The $NADP^+$:F_{420} Oxidoreductase and Purification in *Methanosphaera stadtmanae*

by

Dwayne A. Elias B.Sc. (4-year)

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MASTER OF SCIENCE

Department of Microbiology
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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DWAYNE A. ELIAS

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

MASTER OF SCIENCE

Grandpa,

You always taught us that two of the most important things were hard work and an education.

This thesis is dedicated in your beloved memory.

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Abstract

Methanosphaera stadtmanae (DSM 3091) is a methanogen isolated from the human large intestine. The organism requires H_2 and CH_3OH for methanogenesis and cell growth to proceed, but CO_2 and acetate for growth. The $NADP^+$:F_{420} oxidoreductase is the major enzyme for controlling the intracellular NADPH pool for cellular biosynthesis by catalyzing the electron transfer between F_{420} and $NADP^+$ in methanogens. NAD^+, NADH, and FMN, and FAD could not be used as electron acceptors. Optimal pH for F_{420} reduction was 6.0, and 8.5 for $NADP^+$ reduction. The enzyme from *Methanosphaera stadtmanae* was purified to homogeneity with total protein decreased by over 5200-fold, and an enrichment of over 7700-fold using $(NH_4)_2SO_4$ precipitation, DEAE Cellufine, FPLC, native PAGE, and electroelution. The K_m's were; $NADP^+$ 370 μM, NADPH 142 μM, F_{420} 62.5 μM, and $F_{420}H_2$ 7.7 μM which were different from those in the cell-free extract. The functional enzyme's molecular weight of 148 kDa was determined by gel filtration chromatography and native-PAGE, consisting of α, β, γ subunits of 60 kDa, 50 kDa, 45 kDa as determined by SDS-PAGE.

Properties associated with $NADP^+$:F_{420} oxidation/reduction were studied in several methanogens. Cell extracts were subjected to $(NH_4)_2SO_4$ fractionation (65% supernatant, 0-65% pellet, 90% supernatant, and 65-90% pellet) and assayed for $F_{420}H_2$-dependent $NADP^+$ reduction and F_{420}-dependent NADPH oxidation. In crude extracts, all organisms tested exhibited both activities. In the Methanomicrobiales tested (*Methanospirillum hungatei*, *Methanosaeta concilli*, *Methanolobus tindarius*, *Methanosarcina barkeri*) both activities were recovered in the 90% supernatant. In the Methanococcales tested (*Methanococcus voltae*, *Mc. thermolithotrophicus*) the bulk of the F_{420}-dependent $NADP^+$

oxidation and reduction activities fell in distinct fractions. In *Mc. voltae*, 0-65% pellet K_m for NADPH was 0.3 mM while the 90% supernatant gave 2 mM. In Methanobacteriales, different observations were made with different organisms. In *Methanobacterium* G2R and *Methanosphaera caniculi*, the bulk of both activities were recovered in a single fraction. In *Mb. thermoautotrophicum* Marburg, distinct activities were separated: $F_{420}H_2$-dependent $NADP^+$ reduction (0-65% pellet) and F_{420}-dependent NADPH oxidation (90% supernatant). In *Methanosphaera stadtmanae*, the bulk activity in both directions was recovered in the 65-90% pellet, but, when grown with 2-propanol as an alternate electron source for $NADP^+$ reduction (via $NADP^+$ dependent secondary alcohol dehydrogenase), there was a 10-fold decrease in the $F_{420}H_2$-dependent $NADP^+$ reduction activity only. The above data indicate that: 1) under the conditions tested, the dominant direction was $NADP^+$ reduction, but 2) in several species, redox reactions between these 2 cofactors may involve more than one enzyme.

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List of Abbreviations

ADH	alcohol dehydrogenase
bCFE	boiled cell-free extract
CoA	Coenzyme A
CoM	2-mercaptoethanesulfonate
CFE	cell-free extract
DSM	Deutsche Sammlung von Mikroorganismen
DTT	Dithiothreitol
FDH	formate dehydrogenase
F_{420}	8-hydroxy-5-deazaflavin
$F_{420}H_2$	reduced 8-hydroxy-5-deazaflavin
H_4F	tetrahydrofolate
H_4M	tetrahydromethanopterin
HS-HTP	N-7-mercaptoheptanoyl-*O*-phospho-L-threonine
kDa	kilodalton
Mb.	*Methanobacterium*
Mc.	*Methanococcus*
MES	2-[N-Morpholino]ethanesulfonic acid
MFR	Methanofuran
Mg.	*Methanogenium*
Ml.	*Methanolobus*
MOPS	3-N-[Morpholino]propanesulfonic acid
Ms.	*Methanosarcina*
Msp.	*Methanospirillum*
Msph.	*Methanosphaera*
Mst.	*Methanosaeta*
Mtx.	*Methanothrix*
MV	Methyl Viologen
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced NADP

Chapter I

Literature Review

I.1. Introduction to the Methanogens

Within the prokaryotic organisms which carry out the anaerobic degradation of organic compounds, there lies a group of strictly anaerobic, methane producing archaebacteria known as the methanogens (Keltjens and van der Drift, 1986).

Methane formation occurs in numerous anaerobic habitats such as swamps (Amaral and Knowles, 1994), marshes, lake and river sediments, animal digestive tracts, and geothermal habitats (Blaut, 1994), with optimal temperatures varying from 20°C to 95°C, (Hemming and Blotevogel, 1985). Methanogenesis is the last step in anaerobic decomposition, and therefore plays an essential role in the mineralization of organic material (Garcia, 1990; Blaut, 1994). In spite of their great diversity in morphology, habitat, and nutritional requirements, the methanogens comprise a physiologically similar group of microorganisms which use a limited number of energy substrates including H_2 + CO_2, formate, methanol, methylamines, and acetate, (Blaut, 1994).

Most methanogens can use H_2 as a source of electrons for methanogenesis and thus acts as an electron sink for the anaerobic degradation (Archer and Powell, 1985) of complex organic polymers, (Ziekus, 1977, Balch et. al., 1979). These processes include treatment of wastewater, stabilization of wastewater sludge, anaerobic treatment of sewage, and treatment of solid waste, (McCarty, 1982, Raskin et. al., 1994, Novaes, 1986, Parkin and Owen, 1986, Jewell, 1987, Metcalf et. al., 1991). In view of their unique

physiology, co-factors and co-enzymes, the methanogens play an indispensable role in the anaerobic food chain, (Blaut et. al., 1992).

Interest in anaerobes and methanogens in particular has increased in recent years due to their potential use for the recovery of economically useful byproducts such as CH_4 from biomass fermentations, (Patel et. al., 1984). Methane can serve as a potentially useful biofuel (Daniels et. al., 1984a), but methane released into the atmosphere has also been shown to be in part be responsible for the greenhouse effect, (Oremland, 1988). These organisms should therefore be regarded as an economically important, naturally available resource.

The methanogens can use H_2/CO_2, formate, or acetate which is directly produced by fermentative bacteria, or can act in a syntrophic relationship permitting interspecies H_2 transfer (Garcia, 1990). Cappenberg et. al, (1974) also found interspecies acetate transfer between sulfur-reducing and methane-producing bacteria, sulfur-reducers produce acetate when growing on lactate.

The taxonomy of the methanogens is based on comparative studies of the 16S rRNA oligonucleotide sequences, membrane lipid composition, and antigenic fingerprinting data (Garcia, 1990). Using 16S rRNA to reveal evolutionary relationships, a newer taxonomic system has been proposed by Woese et. al. (1990, 1991), with the most basic division being the "domain", followed by the kingdom. There are three domains; those being 1. Bacteria (Eubacteria), 2. Archaea (Archaebacteria), and 3. Eucarya (Eucaryotes). The domain Archaea is placed as an early branch of the domain Eucarya due to, with few exceptions, a closer molecular resemblance with the eucaryotes than the eubacteria. There are also some aspects shared with Bacteria, and others only shared between Bacteria and

Eucarya. The domain Archaea has been further divided into 2 kingdoms; the Euryarcheota and the Crenarcheota. The methanogens themselves belong to the kingdom Euryarcheota.

I.1.1. An Overview of the Complete Methanogenic Pathway

There is one common methanogenic pathway utilized by methanogens for CO_2 reduction from the oxidation of H_2 or formate (Figure I.1.). Those that use methyl groups (eg. CH_3OH) divide the same pathway into an oxidative and a reductive branch (Figure I.2.), (Blaut et. al., 1992). The overall pathway for CO_2 reduction involves the co-factors methanofuran (MF), 5,6,7,8-tetrahydromethanopterin (H_4MPT), tetrahydrofolate (THF), Co-enzyme M (CoM-SH), and 7-mercaptothreonine phosphate (HS-HTP), (Keltjens and van der Drift, 1986). The reduction of F_{420} in CH_3OH oxidation, or the oxidation of $F_{420}H_2$ for H_2 + CO_2 methanogenesis, can occur between the redox levels of methenyl- and methylene-; and methylene and methyl- H_4MPT, (Blaut et. al., 1992, Thauer et. al., 1993, Thauer, 1997). A major electron carrier for several steps of this pathway is 8-hydroxy-5-deazaflavin, also known as F_{420}.

The roles of MF (Jones et. al., 1985), and H_4MPT as C_1 moieties in CO_2 reduction or formation are quasi-universal in methane formation across substrate and organismal boundaries (Jones et. al., 1985, Escalante-Semerena et. al., 1984, Escalante-Semerena and Wolfe, 1985, Keltjens et. al., 1990). For H_2:CO_2 methanogenesis as in *Mb. thermoautotrophicum*, the last step of methanogenesis begins with the transfer of the methyl group from methyl-H_4MPT to CoM-SH by the methyl-H_4MPT:HS-CoM methyltransferase (Gartner et. al., 1993, Stupperich et. al., 1993, Harms et. al., 1995).

Figure I.1.: Diagrammatic Representation of $H_2 + CO_2$ Methanogenesis.

