NADP] (mM)
Km=0.013 mM

Figure V.5. Dependence of ADH activity for NADPH oxidation on the concentration of acetone. The standard err for Km was 0.0048.

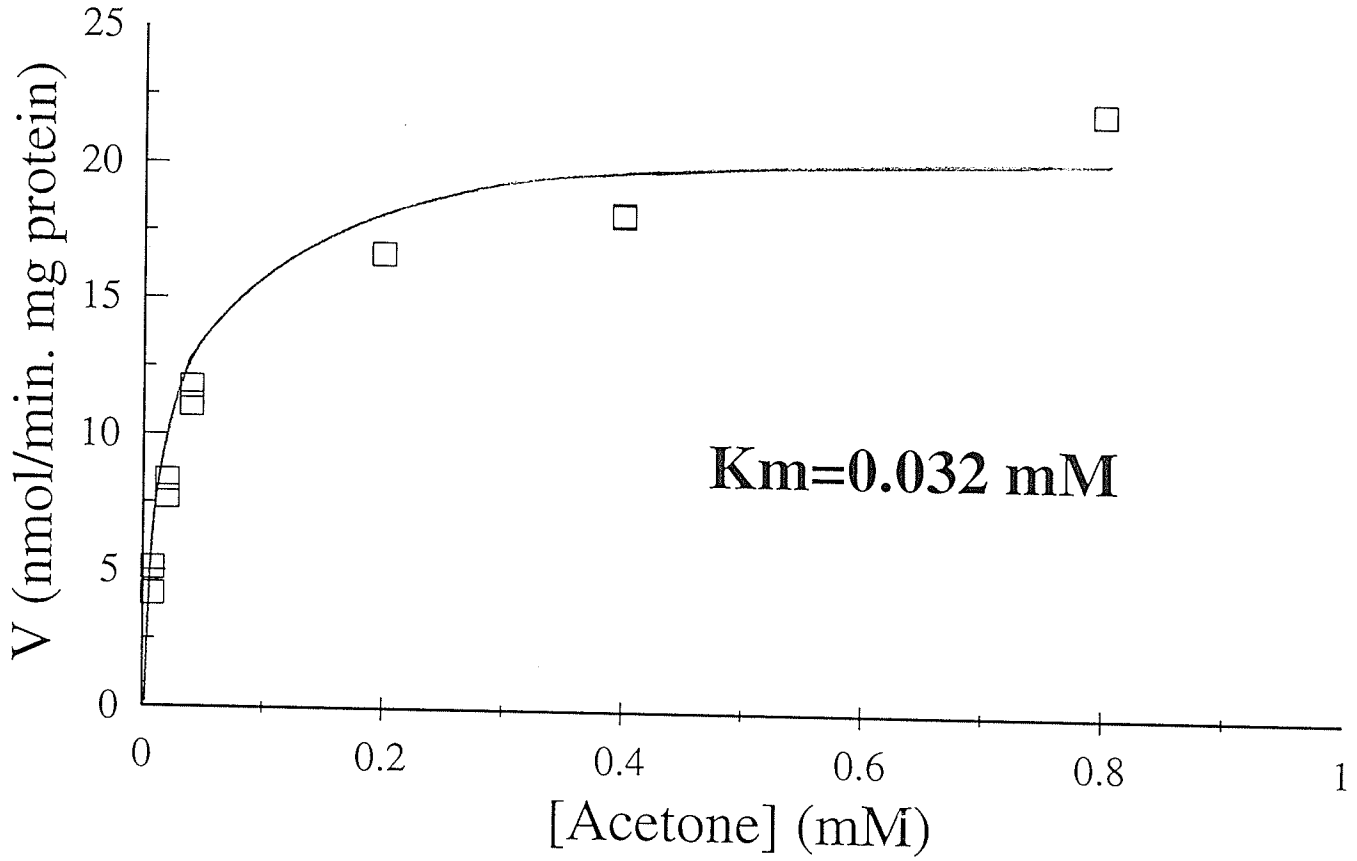

25
20
15
10
5
0
V (nmol/min. mg protein)
0
0.2
0.4
0.6
0.8
1
[Acetone] (mM)
Km=0.032 mM

Figure V.6. Dependence of ADH activity for acetone reduction on the concentration of NADPH. The standard err for Km was 0.0006.

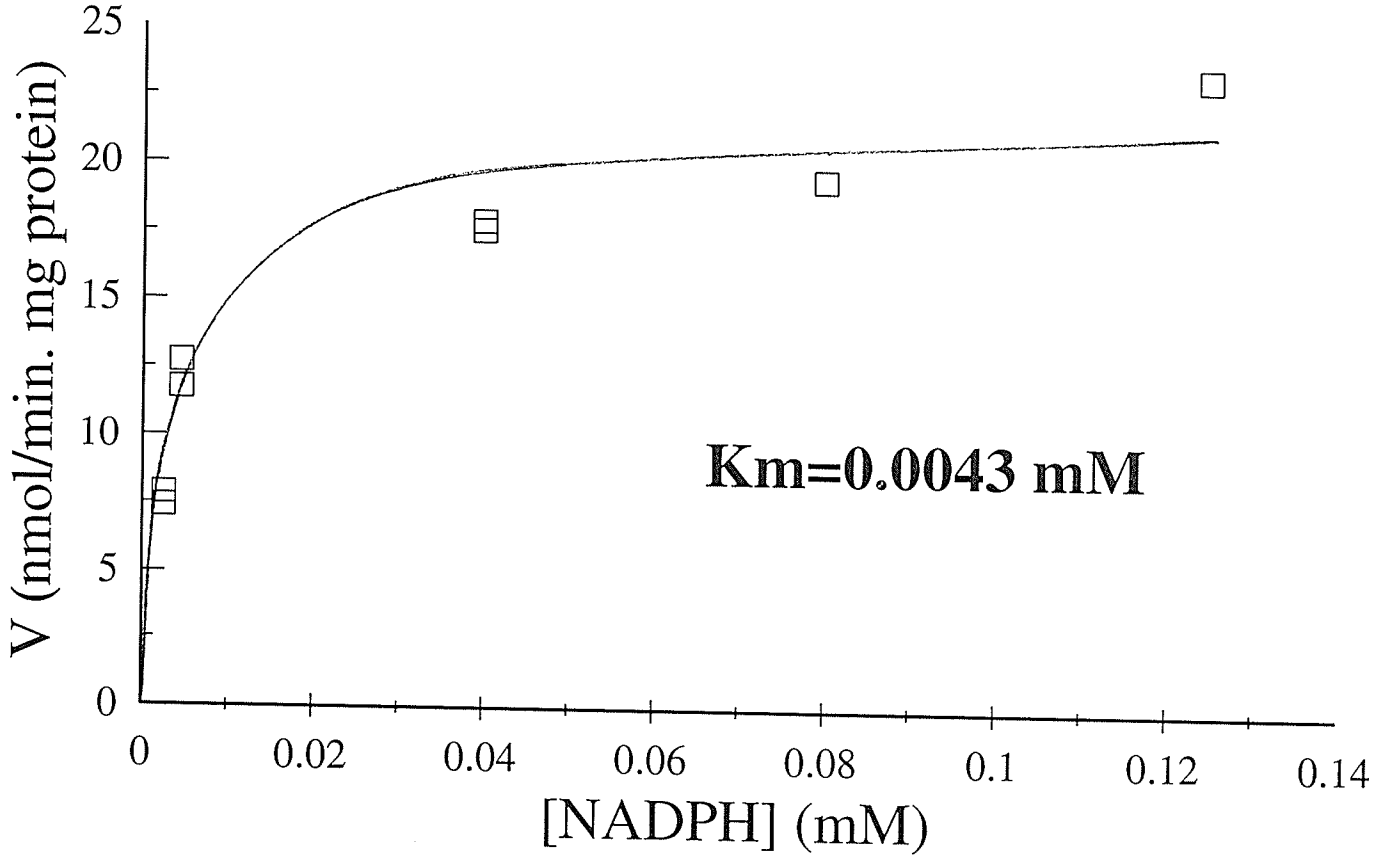
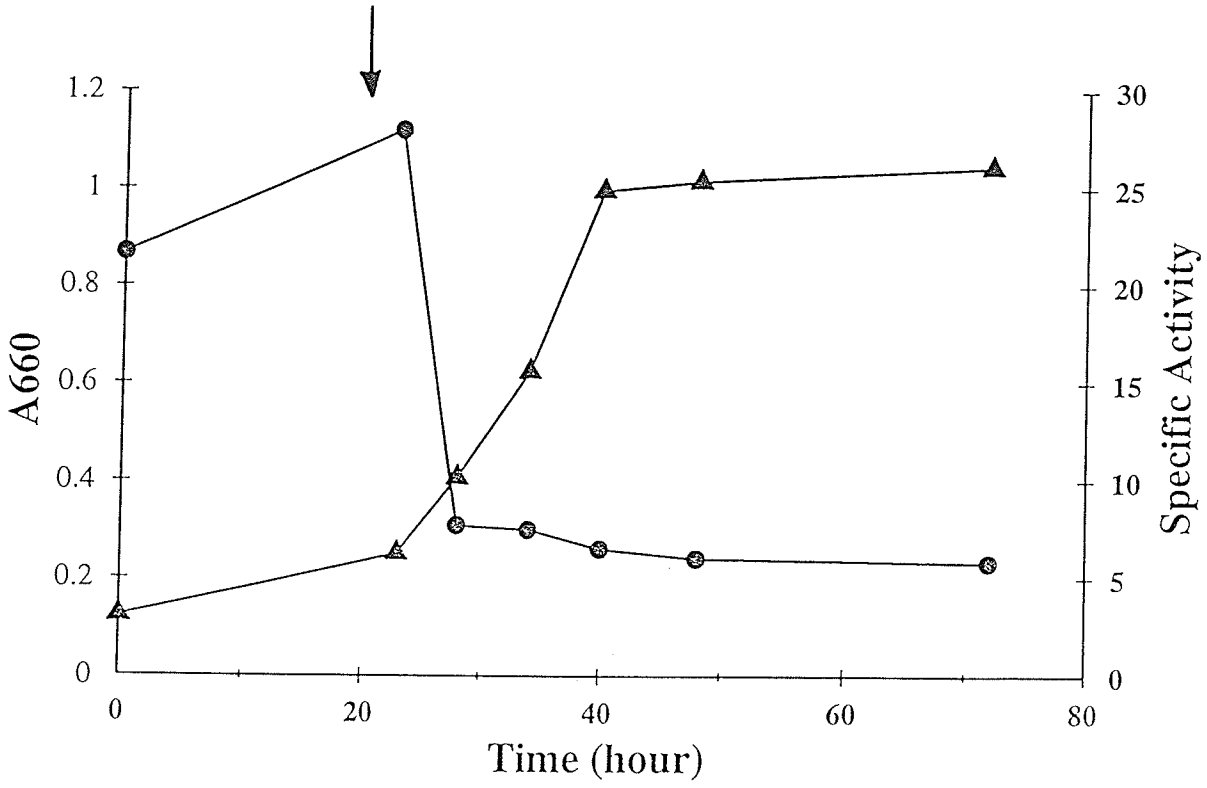