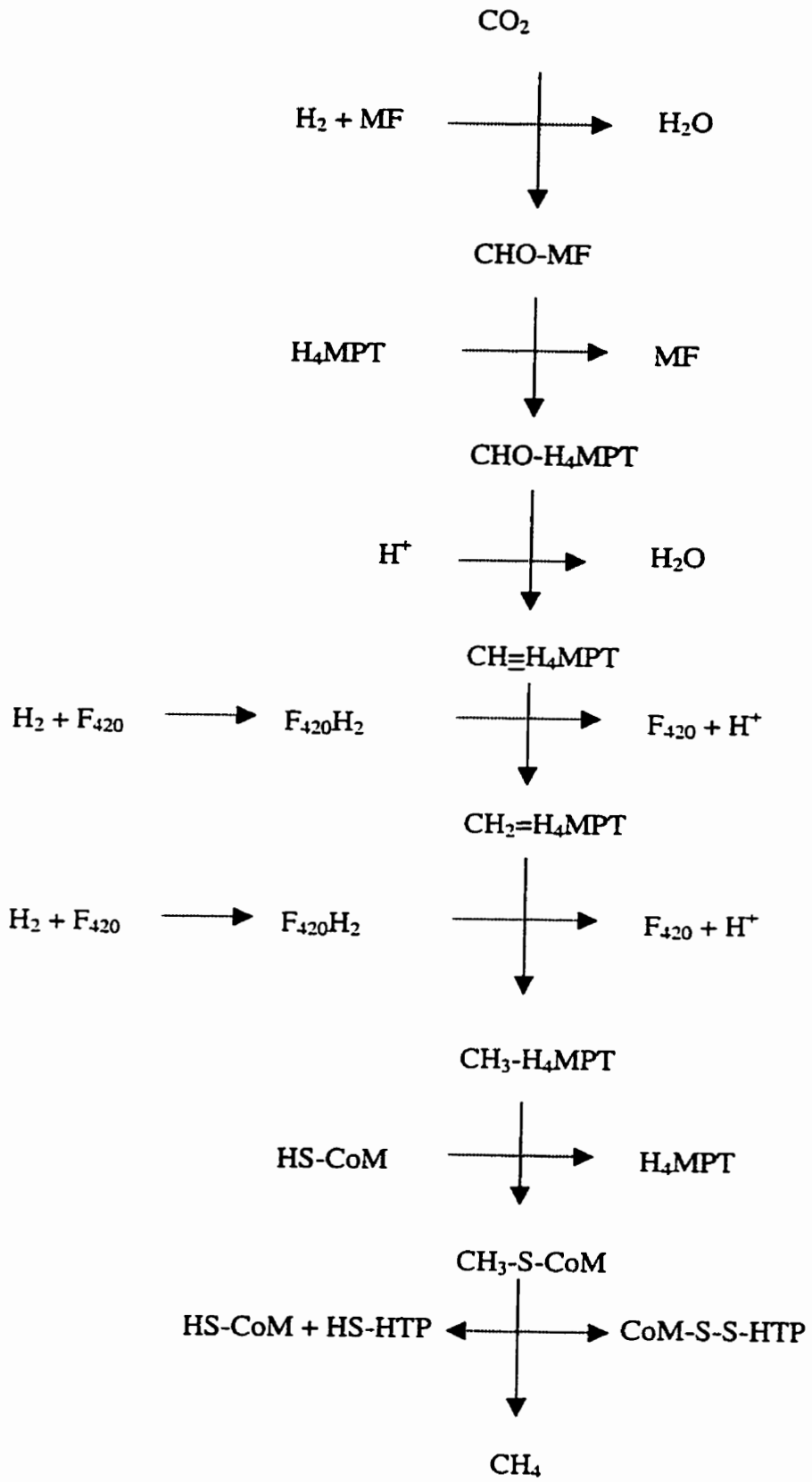
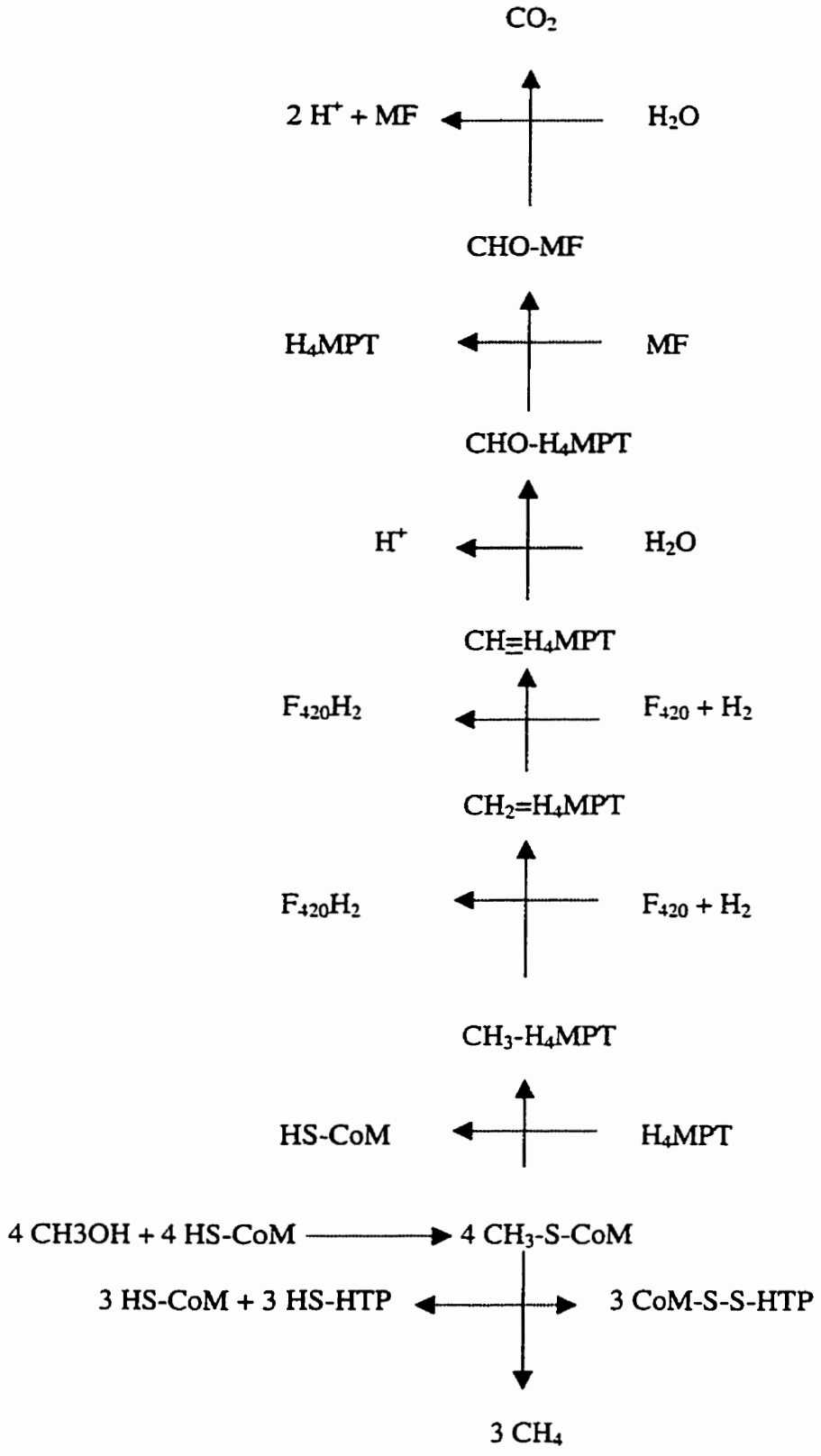

CO2
H2 + MF
H2O
CHO-MF
H4MPT
MF
CHO-H4MPT
H+
H2O
CH≡H4MPT
H2 + F420
F420H2
F420 + H+
CH2=H4MPT
H2 + F420
F420H2
F420 + H+
CH3-H4MPT
HS-CoM
H4MPT
CH3-S-CoM
HS-CoM + HS-HTP
CoM-S-S-HTP
CH4

Figure I.2.: Diagrammatic Representation of CH_3OH Methanogenesis.


CO2
2 H+ + MF
H2O
CHO-MF
H4MPT
MF
CHO-H4MPT
H+
H2O
CH≡H4MPT
F420H2
F420 + H2
CH2=H4MPT
F420H2
F420 + H2
CH3-H4MPT
HS-CoM
H4MPT
4 CH3OH + 4 HS-CoM
4 CH3-S-CoM
3 HS-CoM + 3 HS-HTP
3 CoM-S-S-HTP
3 CH4

In all methanogens, the last step of methanogenesis involves the methylation of CoM-SH with the methyltransferase, followed by the subsequent release as methane via electron donation by HS-HTP on the methylreductase. This results in the formation of a CoM-S-S-HTP heterodisulfide, and reduction of the heterodisulfide is accomplished via the heterodisulfide reductase with either H_2 or $F_{420}H_2$, (Deppenmeier et. al., 1990a, 1990b, 1991). Deppenmeier et. al., (1996) stated that the H_2:heterodisulfide oxidoreductase functions for H_2:CO_2 methanogenesis and the $F_{420}H_2$:heterodisulfide oxidoreductase functions for methanol methanogenesis where $F_{420}H_2$ is generated from methanol oxidation and cytochromes are involved as electron carriers between $F_{420}H_2$ and the heterodisulfide.

I.1.2. H_2 + CH_3OH Methanogenesis

The overall process of H_2 + CO_2 methanogenesis utilizes the indirect transfer of 4 electron pairs in the form of 4 H_2 equivalents per unit of methane formed (equation 1), (Thauer et. al., 1977, Kell et. al., 1980, Daniels et al., 1984b, Blaut et. al., 1992). Methanogenesis from H_2 + CH_3OH uses only one pair of electrons (equation 1), and this can allow for study in isolation of the last reductive step (Blaut et. al., 1986), which is highly favorable energetically (Gottschalk, 1984).

				ΔG° (KJ/CH_4 produced)
Equation 1	$4\ H_2 + CO_2$	------->	$CH_4 + H_2O$	-136.0
	$4\ CH_3OH$	------->	$CO_2 + 3\ CH_4 + 2\ H_2O$	-112.5
	$H_2 + CH_3OH$	------->	$CH_4 + H_2O$	-113.0

In CH_3OH methanogenesis, there is a disproportionation of methanol (Figure I.2.) (van der Meijden et. al., 1983a, 1983b, Deppenmeier et. al., 1996). The oxidative branch uses one of every four CH_3OH entering the methanogenic pathway to yield CO_2 using the C_1 intermediates of H_4MPT, F_{420}, and MF. This is in fact a reverse of the reductive pathway for H_2 + CO_2 methanogenesis. The three other CH_3OH entering the system proceed through the reductive branch of the pathway. They are transferred to CoM-SH, producing CH_3-S-CoM, and the methyl group is then reduced with the sulfur-bound H^+ of HS-HTP using a multienzyme complex known as the methylreductase (Deppenmeier et. al., 1996).

I.1.2.1. Methanol Entry into the Methanogenic Pathway

In *Ms. barkeri*, evidence shows that there are 2 main enzymes involved in the transfer of the methyl group of CH_3OH to HS-CoM for production of CH_3-S-CoM. these are denoted as MT_1 and MT_2. MT_1 is the methanol:5-hydroxybenzimidizolylcobamide methyltransferase, and MT_2 is the Co-methyl-5-hydroxybenzimidizolylcobamide:HS-CoM methyltransferase (Nagle and Wolfe, 1983; van der Meijden et. al., 1983a, 1983b, 1984). MT_1 binds the methyl group of CH_3OH to a corrinoid bound to the enzyme, and is inactivated with oxygen and other oxidizing agents (specific (Müller et. al., 1988a, b; Deppenmeier et. al., 1990, 1991, 1996). Catalytic amounts of H_2 and ATP are required to activate the system. MT_2 transfers the methyl group bound to the corrinoid of MT_1 to HS-CoM forming CH_3-S-CoM. MT_2 is oxygen stable with no requirement for ATP, and is not very substrate (Deppenmeier et. al., 1996; Kengan et. al., 1990; Daas et. al., 1996; Tallant and Krzycki, 1996). It is expected that the entry of methanol into the

methanogenic pathway in *Msph. stadtmanae* would be similar.

I.2. Electron Transfer Reactions in Biosynthesis

I.2.1. Roles of Essential Co-factors

Methanogens utilize C_1 intermediates for conversion of their carbon source. The oxidation of H_2 and the oxidation of CH_3OH to CO_2 for make electrons and reducing equivalents available which are necessary for biosynthesis. Cellular biosynthesis relies directly on the electron transfer reactions involving these reducing equivalents and interaction of the co-factors NAD^+ or NADH, $NADP^+$ or NADPH, and the novel flavin $F_{420}(H_2)$.