25
20
15
10
5
0
V (nmol/min. mg protein)
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
[NADPH] (mM)
Km=0.0043 mM

Figure V.7. Specific Activity of the secondary ADH at various growth stages. (▲), A660; (●), Specific activity in CFE (nmol/min. mg protein). Arrow indicates the time when cells were presurized and shaking started. The oxidation of 2-propanol was assayed as described in section V.2.7 except that the final concentrations of 2-propanol and NADP were 0.1 and 0.04 mM respectively in this experiment.


1.2
1
0.8
0.6
0.4
0.2
0
A660
0
20
40
60
80
Time (hour)
30
25
20
15
10
5
0
Specific Activity

V.3.6 Effect of hydrogen limitation on the expression of secondary ADH in the cells

The difference of enzyme levels shown in figure V.7 could be explained as an effect of hydrogen limitation, since the inoculum cultures were kept at 4 °C for two weeks. Cells settled to the bottom of the bottle which would allow slow occurrence of metabolic activities in the cells in a hydrogen limitation environment. To determine this possibility, regular cultures and cultures grown under hydrogen-limited condition were prepared as described under section V.2.3. The CFE-specific activity (for 2-propanol oxidation) of the secondary ADH in the cells grown under hydrogen-limited condition was 8.5 nmol/min. mg protein, which was about twice as high as that of regular cells (4.5 nmol/min. mg)(assay condition described in figure legend of figure V.7).

V.3.7 Effect of 2-propanol and acetone on the growth of cells

Studies of the toxicity of 2-propanol and acetone to the cells were performed by growing cultures in the presence of various amounts of these chemicals. Growth (A660) was measured after 48 hours of incubation, which was the time of approximate full growth of cells in the regular medium (figure V.7). It was shown that lower concentrations of 2-propanol (< 300 mM) stimulated growth of the cells, while inhibition of growth started at 400 mM; acetone

appeared to be much more toxic to the cells with inhibition of growth occurred at the concentration of 30 mM (figure V.8).

V.3.8 Effect of 2-propanol and acetone on the expression of the secondary ADH

Cells of *Msph. stadtmanae* were inoculated into regular medium containing 400 mM 2-propanol or 14 mM acetone. At these concentrations, cell growth of *Msph. stadtmanae* was not affected (see figure V.8, section V.3.7. Cells were then incubated, and harvested under regular conditions. Results indicated that CFE of 2-propanol- and acetone-treated cells had 3 fold and 2 fold higher enzyme level than that in CFE of control cells (figure V.9).

V.3.9 Methanogenesis from methanol using 2-propanol as the sole electron donor

In the absence of hydrogen, methane was produced from methanol in the presence of 2-propanol (figure V.10). Acetone formation was also detected during the process of methanogenesis in a 1:1 ratio. Cells of *Msph. stadtmanae* were not able to produce methane without the addition of 2-propanol under nitrogen. These results indicated that 2-propanol was oxidized to acetone and provided electrons for methanogenesis. Similarly, 2-butanol supported methanogenesis in whole cells of *Msph. stadtmanae*. However, neither 2-propanol nor

Figure V.8. Effect of acetone and 2-propanol on the growth of cells. (▲), A660 of cultures incubated with various concentratons of acetone; (●), A660 of cultures incubated with various concentratons of 2-propanol.