I.2.1.1. NAD(P)H

Nicotinamide Adenosine Dinucleotide (NAD^+) is used in most organisms in metabolism as an "energy cushion" and provides an energy partitioning as reducing equivalents under unfavorable growing conditions. The redox potential for NADH/NAD^+ is -320 mV (Harris, 1995, Sarma and Mynott, 1973). $NADP^+$ is typically the main electron transfer agent in cell carbon biosynthesis. The redox potential for NADPH/$NADP^+$ is -320 mV (DiMarco et. al., 1990).

Let us now look at the use and electron transfer to NAD^+ and $NADP^+$ in methanogens. While $NADP^+$ can be reduced by the $NADP^+$:F_{420} oxidoreductase, NAD^+ cannot be reduced by F_{420} (Yamazaki and Tsai, 1980a, 1980b, Ziekus et. al., 1977), a hydrogenase (Tzeng et. al., 1975a, Zeikus, 1977, Ziekus et. al., 1977, Sprott et. al., 1987), or formate dehydrogenase (Tzeng et. al., 1975b). There is no known pathway for NAD^+

reduction, and there is no evidence for the presence of a NADPH:NAD^+ transhydrogenase (Daniels et. al., 1984b, McKellar et. al., 1979).

$NADP^+$ on the other hand can be reduced by $F_{420}H_2$ (see next section) which can itself get electrons from the methanogenic pathway (see previous section). In biosynthesis, $NADP^+$ is the preferred cofactor of malate dehydrogenase in *Mb. thermoautotrophicum* (Ziekus et. al., 1977), *Ms. barkeri* (Wiemer and Ziekus, 1978), and *Msp. hungatei* (Sprott et. al., 1979). The isocitrate dehydrogenase in *Ms. barkeri* requires $NADP^+$ (Wiemer and Ziekus, 1979, Keltjens and van der Drift, 1986), but either NAD^+ or $NADP^+$ can be function in gluconeogenic reactions (Ziekus et. al., 1977).

Since some enzymes require NADH, there must be an *in vivo* method of reducing NAD^+. Daniels et. al., (1984b) conceived an idea that enzymes which can use either NAD^+ or $NADP^+$ could act as electron transferases. The example used was the glyceraldehyde-3-phosphate dehydrogenase. In the reducing direction the enzyme has a 10-fold higher affinity for NADPH, while in the oxidizing direction the affinity for NAD^+ and $NADP^+$ is equal. Since $NADP^+$ can be reduced by F_{420} (Daniels et. al., 1984b), the concentration of $NADP^+$ should be lower, while NAD^+ concentration should be higher, such that NADPH could reduce 3-phosphoglyceric acid to glyceraldehyde-3-phosphate. The reduction of NAD^+ could occur. Therefore, it is possible that there could be a regulatory role for NADH in cell metabolism whereby the NAD^+:$NADP^+$ ratio may act as a sensor of cell production of reducing equivalents.

I.2.1.2. $F_{420}(H_2)$

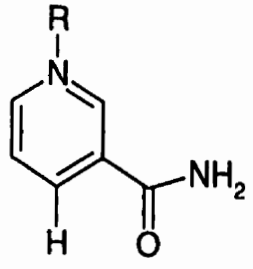
a.). Characterization

The co-factor commonly referred to as F_{420} is essentially a modified flavin with its nearest relative being the flavin mononucleotide (FMN), with carbon substituting for nitrogen at the C_5 position of the heterocyclic ring structure (Figure I.3.) (Daniels et. al., 1984b, Lin and White, 1986, Hemmerich and Massey, 1977). F_{420} is named due to its peak of absorption at 420 nm in basic solutions (Cheeseman et. al., 1972). When F_{420} is reduced to $F_{420}H_2$ with $NaBH_4$, the fluorescent peak at 420 nm disappears, and upon exposure to air, the fluorescence reappears (Keltjens and van der Drift, 1986, Cheeseman et. al., 1972).

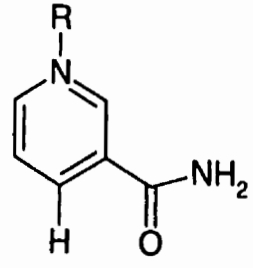
F_{420} itself consists of a core molecule of N-l-lactyl-γ-L-glutamyl phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5'-phosphate. The full structural molecule of F_{420} is the N- [N- [O- (5- (8- hydroxy - 5 - deazaisoalloxazin - 10yl) - 2, 3, 4 - trihydroxy - 4- pentoxylhydroxy - phoshinyl) - L - lactyl - γ - L - glutamic acid (Keltjens and van der Drift, 1986). The basic 5-deazaflavin ring structure is the minimum required for catalytic activity. The structures of biologically produced 5-deazaflavins differ mainly in the number of glumly residues in their N_{10} side chains (Yamazaki et. al., 1982).

The exact functions of these different side chains is however, not well understood. Altering the length of the N_{10} side chain alters the rate, but not the nature of activity with respect to a particular enzyme. The hydroxyl group at C_8 influences the pH activity profile, and methylations at C_8, C_5, and C_7 reduce its activity. Alterations in the ring structure itself, such as altering N_1 to CH, inactivates the coenzyme (Yamazaki et. al., 1982).

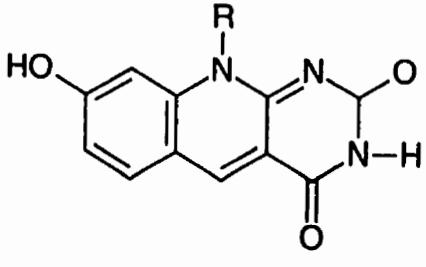
Figure I.3: Structural Comparison of F_{420}, FMN, and NAD^+ showing the structural similarity to FMN and the functional similarity to NAD^+.



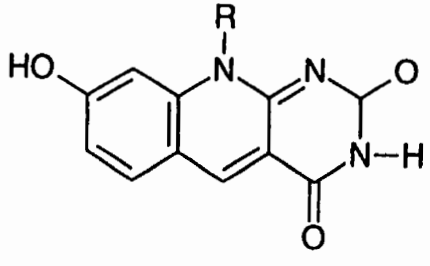
NAD+



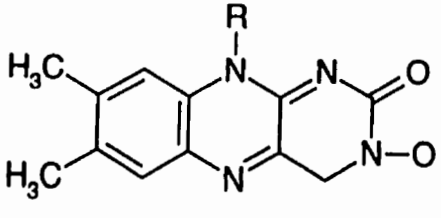
$NADH_2$



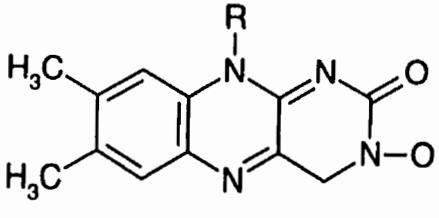
F_{420}



$F_{420}H_2$



FAD+



$FADH_2$

The chromophoric group of F_{420} from *Methanobacterium* strain M.o.H. has been found on *Mycobacterium smegmatis*, *Mycobacterium karlinski*, *Mycobacterium phlei*, *Mycobacterium avium*, *Peptostreptococcus elsdenii*, and *Streptomyces griseus* (Naraoka, 1984). The only difference is that F_{420} has two L-glutamate residues and here there is only one. This appears to reflect a difference in the bonding mode to an apoprotein, (Naraoka, 1984).

As a hybrid of NAD^+ and flavins, F_{420} (8-hydroxy-5-deazaflavin) is chemically equivalent to NAD^+ in being an obligate 2 electron acceptor; i.e. it does not display the semiquinone form of flavins. F_{420} has a redox potential of -340 to -350 mV,(much lower than flavins) (Figure I.3.). The 5-deazaflavin by comparison has a redox potential of -273 mV (Hemmerich and Massey, 1977, Lin and White, 1986).

b.). Cellular Uses

This low redox potential has a major cellular implication of mediating electron transfer between $NAD(P)^+$ and higher 1 and 2 electron acceptors like flavins, and can mediate the reduction of $NAD(P)^+$ with electrons from H_2 or formate oxidation (Hausinger et. al., 1985, Kojima et. al., 1983).

The E^o of NAD^+ and $NADP^+$ is -320 mV. Based on the redox potentials for F_{420} and the 5-deazaflavins, it is expected that the electrons from F_{420} will enter the electron flow at the same position as NADH or NADPH (Lin and White, 1986). This similarity in redox potential between the nicotinamides and F_{420}, and thus similar placement in electron flow, is also consistent with the fact that F_{420} functions as a 2-electron donor like NADH, and not like a flavin (Lin and White, 1986, Hemmerich and Massey, 1977).

A novel function of F_{420} is as a metabolic alarmone to guard against oxygen toxification. When the hydrogenase is exposed to oxygen, the result is formation of oxygen radicals. Upon exposure of the cells to oxygen, F_{420} is converted to its 8-hydroxyadenylylated and 8-hydroxyguanylylated derivatives, and the absorbance spectrum shifts from a peak of 420 nm to 390 nm. The adenosine (guanine) 5'-phosphate is linked to the flavin via the F_{420} 8-hydroxy position. Substitution of the 8-hydroxy group has not been previously observed in the biological chemistry of F_{420}. Because F_{420} is an active hydrogenase substrate, and F_{390} is an inactive hydrogenase substrate, it can act as a metabolic jamming mechanism leading to a shutdown of metabolic operations inside the cell. (Hausinger et. al., 1985).

This process is reversible when anoxic conditions are re-established. The ATP-dependent F_{390} synthetase converts F_{420} to F_{390}, and an F_{390} hydrolase cleaves the AMP or GMP residue to convert F_{390} back to F_{420}. This provides a defense against oxygen toxification by restricting the deleterious effects of oxygen on reduced electron carriers (Vermeij et. al., 1994).

The most widespread function of F_{420} in prokaryotes is photoreactivation via the splitting of thymine dimers. In several cases an 8-hydroxy-5-deazaflavin has been implicated as the chromophore (Eker et. al., 1980, Kiener et. al., 1985, Rokita and Walsh, 1984).

All photolyases found up to 1992 had FAD alone or with methylene tetrahydrofolate (MTHF) (Malhotra et. al., 1992). In *Anacystis nidulans* however, the purified enzyme for photoreactivation contained 8-hydroxy-5-deazaflavin and FAD. Complexes of enzyme-FADH-8-hydroxy-5-deazaflavin and enzyme-$FADH_2$-8-hydroxy-5-deazaflavin carried

out photorepair with equal efficiencies at 440 nm. The $FADH_2$ then repaired the T< >T most likely via electron transfer. The deazaflavin class photolyases were found to be more efficient for 3 reasons: 1) a greater abundance of 430-450 nm photons, 2) a higher extinction co-efficient compared to folate class, and 3) a higher efficiency of transfer via 8-hydroxy-5-deazaflavin than by MTHF to $FADH_2$ (Malhotra et. al., 1992).

In *Streptomyces aureofaciens* mutant S-1308, another function was discovered. The pathway for tetracycline synthesis was not being completed, and was stopped at the step of 5a,11a-dehydrochlortetracycline to 7-chlortetracycline (Miller et. al., 1960). This step involves the hydration of carbons 5 and 11 (McCormick and Morton, 1982).

Jaenchen (1984) studied the synthesis of F_{420} in *Mb. thermoautotrophicum*. The F_{420} became labeled using [U-^{14}C]guanine and [2-^{14}C]guanine but not for [8-^{14}C]guanine. When *Mb. thermoautotrophicum* was grown with[^{14}C]methionine, [^{14}C]phenylalanine or [^{14}C]tyrosine, F_{420} did not become labeled. Stoichiometrically, 1 mol of guanine was incorporated per mol of F_{420} formed. The most likely interpretation of these data is that the pyrimidine ring of the deazaflavin is biosynthetically derived from the pyrimidine ring of the purine. The observation that F_{420} becomes labeled in the presence of [2-^{14}C]guanine but not with [8-^{14}C]guanine suggests that this reaction may also occur in deazaflavin biosynthesis.

I.2.3. Biosynthesis: Electron Transfer Reactions in Methanogens

I.2.3.1. Biosynthetic Carbon Metabolism

Experiments with *Mb. thermoautotrophicum* (Stupperich et. al., 1983a, Stupperich and Fuchs, 1983, Stupperich and Fuchs, 1984a, Stupperich and Fuchs, 1984b, Holder et.

al., 1985, Länge and Fuchs, 1985, Rühlemann et. al., 1985) and *Ms. barkeri* (Kenealy and Zeikus, 1982) have shown unequivocally that acetyl-CoA is the first condensation product in cell carbon synthesis (Keltjens and van der Drift, 1986). The CO-dehydrogenase is a key enzyme in cell carbon synthesis (Keltjens and van der Drift, 1986).

All carbon that is used for biosynthesis eventually is fixed to acetyl-CoA. From acetyl-CoA, pyruvate is formed via the pyruvate oxidoreductase, using F_{420} as the electron acceptor, as no pyruvate dehydrogenase complex has been found. The pentose-phosphate pathway, Embden-Meyerhoff pathway, and Entner-Doudoroff pathway all function to oxidize pyruvate to glucose, generate NADPH, tetrose and pentose sugars (Danson, 1989).

All methanogens are capable of synthesizing pyruvate from acetyl-CoA, as has been shown in *Mb. thermoautotrophicum*, *Ms. barkeri*, *Mc. maripaludis*, *Mc. voltae*, *Msp. hungatei*, and *Mtx. concilii*, (Ziekus et. al., 1977, Wiemer and Ziekus, 1979, Sheih and Whitman, 1987, Ekiel et. al., 1983, Ekiel et. al., 1985b, Evans, 1985). Ziekus et. al., (1977) demonstrated that F_{420} is reduced by CoA-dependent α-ketoglutarate dehydrogenases in *Mb. thermoautotrophicum*.

While no methanogen possesses a complete Krebs cycle, *Mb. thermoautotrophicum*, *Msp. hungatei*, *Mc. voltae*, and *Mc. maripaludis* lack isocitrate dehydrogenase and use the Krebs cycle in a reductive direction to synthesize α-ketoglutarate from oxaloacetate via malate, fumarate, succinate, and succinyl-CoA, (Fuchs and Stupperich, 1978, Ekiel et. al., 1983, Ekiel et. al., 1985a, Sheih and Whitman, 1987). *Ms. barkeri* and *Mtx. concilii*, which do not possess the α-ketoglutarate dehydrogenase, use the oxidative direction of

the cycle to form α-ketoglutarate from oxaloacetate and acetyl-CoA by citrate synthase, acotinase, and isocitrate dehydrogenase, (Daniels et. al., 1984b, Stupperich et. al., 1983b, Wiemer and Ziekus, 1979, Ekiel et. al., 1985b).

In all methanogens, hexoses are derived from phosphoenolpyruvate, which is converted to glucose via gluconeogenesis, (Blaut et. al., 1994). Pentoses for nucleotide synthesis are derived from galactose-3-phosphate and fructose-1,6-bisphosphate, (Fuchs and Stupperich, 1980, Ekiel et. al., 1983).

I.2.3.2. NADPH Formation in Methanogens

a.). From $F_{420}H_2$

The reduction of $NADP^+$ to NADPH can occur in different ways. In the context of this work, the main electron donor to $NADP^+$ is F_{420}, which can be reduced to $F_{420}H_2$ in a number of ways.

Many methanogens possess the F_{420}-dependent hydrogenase. With the F_{420}-dependent hydrogenase, the electrons from hydrogen oxidation can be transferred directly to F_{420}, and then on to reduce $NADP^+$ via the $NADP^+$:F_{420} oxidoreductase. Generation of $F_{420}H_2$ can also occur by the methanogenic pathway from CH_3OH to CO_2. In either case the electrons for $NADP^+$ reduction come from F_{420} as the electron transfer agent, (Kell et. al., 1980).

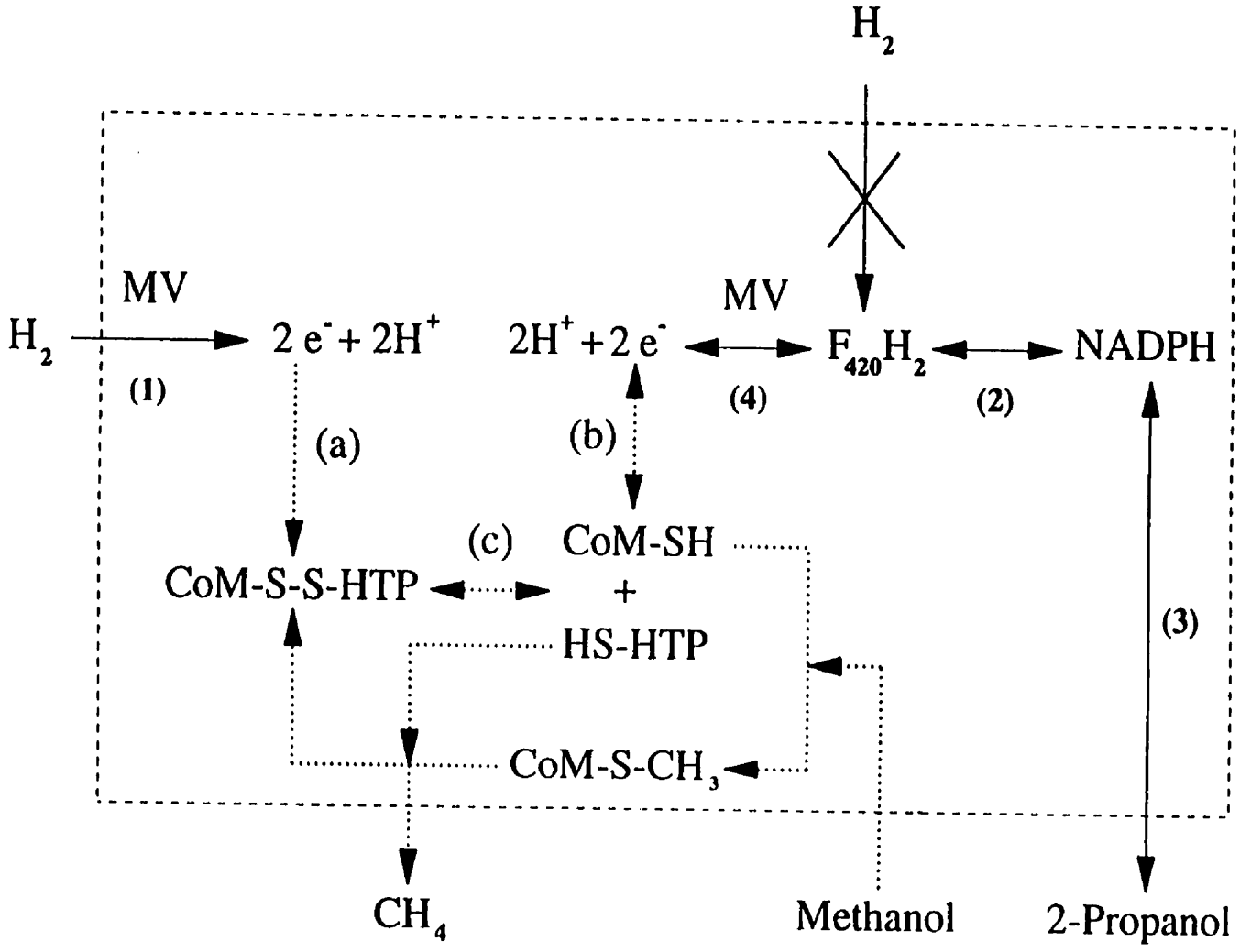
In the case where the F_{420}-dependent hydrogenase is replaced with an F_{420}-independent hydrogenase, as in *Msph. stadtmanae*, the picture becomes less clear. Wong et al., (1994) speculate that hydrogen is oxidized by the F_{420}-independent hydrogenase, and after reduction of the CoM-S-S-HTP heterodisulfide by an unknown electron carrier,

it is oxidized with the subsequent reduction of F_{420} via the F_{420}-dehydrogenase. The F_{420}-dehydrogenase reduces F_{420} to $F_{420}H_2$ which can then reduce $NADP^+$ via the $NADP^+$:F_{420} oxidoreductase (Figure I.4.). This is not without merit as Deppenmeier et. al., (1991) showed that the F_{420}-dependent hydrogenase reduced F_{420} to $F_{420}H_2$ which was used to also reduce the heterodisulfide in the methanogen Gö1.

An alternate route is the reduction of F_{420} and eventually $NADP^+$ through the oxidation of formate. In *Mc. vannielii* for example, such a system exists (Tzeng et. al., 1975a, Jones and Stadtman, 1980). This occurs by reduction of a 5-deazaflavin via the 5-deazaflavin:Formate Dehydrogenase. The reduced 5-deazaflavin then is able to reduce $NADP^+$ via the $NADP^+$:5-deazaflavin oxidoreductase. While the $NADP^+$:5-deazaflavin oxidoreductase specifically utilizes the 5-deazaflavin as a co-factor, the formate dehydrogenase can use FMN, FAD, and the 8-hydroxy-5-deazaflavin (Yamazaki and Tsai, 1980, Jones and Stadtman, 1980).

In *Mb. formicicum*, the F_{420}-dependent hydrogenase and formate dehydrogenase are membrane associated protein components in the formate hydrogen-lyase system. These are used for the oxidation of formate in the absence of hydrogen. $NADP^+$ was not required for formate hydrogen-lyase activity, nor were any other intermediate electron carriers besides F_{420} (Baron and Ferry, 1989). It is then conceivable that the reduced F_{420} reduces $NADP^+$ via the $NADP^+$:F_{420} oxidoreductase.

Figure I.4.: The route of electron flow for the reduction of $NADP^+$ in *Msph. stadtmanae* by Wong et. al., (1994). Solid lines represent reported paths, while dotted lines represent proposed paths. Bracketed numbers are; (1) MV-hydrogenase, (2) $NADP^+$:F_{420} oxidoreductase, (3) secondary alcohol dehydrogenase, (4) F_{420} dehydrogenase. Letters represent; (a) electron path from H_2 to CoM-S-S-HTP, (b) electron path from F_{420} to CoM-S-S-HTP, (c) heterodisulfide reductase. Reprinted with permission.