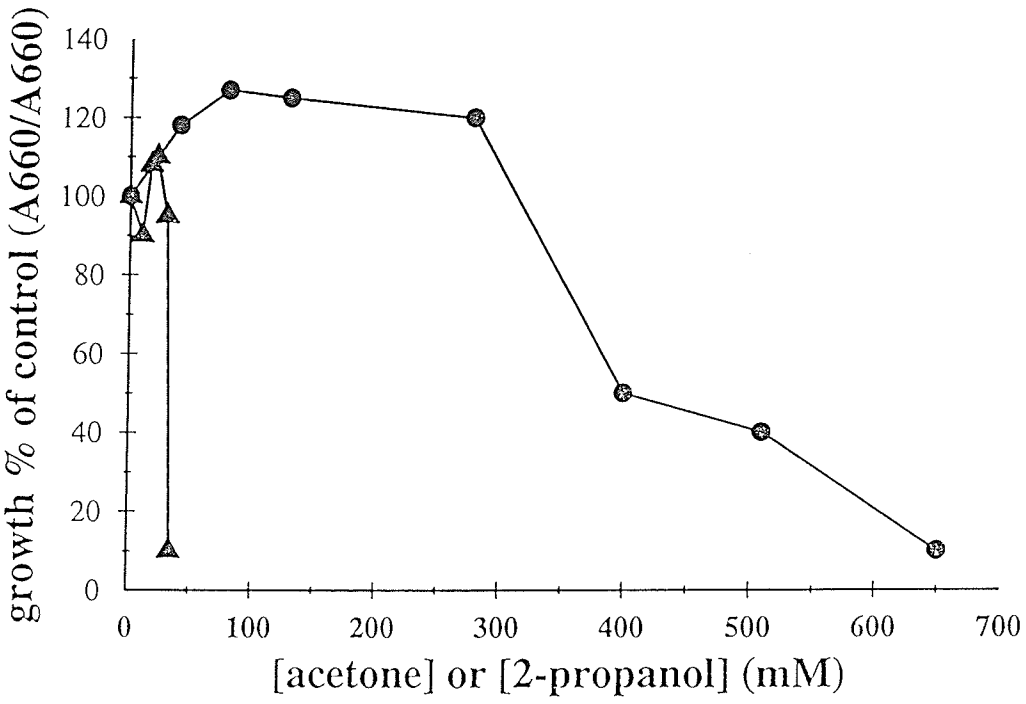

growth % of control (A660/A660)
140
120
100
80
60
40
20
0
0
100
200
300
400
500
600
700
[acetone] or [2-propanol] (mM)

Figure V.9. Effect of 2-propanol and acetone on the expression of secondary ADH.

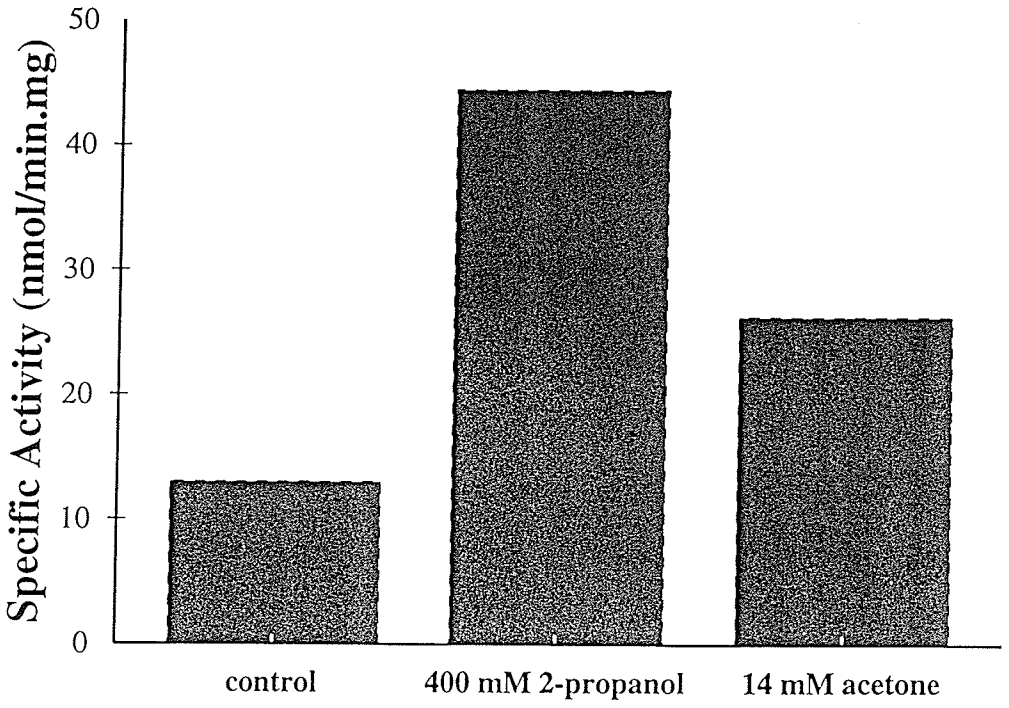
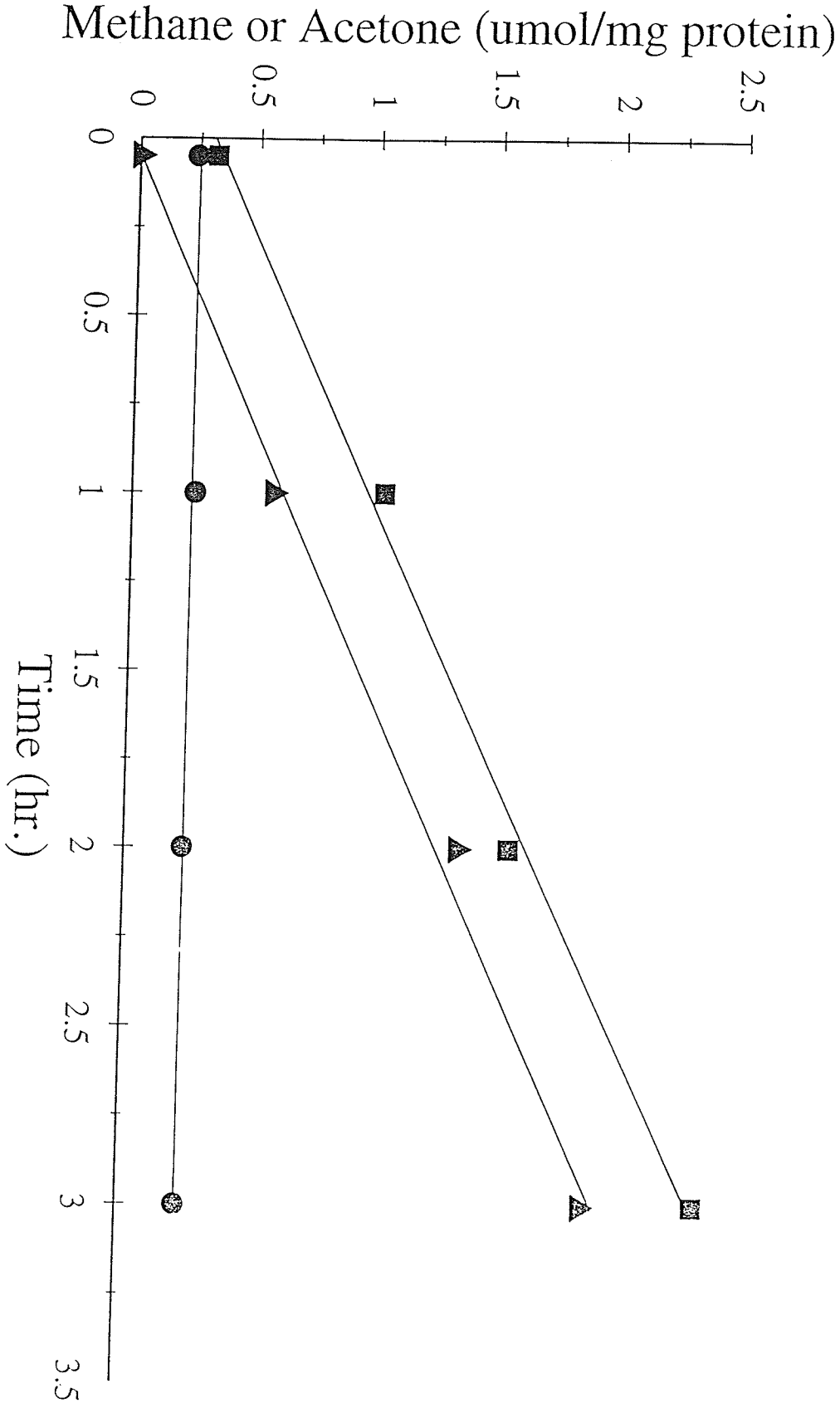

Specific Activity (nmol/min.mg)
50
40
30
20
10
0
control
400 mM 2-propanol
14 mM acetone

Figure V.10. Methanogenesis from methanol under nitrogen in cell suspensions. (■), methane formation from methanol and 2-propanol; (▲), acetone levels in cell suspensions added with methanol and 2-propanol; (●), methane formation in the absence of 2-propanol.