H2
MV
H2
2 e- + 2H+
(1)
(a)
2H+ + 2 e-
MV
F420H2
(4)
(2)
NADPH
(b)
(c)
CoM-SH
+
HS-HTP
CoM-S-S-HTP
(3)
CoM-S-CH3
CH4
Methanol
2-Propanol

b.). Other Sources

Alternative routes for the production of NADPH include the alcohol dehydrogenase (ADH). This can be a 1-step reaction pathway to the formation of NADPH as seen in *Msph. stadtmanae*, which possess a secondary ADH (Wong et. al., 1994). Here the ADH is specific for 2-propanol and 2-butanol, and can only use $NADP^+$ as the electron acceptor. In *Mb. palustre*, a similar enzyme has been found (Zellner et. al., 1989). *Methanogenium organophilum* also possesses an $NADP^+$-dependent 2°-ADH which uses 2-propanol. However it was also found while that *Mg. thermophilum* and *Msp. hungatei* possess a 2°-ADH, they are F_{420}-dependent (Widdel et. al., 1988, Widdel and Wolfe, 1989). Other ADH's have been found which use a variety of alcohol's for oxidation (Zellner and Winter, 1987), with the main electron acceptor being F_{420} (Eirich and Dugger, 1984). Zellner and Winter (1987) found a number of methanogens that utilize a 2°-ADH, but it was not stated whether these enzymes were co-factor dependent.

The isocitrate dehydrogenase reaction is the main reaction to reduce $NADP^+$ in the context of the Krebs cycle, while others act to oxidize NADPH. NADPH provides sufficient reducing potential for CO_2 reduction to cellular carbon in *Mb. thermoautotrophicum*, and is evidenced by the requirement of the glyceraldehyde-3-phosphate dehydrogenase for $NADP^+$, (Zeikus et. al., 1977).

I.3. Description and Biosynthetic Importance of F_{420}-dependent $NADP^+$ Oxidoreductases in Methanogens

In most hydrogenotrophic methanogens, the bulk of the electrons for carbon reduction for biosynthesis are the result of H_2 oxidation. These electrons are usually passed to F_{420}

prior to being transferred to $NADP^+$ for reduction, as F_{420} is accepted as a major electron transfer agent within the cell. The $NADP^+$:F_{420} oxidoreductase is the major enzyme for regulating the size of the intracellular $NADP^+$ pool for biosynthesis by catalyzing the electron transfer between $F_{420}(H_2)$ and NADP(H). Therefore, the $NADP^+$:F_{420} oxidoreductase is a crucial enzyme for biosynthesis, and its characterization is important. Many different methanogens possess an $NADP^+$ reductase or oxidoreductase. Yamazaki and Tsai, (1980) purified the $NADP^+$:5-deazaflavin reductase in *Mc. vannielii* (Table I.1.). They showed that the k_{cat} for the forward reaction was 24 times that of the reverse reaction, and concluded that the reduction of $NADP^+$ is favored at pH 7.0, which is expected to be near the physiological pH. The purification procedure was done at 4°C, with $(NH_4)_2SO_4$ treatment, an agarose-hexane-$NADP^+$ affinity column eluted with a linear gradient of $NADP^+$, DEAE Cellulose and Sephacryl S-200 gel filtration chromatography.

After the first $(NH_4)_2SO_4$ step, 53% of the initial activity was retained, and was later calculated that the reductase content was 0.09% of the soluble protein of the cell. At a low concentration, (66 μg/ml), the purified enzyme lost 84% of its activity in 2 days at 4°C. When 20% ethylene glycol (v:v) was added to the buffer, only 22% activity was lost after 2 days, and no activity lost thereafter.

Sulfhydryl groups were present and essential for catalytic activity as demonstrated by loss of activity with the addition of iodoacetamide, and *p*-chloromercuriphenyl sulfonate. The purified enzyme did not use NAD^+, NADH, FAD, FMN, or Riboflavin in the presence of NADPH, and could not catalyze reduced 5-deazaflavin oxidation.

Yamazaki et. al. (1980) further demonstrated that $NADP^+$ reduction in *Mc. vannielii*

Table I.1.: Comparison of $NADP^+$ Reductases

	Mb. thermoautotrophicum ΔH[1]	*Mc. vannielii*[2]	*Arch. fulgidus*[3]	*Strep. griseus*[4]
optimum temp.	60°C	17 - 20°C	80°C	N.A.
# of subunits	4 x 28 500	2 x 43 000	2 x 28 000	2 x 21 000
molec. wght.	112 000	85 000	55 000	42 000
K_m's $NADP^+$	40 µM	10 µM	40 µM	14.4 µM
$F_{420}H_2$	128 µM	8 µM	20 µM	12.9 µM
NADPH	N.A.	N.A.	N.A.	19.5 µM
F_{420}	N.A.	N.A.	N.A.	3.4 µM
optimal pH				
$NADP^+$ reduction	N.A.	7.9	8.0	7.9
NADPH oxidation	N.A.	4.8	5.5	5.9

N.A.-data not given in publication.
1- Jacobsen and Walsh, (1984).
2- Yamazaki et. al., (1980).
3- Kunow et. al., (1993).
4- Eker et. al., (1989).

was a direct hydride transfer from C_5 of the 8-hydroxy-5-deazaflavin to C_4 of $NADP^+$. Using ^3H labeled substrates, 97% of the ^3H used was recovered, and of this, 49% was found in NADPH and 48% was found in 8-hydroxy-5-deazaflavin, showing the reaction was S-specific with respect to the nicotinamide nucleotide. The ^3H transfer occurred only between (4S)-[4-^3H] NADPH and 8-hydroxy-5-deazaflavin, producing reduced 8-hydroxy-5-deazaflavin in the form of [5-^3H] 8-hydroxy-5-deazaflavin. Therefore, the enzyme used the same face of the 8-hydroxy-5-deazariboflavin ring system for stereospecific transfer of H^+ as the hydrogenase of *Mc. vannielii*, (Yamazaki et. al., 1985).

Yamazaki et. al. (1982) studied the reactivity of different substrates on the $NADP^+$:5-deazaflavin oxidoreductase in *Mc. vannielii*. The basic heterocyclic structure of the co-factor 2,4-dioxopyrimido [4,5b] quinoline was the minimal required since neither riboflavin nor 1,5-dideazariboflavin were reduced. The importance of C_5 as the electron entry site was shown when 5-methyl-deazariboflavin was not reduced, and also inhibited 5-deazariboflavin reduction. Also, the replacement of CH at position 5 by N as in riboflavin or N-1 by CH as in 1,5-dideazariboflavin renders these analogues inactive.
An F_{420}-dependent $NADP^+$ reductase has also been found in *Mb. thermoautotrophicum*, as reported by Eirich and Dugger (1984).

Purification in *Mb. thermoautotrophicum* began with anaerobic cell lysis in a French press. The CFE was applied to DEAE Cellulose, Blue Sepharose CL-6B, Sephadex G-200, and Red Sepharose CL-6B columns (Table I.1.) (Eirich and Dugger, 1984).

The enzyme was enriched over 3000 times with a recovery of 65% and calculated to be 0.03% of the soluble protein of the cell with an optimal pH of 8.0. The enzyme was stable for 10 days at 4°C when stored in 2-mercaptoethanol (Eirich and Dugger, 1984).

Upon comparison Eirich and Dugger (1984) found that there were significant differences between the oxidoreductases of *Mb. thermoautotrophicum* and *Mc. vannielii*. Firstly, there is approximately 3 times less $NADP^+$ oxidoreductase per milligram total soluble protein in *Mb. thermoautotrophicum* than in *Mc. vannielii*. Other differences involved optimum temperature, molecular weight, and K_m's (Table I.1.).

Eirich and Dugger (1984) site two possible reasons for these differences; first it's a result of phylogenetic differences between the two organisms. *Methanobacterium* is in the order Methanobacteriales, and family Methanobacteriales, while *Methanococcus* is in the order Methanococcales and family Methanococcaceae. Therefore, these two organisms have quite different 16S rRNA cataloging, and cell wall composition. Secondly, *Methanobacterium* is a thermophile, and *Methanococcus* is a mesophile, therefore *Methanobacterium* may have a more heat stable tetrameric structure. Of course, a third possibility is that the differences are a result of both of the above reasons. However, non-methanogenic archaebacteria also contain the enzyme.

Archeoglobus fulgidus is hyperthermophilic, sulfate-reducing archeon belonging to the kingdom Euryarcheota (Woese et. al., 1990, 1991). The organism has biochemical characteristics similar to the methanogens including F_{420}, MF, and H_4MPT. None of the metabolic reactions involving lactate to 3 CO_2 involve NAD^+ or $NADP^+$, but NAD^+ and $NADP^+$ are required for biosynthesis. An $NADP^+$:F_{420} oxidoreductase has been found, (Kunow et. al., 1993) (Table I.1.).

Purification of the enzyme was achieved in the rather typical manner. Cell lysis with a French press, followed by centrifugation at 120 000 x g for one hour, and Blue-Sepharose CL-6B, Mono Q HR 10/10, and 2',5'-ADP Agarose chromatography. The enzyme was

found to be oxygen-insensitive, with a 3600-fold enrichment, with 60% recovery. This represented approximately 0.03% of total cell protein. FMN, FAD, NAD^+, and Methyl Viologen (MV) could not be used. The optimal pH for $NADP^+$ reduction was 8.0, and 5.5 for NADPH oxidation. Activity was stimulated 3-fold with the addition of 2 M $(NH_4)_2SO_4$, and 1 M K_2HPO_4, Na_2HPO_4, or K_2SO_4 could substitute for this effect. The electron transfer was determined to be a Si-face specific hydride transfer from the C_5 of F_{420} to C_4 of $NADP^+$. Therefore, this is the same reaction as seen in *Mc. vannielii*, (Yamazaki et. al., 1980, 1982), and *Mb. thermoautotrophicum*, (Jacobsen and Walsh, 1984).

Streptomyces griseus also possesses an $NADP^+$:8-hydroxy-5-deazaflavin oxidoreductase (Table I.1.). Here the purification procedure consisted of cell lysis with sonication, and centrifugation. The enzyme was purified using Sepherosil, Red-Sepharose, Sepharose CL-6B, and QAE-Sephadex A-50 chromatography. SDS-PAGE showed a greater than 90% purification of the enzyme, with an enrichment of 590-fold, with a 72% overall recovery. The enzyme appeared to be very specific for $NADP^+$ and 8-hydroxy-5-deazaflavin, and did not use NAD^+ or NADH (Eker et. al., 1989).

Maximal activity was found at 0.32 M NaCl. There was a significant loss of activity at low ionic strengths; the 1/2 life was less than 9 hours at 0.08 M NaCl, and greater than 6000 hours at 1 M NaCl. Ethylene glycol was found to enhance stability by approximately 83-fold, while detergents inhibited activity. With respect to the 8-hydroxy-5-deazaflavin, the N-side chain had a limited influence on reactivity to the enzyme, but decreasing the size of the side chain decreased the reactivity. A side chain of N=10 was found to be maximal for reactivity. The *in vivo* direction for the enzyme was determined

to be for the reduction of the 8-hydroxy-5-deazaflavin, (Eker et. al., 1989).