Methane or Acetone (umol/mg protein)
0
0.5
1
1.5
2
2.5
Time (hr.)
0
0.5
1
1.5
2
2.5
3
3.5

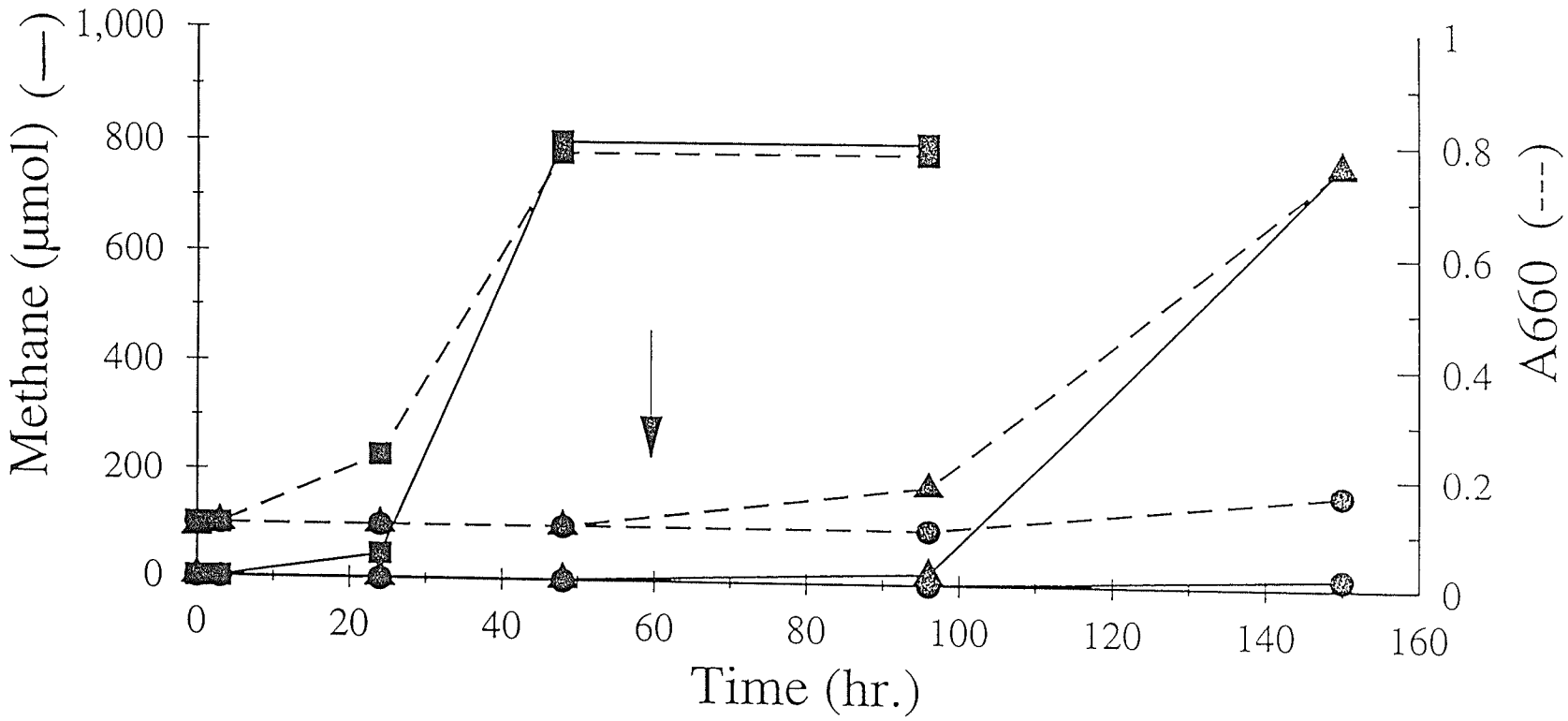
2-butanol supported cell growth in the presence of methanol under N_2/CO_2.

V.3.10 Effect of 2-propanol on the survival of cells under a hydrogen-deficient condition in the presence of methanol

Inoculum taken from a fresh culture at the stage of exponential growth was injected into medium containing no 2-propanol or 100 mM 2-propanol under N_2/CO_2 in the presence of methanol. Control cultures were made with medium under the gas phase of H_2/CO_2. All cultures were then incubated at 37 °C, and methane and OD were measured at different time points.

Cultures in the absence of 2-propanol under N_2/CO_2 failed to produce methane while in the presence of 2-propanol, cells were able to produce methane within the first 24 hrs (0.6 μmol total or 40 μM in the gas phase; this increase is not large enough to be visible)(figure V.11). The methane levels remained the same after 24 hrs incubation. Growth and methanogenesis started in culture preincubated with 2-propanol and under N_2/CO_2 after gas phase was changed into H_2/CO_2 at 60 hrs, and reached to the level of positive control at 150 hr. Cultures preincubated under N_2/CO_2 in the absence of 2-propanol had little growth and much lower methane level at 150 hr (figure V.11). These data indicated that the activity of secondary ADH in the cells was helpful for maintaining cell viability under hydrogen limited conditions.

Figure V.11. Methanogenesis and cell growth in cultures under different conditions. (____), methane levels; (....), A600; (▲), incubated with methanol and 2-propanol under N_2/CO_2; (●), incubated with methanol only under N_2/CO_2; (■), incubated with methanol under H_2/CO_2. Arrow indicates the change of gas phase from N_2/CO_2 to H_2/CO_2.


Methane (µmol) (—)
1,000
800
600
400
200
0
0
20
40
60
80
100
120
140
160
Time (hr.)
1
0.8
0.6
0.4
0.2
0
A660 (---)

V.4 Discussion

The secondary ADH in *Msph. stadtmanae* CFE could only use secondary alcohols as its substrates and NADP as the electron acceptor. Cells of *Msph. stadtmanae* were shown to produce methane from methanol and 2-propanol. The transport of electrons provided by 2-propanol for methanogenesis might also involve other enzymes, such as F_{420}:NADP oxidoreductase and F_{420} dehydrogenase, as suggested by Wong et al. (1994).

When the CFE was exposed to air, the secondary ADH activity decreased to 35% of the original activity within 4 hours. The residual activity then remained relatively stable under air. Attempts to recover the secondary ADH activity in the air-treated CFE by removing air from the CFE were not successful, indicating that the change caused by air exposure was probably not reversible. So far, there are no data pertaining to air sensitivity of secondary ADH in methanogens. It was reported that in *Clostridium beijerinckii*, the secondary ADH activity was air-sensitive in the unpurified enzyme. Up to 50% loss of activity was caused within the first 30 min of air exposure in the CFE, and the remaining activity was then stable (Ismaiel et al. 1986). Interestingly, the purified secondary ADH of *C. beijerinckii* was air stable. It was suggested that the formation of H_2O_2 or oxygen radicals in CFE upon exposure to oxygen might occur and subsequently destroy the enzyme activity (Stadtman and Oliver 1991). The mechanism of air sensitivity in the secondary ADH of *Msph. stadtmanae* might be similar to that in

C. beijerinckii. Further experiments using purified enzyme need to be performed to uncover the mechanism of this air sensitivity.

The optimal pH of the secondary ADH in CFE of *Msph. stadtmanae* was 9.2 and 8.6 for the oxidation of 2-propanol and reduction of acetone respectively, while in *Mg. organophilum* CV (Frimmer and Widdel 1989) and *Mg. thermophilum* TCI (Widdel and Wolfe 1989), the pH optima were 10 and 4.2 respectively for 2-propanol oxidation. The Kms of ADH in CFE of *Msph. stadtmanae* for NADP, NADPH, 2-propanol and acetone were also determined. It was shown that the enzyme had a higher affinity for acetone and NADPH than for 2-propanol and NADP. The fact that acetone is one of the major fermentation products of certain microorganisms (George et al. 1983) and that Km of the enzyme for acetone was lower than for 2-propanol suggested that the enzyme might possibly function as an acetone reducer in the natural environments. This is consistent with the observation that acetone was much more toxic to the cells than 2-propanol and the activity for acetone reduction was about twice as high as that of 2-propanol oxidation at approximate physiological pH.

Cells of *Msph. stadtmanae* could not grow on 2-propanol+methanol, different from other methanogens that are known to possess ADH (Frimmer and Widdel 1989). One explanation for this is that cells of *Msph. stadtmanae* contained a much lower level of secondary ADH than other methanogens, such as in *Mb. palustre* and *Mg. liminatans* (Bleicher and Winter 1991). The specific activities of CFE from these two methanogens were 5 and 0.7 μmol/min.mg

protein respectively, which are about 50 to 357 fold higher than the specific activity in CFE of *Msph. stadtmanae* (14 nmol/min. mg from figure V.1).

However, the secondary ADH in *Msph. stadtmanae* might function primarily in the direction of 2-propanol oxidation. As major products of some anaerobic eubacteria, 2-propanol and 2-butanol might be present in the natural environment (Ismaiel et al. 1993, George et al. 1983, Kutzenok and Aschner 1952), and provide electrons for methanogenesis from methanol. This possibility was supported by the results that 2-propanol was used for methanogenesis and the presence of 2-propanol increased the ability of cells to survive hydrogen deficient conditions. Although cells of *Msph. stadtmanae* could not grow with methanol and 2-propanol in the absence of hydrogen, cell growth was stimulated by the addition of 2-propanol in normal cultures. This growth stimulation might be contributed by the secondary ADH which oxidized 2-propanol and reduced NADP, providing the cells the electrons for biosynthesis.

The secondary ADH level in *Msph. stadtmanae* increased with the addition of either substrates and under hydrogen limitation. In several other methanogens including *Mg. thermophilum, Mg. organophilum, Msp. hungatei* and *Mb. bryantii,* it was found that both substrates (2-propanol, 2-butanol, and acetone, etc.) and non-substrates (tertiary butanol and pentanol) stimulated the expression of secondary ADH (Widdel and Wolfe 1989). In *Mg. thermophilum* TCI, the secondary ADH was also expressed under H_2 limitation conditions. In *Mg. organophilum* and *Mb. bryantii,* the

presence of a primary alcohol (1-propanol or 1-butanol) also caused the expression of the secondary ADH (Widdel and Wolfe 1989).