I.4. Background on *Msph. stadtmanae*

I.4.1. Whole Cells

A methanogen was isolated from human feces (Miller and Wolin, 1983), and later identified as *Msph. stadtmanae* (Miller and Wolin, 1985). *Msph. stadtmanae* (DSM 3091) is a 1.0 μm, non-motile, Gram positive spherical obligate methanol-utilizing methanogen. The organism has a pH optimum of 6.8 to 7.0, with an optimum temperature range of 36°C to 40°C. For methanogenesis, *Msph. stadtmanae* requires methanol and hydrogen, but not CO_2, (Miller and Wolin, 1985) and utilizes the oxidation of hydrogen via an F_{420}-independent hydrogenase to reduce methanol to methane (Wong et. al., 1994). Other methanogens are capable of reducing methanol to methane, but can do so in the absence of hydrogen, (Balch et. al., 1979), whereas *Msph. stadtmanae* absolutely requires H_2 to survive (Miller and Wolin, 1985).

It requires acetate, CO_2, isoleucine, ammonium, and thiamine for growth, and biotin has been shown to be stimulatory. No cytochromes have been detected, and despite its non-conventional metabolism, has been placed in the family Methanobacteriacae partly on the basis of its serine containing pseudomurein cell wall structure (Miller and Wolin, 1985).

There are certain novelties about *Msph. stadtmanae* that makes the organism a worthwhile candidate for study. The first is based on the work of Deppenmeier et. al. (1990a), where the following speculations were made. Methanogens growing at the expense of CO_2 rely on the H_2-dependent system, and those growing on methanol or

methylamines use the $F_{420}H_2$-coupled system for the reduction of the CoM-S-S-HTP heterodisulfide. The $F_{420}H_2$ is generated by the methyl group oxidation of CH_3OH to CO_2 and is then available for the terminal electron step of methanogenesis. As well, the analogy was made that methanogens which oxidize methyl groups contain cytochromes, and are involved in $F_{420}H_2$:heterodisulfide oxidoreductase activities, (Deppenmeier et. al., 1990b) whereas organisms such as *Mc. thermolithotrophicus* do not have cytochromes, and use the H_2:heterodisulfide oxidoreductase. The $F_{420}H_2$:heterodisulfide oxidoreductase complex was later resolved into a three component electron transport system including F_{420} dehydrogenase (Deppenmeier et. al., 1996).

The interesting development with respect to this analogy is that *Msph. stadtmanae* has no cytochromes, yet it does reduce the methyl group of methanol to methane (Miller and Wolin, 1985).

As stated previously, in most methanogens the electrons from the oxidation of hydrogen are normally transferred to the obligate 2 electron carrying cofactor F_{420} via an F_{420}-dependent hydrogenase. Since the enzyme is absent in this organism, and F_{420} is found in small amounts, an alternate electron transfer route has been proposed by Wong et. al. (1994).

The proposed scheme functions in the following manner. It is via an F_{420}-independent hydrogenase that electrons from hydrogen oxidation are proposed to flow to the CoM-S-S-HTP heterodisulfide. To provide electrons for biosynthesis, the heterodisulfide is reduced to CoM-SH and HS-HTP by H_2, and the electrons then transferred to F_{420} via the F_{420}-dehydrogenase. The reduced F_{420} binds to the $NADP^+$: F_{420} oxidoreductase and transfers electrons for the reduction of $NADP^+$. It is expected both because of the usage of

$NADP^+$ and the results of the present work discussed below that the $NADP^+$:F_{420} oxidoreductase enzyme is utilized in biosynthesis.

A further consideration in *Msph. stadtmanae* is that although F_{420} is present, the levels found are considerably lower than that found in other methanogens (Table I.2.). Using fluorescence measurements, the levels of F_{420} in *Msph. stadtmanae* were approximately 3% of that found in *Mb. thermoautotrophicum* strain Marburg, (Wong et. al., 1994). Due to these low levels of F_{420} found in *Msph. stadtmanae*, and the absence of an F_{420}-dependent hydrogenase, the question of the true role(s) of F_{420} in *Msph. stadtmanae* was posed.

Also, although trace amounts of the enzymes 5,10-methylenetetrahydromethanopterin dehydrogenase and CO_2 reductase, which are responsible for the C_1 methanogenic conversion steps involving H_4MPT and MF have been found, there is no evidence to place the C_1 carrier MF itself in *Msph. stadtmanae* (Schwöner and Thauer, 1991; van der Wijngaard et. al., 1991; Choquet et. al., 1994; Lin and Sparling, 1995). The low activity of these enzymes is in agreement with a role of only purine and amino acid metabolism. Therefore, it is plausible to presume that the inability of *Msph. stadtmanae* to reduce CO_2 to CH_4 is due to the absence, or the presence of an inactive CO_2 reductase system. Also, CH_3OH reduction to CH_4 is via a similar route as seen in *Ms. barkeri* (van der Wijngaard et. al., 1991).

Since *Msph. stadtmanae* can only reduce CH_3OH, and H_2 is obligatory, all the energy conserved by the cell must come from the last reductive step of methanogenesis, (Sparling et. al., 1993a). Sparling et. al., (1993a) tested the effects of TCS, DCCD, and Na^+ in methanogens. The rate of methanogenesis was not significantly affected by the

Table I.2.: Variation in F_{420} Concentrations among Methanogens

Organism	5-Deazaflavin (nmol/g dry wght)
Methanobacterium thermoautotrophicum ΔH	3,800[a,c]
Methanobacterium thermoautotrophicum Marburg	2,950[b]
Methanospirillum hungatei	3700[c]
Methanobacterium formicicum	2400[a]
Methanococcus thermolithotrophicus	13,550[b]
Methanosarcina barkeri	190[a,c]
Methanosphaera stadtmanae	80[b]
Halobacterium volcanii	120[a]
Thermoplasma strain 122-1B3	5[a,c]
Sulfolobus sofataricus	1.1[a,c]
Streptomyces aureofaciens	20[z]
Streptomyces griseus	21[a]
Mycobacterium avium	50[a]

[a] - Lin and White (1986).
[b] -Wong et al., (1994).
[c] -DiMarco et al., (1990).

addition of Na^+ (0.4mM to 37mM) or K^+ (0.4mM to 20mM). TCS dramatically reduced the ATP content, and decreased the membrane potential. TCS increased the rate of methanogenesis, while DCCD decreased methanogenesis. TCS could partially reverse the effects of DCCD (Sparling et. al., 1993a), and similar effects were seen in the presence of H_2 + CH_3OH in *Ms. barkeri*, (Blaut and Gottschalk, 1984). Methanogenesis was stimulated by TCS or gramicidin, indicating a capacity to uncouple methane formation from the maintenance of the membrane potential. Therefore, there is a coupling of CH_3OH reduction with ATP synthesis via a proton motive force, (Sparling et. al., 1993a).

Methanosphaera, a member of the Methanobacteriales, is the only genus outside of the Methanomicrobiales that can reduce methanol to methane, (Biavati et. al., 1988, Miller et. al., 1985). It is uniquely suitable for the study of 1-carbon biosynthetic intermediates because the energy substrate, methanol, is not significantly involved in biosynthesis and all of the enriched carbon from sources such as ^{13}C-acetate and $^{13}CO_2$ are incorporated into cell carbon. Approximately 95% of cell carbon comes from CO_2 and acetate (Miller et. al., 1995, Choquet et. al., 1994).

In *Msph. stadtmanae*, there is no competition between the biosynthetic and catabolic uses of CO_2 and acetate, as opposed to other methanogens that produce CH_4 from CO_2 and/or acetate, (Miller et. al., 1995).

Msph. stadtmanae does not require formate and when omitted, the C_2 of purines comes mainly from [^{13}C]acetate, and the C_4 and C_6 of purines from CO_2. The C_2 of pyrimidines comes from CO_2, and C_4, C_5, C_6 from C_4, C_3, and C_2 of aspartate, respectively. The C_2 of the purine ring comes from formate, and from the C_2 of acetate when formate is absent. The carbon carrier is likely to be H_4MPT because the C_2 also

comes from CH_3OH in the absence of formate. Because *Msph. stadtmanae* produces methenyl-H_4MPT and formyl-H_4MPT, (Schwöner and Thauer, 1991, van der Wijngaard et. al., 1991), this supports a biosynthetic role for H_4MPT in purine biosynthesis, (Choquet et. al., 1994).

Considerable work has been done on the amino acid biosynthesis in *Msph. stadtmanae* using ^{13}C labeling (Miller et. al., 1995; Choquet et al., 1994). With the exception of leucine and isoleucine, all the amino acids were incorporated with ^{13}C, (Miller et. al., 1995). Leucine and isoleucine could not be labeled, due to a Na^+-mediated active transport system for leucine (Sparling et al., 1993b).

Neither *Msph. stadtmanae* nor *Mc. voltae* are able to synthesize acetyl-CoA from 2 CO_2, (Choquet et. al., 1994). Pyruvate is formed by reductive carboxylation of acetate and pyruvic acid, and further metabolized to alanine, serine, and cysteine (Miller et. al., 1995). The methyl groups of acetate were equally distributed among the β- and γ- carbons of glutamate, arginine, and proline (Miller et. al., 1995). Therefore, α-ketoglutarate is produced from oxaloacetate via succinate by the incomplete oxidative Krebs cycle, (Miller et. al., 1995). This is the first demonstration in a methanogen for methyl group synthesis from methionine from the methyl group of acetate; therefore the β-carbon of serine is the precursor of the methyl group, (Miller et. al., 1995).

I.4.2. The $NADP^+$:F_{420} Oxidoreductase

Preliminary work has been done on the $NADP^+$:F_{420} oxidoreductase in *Msph. stadtmanae* in our lab by undergraduates (K. Terrick, D. Juck). Therefore, a short summary of this work seems appropriate before proceeding with the present work.

Total activity in the CFE increased linearly upwards of 5-fold when incubated for up to 5 days. The optimum pH for oxidation of NADPH was 6.0. The K_m for NADPH (the reduction of F_{420}) in the CFE was 0.238 mM NADPH, with a V_{max} was 0.02 μmol F_{420}/min/mg protein, (D. Juck, unpublished).

The optimum pH for the reduction of $NADP^+$ was 8.5, with a K_m for $NADP^+$ of 0.034 mM, and a V_{max} of 0.067 μmol F_{420}/min/mg protein. Although F_{420}, $NADP^+$, and NADPH showed first-order kinetics, the reduction of $F_{420}H_2$ appeared to be non-first order (K. Terrick, unpublished).

Due to a 20-fold difference in K_m values (F_{420} 0.0124 mM, and NADPH 0.238 mM), the cellular levels of NADPH may be much higher than F_{420}. This would indicate that NADPH is required far more in biosynthetic reactions than F_{420}, and that another role of F_{420} may be as a cofactor for reactions involving the maintenance of required cellular levels of NADPH. NAD^+ and NADH cannot replace $NADP^+$/NADPH in this reaction. The enzyme in the CFE is air stable over time, but does show temperature sensitivity at 60°C (i.e. activity is lost), but is relatively stable at 40°C and 23°C over 50 hours, with an activity optimum of 37-42°C, (D. Juck, unpublished).

It has been previously found that there is an approximate 10-fold increase in total activity and a concomitant 30-fold increase in specific activity in the $NADP^+$:F_{420} oxidoreductase of *Msph. stadtmanae* when the CFE is exposed to a 65-90% saturation treatment of $(NH_4)_2SO_4$. Along with this increase, there was a loss of stability in the enzyme. The enzyme in the CFE was still active and relatively stable. With the 65-90% $(NH_4)_2SO_4$ pellet, there was complete loss of activity within 36 hours. Rudimentary purification steps were previously attempted using the 65-90% $(NH_4)_2SO_4$ pellet and

DEAE Cellulose ion exchange in a positive KCl gradient. Elution occurred at approximately 0.5 M KCl, but the enzyme denatured shortly thereafter, (K. Terrick, unpublished).

I.5. Objectives

The initial project proposal, based on knowledge of the enzyme as of July, 1994 was to purify the $NADP^+$:F_{420} oxidoreductase and biochemically characterize the enzyme to yield its function(s) in the metabolism of *Msph. stadtmanae*. An important point to note is that prior to the present work, a peak activity for the enzyme during exponential growth was not detected. This was not through fault of the previous workers, but simply due to the fact that the short time span of the peak activity (4 hours) was never detected. Indeed, if the peak activity were to be over-looked, previous and present results would be highly similar.

However, due to both the literature to date and the findings of the undergraduate students, it was uncertain whether the $NADP^+$:F_{420} oxidoreductase of *Msph. stadtmanae* would be similar to the enzymes found in *Mc. vannielii* and *Mb. thermoautotrophicum*. In neither case was there a reported increase in total or specific activity as seen in *Msph. stadtmanae* when exposed to a 65-90% saturation treatment of $(NH_4)_2SO_4$. As well, *Msph. stadtmanae* requires methanol as an obligate energy source, whereas *Mc. vannielii* and *Mb. thermoautotrophicum* do not. Considering the biosynthetic significance of the $NADP^+$:F_{420} oxidoreductase, this difference in energy sources may cause the organism to use an enzyme with completely different properties. The methanogenic pathway is essentially reversed and therefore so is the intracellular concentration of $F_{420}H_2$ with

respect to H_4MPT.

This prompted a more in depth set of objectives for the project. First was to confirm the unusual findings of the undergraduate students. These unusual findings included the increase in total activity upon incubation of the enzyme at 4°C, and the concomitant increases in total and specific activities with $(NH_4)_2SO_4$ treatment. As well as the fact that while F_{420}, $NADP^+$, and NADPH gave first-order K_m's, $F_{420}H_2$ did not.

Experiments were designed to repeat the earlier findings of the undergraduates, and also try to explain the increase in total activity seen with incubation of the CFE, the increase in total and specific activity in the 65-90% $(NH_4)_2SO_4$ pellet, and the loss of stability in the enzyme after the 65-90% $(NH_4)_2SO_4$ treatment. There was a possibility that these findings were related to the loss of some non-covalently bound unit on the enzyme which disassociated with incubation and was cleaved with the 65-90% $(NH_4)_2SO_4$ treatment, and experiments were designed to test this possibility. In doing these and other experiments, the hope was to characterize the enzyme in its partially purified state as completely as possible.

Once the characterization of the enzyme was completed, the second objective was to purify the enzyme and compare it with those mentioned above. This would test the thought that a different energy source, and thereby a different intracellular substrate concentration would yield a different type of enzyme.

Finally, due to the unusual kinetics of the pre-purified enzyme as stated above, and the lack of literature on similar enzymes in other methanogens, the enzyme in *Msph. stadtmanae* was compared to that of other methanogens available in our lab. These organisms varied in energy sources and electron donors. This would allow a wider

comparison of the enzyme(s) present within three families of methanogens, as well as a comparison between families. Since *Msph. stadtmanae* can use 2-propanol as an alternate source for $NADP^+$ reduction via the $NADP^+$-dependent secondary alcohol dehydrogenase, it was tested in both the presence and absence of 2-propanol. All organisms would be tested in the CFE, and the supernatant and pellet of the 0-65% and the 65-90% $(NH_4)_2SO_4$ treatment. Among the objectives here was to test for the presence of an $NADP^+$:F_{420} oxidoreductase, or the presence of two separate enzymes for $NADP^+$ reduction and NADPH oxidation.

Chapter II

Kinetic Characteristics of the $NADP^+$:F_{420} Oxidoreductase in the CFE and after $(NH_4)_2SO_4$ Precipitation in *Methanosphaera stadtmanae*

II.1. Introduction

Initial kinetic experiments were performed prior to attempting purification of the enzyme due to previous findings of an increase of total and specific activity of 10- and 30-fold, respectively after $(NH_4)_2SO_4$ precipitation (Chapter I.4.2.). It was felt that since the previous work yielded such unusual preliminary characteristics that a more in depth investigation and understanding should be performed before attempting purification of the enzyme. This was to 1.) be able to repeat and thereby validate the earlier findings, and 2.) to determine some characteristics of the enzyme that might aid in purification later.

II.2. Materials

III.2.1. Chemicals and Materials

All chemicals and buffers were of reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO). The culture bottles used (1 L. volume) were purchased from Corning. Assay tubes and stoppers were purchased from Canlab.

II.2.2. Growth Medium and Harvesting of Cultures

II.2.2.1. *Methanosphaera stadtmanae*

Methanosphaera stadtmanae (DSM 3091) was grown in 1 Litre Pyrex bottles at 500 ml volumes using the following formulations (Sparling et. al., 1993b, modified from Miller and Wolin, 1985):

Tryptone	2.0 g/L
Yeast extract	2.0 g/L
K_2HPO_4	2.8 g/L
$(NH_4)_2SO_4$	0.3 g/L
NaCl	0.61 g/L
$MgSO_4$-7 H_2O	0.13 g/L
$CaCl_2$-H_2O	0.08 g/L
NaAcetate	0.5 g/L
$NaSeO_4$ (0.17 g/ ml stock solution)	0.5 ml/L
$NaCO_3$	1.5 g/L
NH_4Cl	1.0 g/L
Vitamin Supplement	15 ml/L
Mineral Elixir	15 ml/L
Resasurin (25mg/100 ml stock solution)	1 ml/L

200 mM N_a2S-$9H_2O$ was added to the medium from a stock solution after the medium was made anaerobic with gassing under H_2/CO_2 (80:20, v/v). Sterile methanol was added after autoclaving from a stock of 40% methanol (v/v) to a final concentration of 0.4% (v/v).

The vitamin supplement consisted of: (Wolin et. al., 1963):

Pyridoxine-HCl	10 mg/L
Riboflavin	5 mg/L
Thiamine	5 mg/L
Nicotinic Acid	5 mg/L
p-Aminobenzoic acid	5 mg/L
Lipoic acid	5 mg/L
Biotin	2 mg/L
Folic acid	2 mg/L
Cyanocobalamin	1 mg/L

The mineral elixir consisted of: (Belay et al., 1984):

Trisodium nitroloacetate	2.02 g/L
$FeCl_3$-6 H_2O	0.21 g/L
$CoCl_2$-6 H_2O	0.20 g/L
$MnCl_2$-4 H_2O	0.10 g/L
$ZnCl_2$	0.10 g/L
$CaCl_2$-2 H_2O	0.05 g/L
$CuSO_4$-2 H_2O	0.05 g/L
$NaMoO_4$-2 H_2O	0.50 g/L
$NiCl_2$-6 H_2O	0.10 g/L

The pH of the medium solution was adjusted to between 6.8 and 7.0 (Acumet 950 pH/ion Meter, Fisher Scientific) before gassing with CO_2, and dispensed in 500 ml volumes. The medium was dispensed into 1 Litre high pressure Pyrex bottles, and sealed

with black butyl rubber stoppers which were wired onto the bottles with stainless steel wire. The gas phase of the bottles was then evacuated and replaced with a 80:20 H_2/CO_2 atmosphere (5 p.s.i.) using cycles of 15 minutes vacuum, 10 minutes gas for three cycles with the last cycle leaving the bottles under positive pressure (Balch et al., 1979; Daniels et al., 1986). The anaerobic medium was reduced using 5 ml per 500 ml of medium of Na_2S-9 H_2O. The bottles were autoclaved, and the sterile methanol was added. Before use, all sterile syringes were rinsed inside with 2 mM sterile anaerobic Na_2S-9 H_2O to scrub out any oxygen while ensuring sterility.

For inoculation, bottles were given a 10% inoculum from a late-log phase culture stored at 4°C. The cultures sat at 37°C for 12 hours, and were then aseptically gassed to 20 p.s.i. with 80 H_2: 20 CO_2 and shaken for 12-24 hours at 37°C.

Methanosphaera stadtmanae was harvested aerobically after being gassed with three cycles of nitrogen. The bottles were opened and the cultures centrifuged for 20 minutes at 27 000 x g. The supernatant was poured off and the pellet was dissolved in a small amount of harvesting buffer (20 mM Tris-Cl, 2mM DTT, pH 7.5). The cells were lysed aerobically using an Aminco French Pressure Cell Press at 36 000 to 40 000 p.s.i. 2 to 3 times. After pressing, the cells were then centrifuged at 27 000 x g for 20 minutes. The CFE was then either used for analysis, or treated with $(NH_4)_2SO_4$ for further analysis.

II.2.2.2. *Methanococcus thermolithotrophicus*

Methanococcus thermolithotrophicus (a gift from L. Daniels) was grown in 500 ml volumes in the 1 Litre Pyrex bottles using the following formulation (Sparling and Daniels, (1986):

KCl	0.34 g/L
NH_4Cl	1.00 g/L
$CaCl_2$-2 H_2O	0.10 g/L
K_2HPO_4	0.25 g/L
NaCl	11.63 g/L
Mineral Elixir	10 ml/L
$NaWO_4$ (0.23g/100 ml stock solution)	1.0 ml/L
Na_2SeO_4 (0.17g/100 ml stock solution)	1.0 ml/L
1,4-piperazinediethanesulfonic acid	11.90 g/L
2-(N-morpholino)-ethanesulfonic acid	9.75 g/L
N-[2-hydroxyethyl]piperazine-N'-2-ethanesulfonic acid	15.12 g/L
Resasurin (25mg/100 ml stock solution)	1.0 ml/L
HCOONa	20.40 g/L.

The pH of the medium was adjusted to between 5.8 and 6.2 with KOH pellets, and 500 ml of the medium solution was added to the Pyrex bottles and sealed with black butyl rubber stoppers and sealed with stainless steel wire. The gas phase of the bottles was evacuated and replaced with a 80:20 N_2/CO_2 atmosphere (5 p.s.i.) using cycles of 15 minutes vacuum, 10 minutes gas for three cycles with the last cycle leaving the bottles under positive pressure (Balch et al., 1979; Daniels et al., 1986). The anaerobic medium was then reduced using 5 ml per 500 ml of medium of Na_2S-9 H_2O. The bottles were autoclaved, and once sterile, 1% $MgCl_2$-6 H_2O (5 ml of a 50.8 g/100 ml sterile anaerobic stock solution) was added by sterile syringe (Belay et al., 1984). Bottles were given a 10% inoculum from the exponential phase cultures stored at 4°C. These were incubated in

a water bath at 60°C for approximately 8 hours with no agitation, venting aseptically every 2-3 hours.