In conclusion, the physiological roles of the secondary ADH in *Msph. stadtmanae* might be for the reduction of acetone or the oxidation of 2-propanol, based on the preliminary characterization of the enzyme and growth tests under various conditions. Further studies to aim at purifying the enzyme and characterization using the purified enzyme would provide further insight into the functions of the enzyme.

Chapter VI. General conclusion

The major thrust of the work reported in this thesis was to investigate the use of pterins (specifically H_4M and H_4F as $_1C$ carriers for biosynthesis). The first approach was to investigate C_1 metabolism in *Msph. stadtmanae*, since this organism possesses most of the enzymes known to function in the common methanogenic pathway in other organism and H_4M, yet is unable to generate methane from CO_2. By using ^{14}C short-term labelling in *Msph. stadtmanae* it was indicated that the break of the common methanogenic pathway was at the formyl- or methenyl- level, since $^{14}CH_4$ could be produced from ^{14}C-formaldehyde or ^{14}C-serine while no $^{14}CH_4$ could be detected from $^{14}CO_2$ or ^{14}C formate plus H_2.

Such data also indicated the involvement of H_4M via SHMT in methanogenesis from serine, and via methanol since methanol was incorporated into proteins and RNA. This is supported by the fact that enzymes below the level of methylene-H_4M in the common methanogenic pathway were all present in this organism. The facts that FDH activity was present and that ^{14}C-formate was incorporated specifically into histidine and RNA would suggest that formate might enter the C_1 pool through a non-H_4M pathway. The very low level of FDH activity detected , however, made it difficult to attempt to study other enzyme activities that would be needed for biosynthesis from formate.

A second approach was followed based on the ^{13}C work (refer to chapter I of this thesis) which indicates that some methanogens

do not have a connection between methanogenesis and C_1 biosynthetic pathways. An enzyme that would be easy to assay is SHMT since its H_4M activity could also be verified through methanogenesis from serine+H_2. It was expected that those organisms which did not connect C_1 biosynthesis and methanogenesis would have a H_4M independent SHMT which is what was observed. In *Mb. thermoautotrophicum* Marburg and *Msph. stadtmanae,* members of *Methanobacteriales,* the activities were found to be H_4M dependent. In representatives of *Methanococcales,* the enzyme activity in *Mc. thermolithotrophicus* was also H_4M dependent, however, no activity was detected in *Mc. voltae*. In all four members of *Methanomicrobiales* tested, including *Msp. hungatei* GP1, *Mst. concilii, Ml. tindarius* and *Ms. barkeri* Fusaro, the enzyme was strictly H_4F dependent. However, whether or not H_4F is truly the cofactor used in these organisms is difficult to ascertain since the presence of folic acid in methanogens could not be unambiguously determined with the available bioassay and radioimmunoassay methods.

The H_4F dependent SHMT in *Msp. hungatei* was purified and further characterized using the purified enzyme. The enzyme resembled its bacterial counterparts in the structure, molecular mass and Kms. The N-terminal sequence comparison showed greater homology between *Msp. hungatei* and several bacterial and eucaryotic sources than between *Msp. hungatei* and *Mb. thermoautotrophicum*. To further understand the difference, especially the variation in pterin specificity between SHMT of *Msp. hungatei* and that of other

sources, a complete sequence of the enzyme in *Msp. hungatei* would be helpful for comparison.

The variation of pterin specificity for SHMT among different orders of methanogens, and the formate incorporation pathway occurred in members of *Methanobacteriales* remain to be the interesting fields for further investigation. It is still not known whether these variations observed represent general variations among different groups of methanogens or not. Further enzymatic studies on both FDH and SHMT of other methanogens would answer this question.

So far, there is no data available on the synthesis of thymine in methanogens (neither the C_1 carrier nor the C_1 source). In many other organisms, H_4F is involved as the C_1 carrier while formate is the major C_1 source (Blakley 1969). Further studies in methanogens of C_1 biosynthesis-related enzymes, such as the dihydrofolate (or dihydromethanopterin) reductase and thymidylate synthetase, which are required for the synthesis of thymidylate in bacteria and eucaryotes (Blakley 1969), would be helpful to clarify the situation in methanogens. ^{13}C-NMR studies of thymine synthesis, using ^{13}C formate and/or 2-^{13}C-acetate in methanogens especially representatives of *Methanobacteriales* (where formate might enter the C_1 pathway, such as in *Mb. thermoautotrophicum* and *Msph. stadtmanae*) and *Methanomicrobiales* (some of which were found to possess H_4F dependent SHMT) would also be informative to uncover the biosynthetic pathway of thymine in methanogens.

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