Methanococcus thermolithotrophicus was harvested anaerobically, in the anaerobic glove box and centrifuged at 27 000 x g for 20 minutes. The remaining harvesting was carried out aerobically. The pellets were dissolved in 8-10 ml of 40:60 (v:v) 50mM Tris-HCl/Methanol solution heated to 80°C, and allowed to sit for approximately 30 minutes with frequent vortexing. This resulted in cell lysis, and the extract was then used for harvesting and purification of F_{420} (Daniels et al., 1985).

II.2.3. Purification and Reduction of Factor F_{420}

The heated cell lysate was centrifuged at 27000 x g for 20 minutes to pellet membrane fractions and other cellular debris. The supernatant was loaded onto a 2.9 cm X 11.4 cm DEAE Sephadex-A 25 column and equilibrated with 50 mM Tris pH 8.0 (Daniels et al., 1985). Contaminating species present in the extract were eluted from the column using 50 ml bumps of 0.3M and 0.6M NH_4HCO_3. The distinct yellowish-green band of F_{420} was eluted from the column using 1M NH_4HCO_3 (volume sufficient to elute all visible F_{420}). Fractions were collected in 5 ml fractions and tested spectrophotometrically (Milton Roy Spectronic 3000 Array Spectrophotometer) for purity (Cheeseman et. al., 1972). Further purification was obtained by repeating the column purification and analysis on clean Sephadex-A 25 column (2.2 x 19.8 cm) after boiling off all of the NH_4HCO_3 using a Rotovapor-R (Buchi) at 80°C. Two columns were kept at all times, one for each of the purifications. The resultant F_{420} was concentrated to an absorbance of approximately 0.6 at 400 nm using 0.100 ml of F_{420} in 2 ml of assay buffer (see later). Concentration was

determined by using the absorbance found and calculating equation 2 where l = 1 cm. and e = the molar extinction coefficient = 25.9 mM^{-1} cm^{-1} (Jacobsen et al., 1982).

Equation 2 Absorbance = e c l

The reduction of purified F_{420} was carried out as follows; a N_2 cannula was inserted into a test tube containing 2 ml of F_{420} and a micro stir flea. A pH meter probe was inserted below the F_{420} level. Using a tube connected to a syringe, 0.1 ml of a 1M $NaBH_4$ stock solution gassed under H_2 was added directly to the F_{420}. The pH of the solution was lowered to below pH 1.0 then immediately returned to pH 8.0 using concentrated HCl and concentrated KOH respectively, also added via a tube directly to the solution. The F_{420} is considered reduced when it remained colorless at pH 8.0. The tube was then stoppered, crimp top sealed, and gassed under nitrogen (modified from Deppenmeier et al., 1990).

II.2.4. Determination of Protein Content

Protein concentration was determined using a modified Bradford assay (Bradford, 1976). Standard curves were prepared using bovine serum albumin at 0 mg/ml, 20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml, and 100 mg/ml The samples were diluted to 1:10 and aliquoted. The final assay tubes contained 200 µl of the 1:10 diluted sample and 1.8 ml of filtered Bradford Assay Solution.

II.2.5. $(NH_4)_2SO_4$ Precipitation Protocol

Precipitation of proteins and enrichment of CFE for a particular protein can be achieved using $(NH_4)_2SO_4$. After lysis of cells, the CFE was subjected to a 65% saturation of $(NH_4)_2SO_4$ at approximately 0°C. This was achieved by dissolving the $(NH_4)_2SO_4$ while on ice. Once dissolved, the 65% $(NH_4)_2SO_4$ solution was centrifuged at 47 000 x g for 20 minutes. The 0-65% $(NH_4)_2SO_4$ pellet was discarded, and the 65% $(NH_4)_2SO_4$ supernatant was further saturated to 90% and the centrifugation repeated. The 90% $(NH_4)_2SO_4$ supernatant was discarded and the 65-90% $(NH_4)_2SO_4$ pellet kept as this fraction contained the major amount of activity. The 65-90% $(NH_4)_2SO_4$ pellet was resuspended in 2 ml of 20 mM Tris-Cl, pH 8.1.

II.2.6. Analysis of Enzymatic Activity

The activity of the $NADP^+$:F_{420} oxidoreductase of *Msph. stadtmanae* was measured in freshly pressed cells. The assays were performed in 5 ml Corning Pyrex assay tubes containing 2 ml of assay buffer (NaCitrate, 1.4706 g/100 ml; KH_2PO_4, 0.6805 g/100 ml) (Yamazaki et al., 1980). The pH of the buffer solution was 6.0 for the reduction of F_{420} and 8.5 for the reduction of $NADP^+$. The assay tubes were then sealed with black butyl rubber stoppers and gassed under nitrogen (Daniels and Wessels, 1984). All components of the assay, (CFE, 65-90% $(NH_4)_2SO_4$ pellet, F_{420} (or $F_{420}H_2$), $NADP^+$ (or $NADPH+H^+$) were added to the anaerobic assay tubes using Hamilton microlitre syringes. The final ingredient added was always $NADP^+$ (or $NADPH+H^+$). The $NADP^+$ or NADPH were added from a 6 mM stock solution, giving a final concentration of 0.27 mM. The F_{420} and $F_{420}H_2$ were concentrated to an absorbance of 0.6 at 400 nm. This corresponded to an

initial concentration of 0.495 mM and a final concentration of 0.023 mM. After addition, the tubes was gently mixed and cleaned with a Kimwipe, and the increase or decrease in absorbance at 400 nm was measured. The change at 400 nm was measured spectrophotometrically (NovaspecII 4040, LKB Biochrom) and charted (Fischer Recordall series 5000 chart recorder). Absorption changes were measured at 400 nm rather than at 420 nm to eliminate any possible distortions due to temperature or pH changes in the F_{420} molecule (Jacobsen et al., 1982).

The change in absorption at 400 nm was determined by drawing a line with a pencil and ruler to extend the line recorded by the chart recorder. The change in absorption was then determined by calculating the slope of the line made by the chart recorder. This made a right angle triangle with the hypotenuse representing the rate of change in absorption, the base representing time, and the vertical component representing the total change in absorption. When this was calculated, it gave the change in absorption in min^{-1} (eqn. 3).

Equation 3

example: chart speed = 5" /minute

length of base of triangle = 10"

vertical component = 0.980 - 0.530 = 0.450

Therefore, 0.450 = absorption change over 2 minutes = 0.450 / (5"/minute / 10")

$= 0.225\ min^{-1}$

II.2.7. Determination of Peak Enzymatic Activity over the Growth Phases

The total and specific activity of each sample was determined to find the peak activity of the $NADP^+$:F_{420} oxidoreductase along the growth curve. Activity was verified in the direction of NADPH oxidation. Samples were taken at various times over 86 hours post-inoculation, given the previous growth curve work showing exponential growth ranged from 20 to 40 hours post-inoculation. The peak activity was seen at 36 hours and corresponded to an optical density of 0.5 at 660 nm. The experiment was then redone with extra time points around the peak activity found. This was done in order to reconfirm the peak activity, and to give an accurate representation of the peak activity curve as growth of the culture progressed.

Finally, each of the fractions of CFE, 0-65% $(NH_4)_2SO_4$ supernatant, 0-65% $(NH_4)_2SO_4$ pellet, 65-90% $(NH_4)_2SO_4$ supernatant, 65-90% $(NH_4)_2SO_4$ pellet, 90-100% $(NH_4)_2SO_4$ supernatant, and 90-100% $(NH_4)_2SO_4$ pellet were tested at key points along the growth curve. This was done to confirm that the 65-90% $(NH_4)_2SO_4$ pellet indeed possessed the highest activity throughout the growth period.

II.2.8. Comparison of $(NH_4)_2SO_4$ Treatments

Because of the increase in total activity observed in the 65-90% $(NH_4)_2SO_4$ pellet versus the CFE, a comparison was made between the two-step method of obtaining the 65-90% $(NH_4)_2SO_4$ pellet from the CFE (described above), and a one-step process to obtain a 0-100% $(NH_4)_2SO_4$ pellet, and 0-100% $(NH_4)_2SO_4$ supernatant to determine whether a protein component had separated from the enzyme causing a shift in activity. These treatments were done at different points along the growth curve, with A_{660}

absorbances of 0.27, 0.33, 0.45, 0.54, 0.77. This was done to check that: 1) the highest activity was indeed in the 65-90% $(NH_4)_2SO_4$ pellet, 2) the total and specific activity in the 65-90% $(NH_4)_2SO_4$ pellet was at least comparable to the 0-100% $(NH_4)_2SO_4$ pellet, and 3) to give an indication of whether a possibly non-covalently bound cofactor or protein subunit was being lost in the $(NH_4)_2SO_4$ treatment.

II.2.9. Determinations of K_m and V_{max} in the CFE and 90% $(NH_4)_2SO_4$ pellet

The K_m and V_{max} for both the CFE and the 65-90% $(NH_4)_2SO_4$ pellet were measured. This was done to compare and contrast the properties of the enzyme between the initial and the purified enzyme. The assays were carried out anaerobically as in all assays and concentrations used were 0.027-0.27 mM (10-100 µl) $NADP^+$ or $NADPH+H^+$, 0.0023-0.023 mM (10-100 µl) F_{420} or $F_{420}H_2$, and 50 µl of sample (either CFE or 65-90% $(NH_4)_2SO_4$ pellet).

II.2.10. Protease Inhibitors Studies

Proteinase inhibitor studies were also performed in the CFE and the 65-90% $(NH_4)_2SO_4$ pellet to determine if the degradation of a subunit caused the observed increase in total activity over storage at 4°C. The proteinase inhibitors used were 1 mM PMSF, 5 mM EDTA, and a combination of both against a control; EDTA is a metal chelator (Harris and Angal, 1994), and PMSF is a serine protease inhibitor (Harris and Angal, 1994). This was done for both the total and specific activity, and performed at 0, 48, 72, and 96 hours post-incubation. As with all assays performed here, the assay was

conducted in anaerobic tubes, with the mixture consisting of 2.0 ml of assay buffer pH 6.0, 0.023 mM F_{420}, 0.27 mM NADPH+H^+, and 50µl of sample.

II.2.11. NH_4^+ or High Salt Activation in the 65-90% $(NH_4)_2SO_4$ pellet

In order to eliminate the possibility that the enzyme was being activated by either NH_4^+, or the high concentration of salt used to achieve the 65-90% $(NH_4)_2SO_4$ pellet, 500 mM NH_4Cl and 500mM KCl were added to the CFE. The NH_4Cl was used to check for NH_4^+ activation, and the KCl for high salt activation at 500mM since it was calculated that the concentration of $(NH_4)_2SO_4$ left in the pellet after centrifugation and removal of the supernatant was ~400 mM. The CFE was suspended in 20mM Tris-HCl as the buffer. After cell lysis and centrifugation, aliquots of 5ml were made, with the appropriate agent included. These were assayed for activity and protein content in the usual manner.

II.2.12. Testing for a Regulatory Co-factor or Co-enzyme

After determining that neither high salt nor NH_4^+ was activating the 65-90% $(NH_4)_2SO_4$ pellet, it was decided to test whether a co-factor being lost resulted in the activation of the enzyme. If the activity (both total and specific) in the 65-90% $(NH_4)_2SO_4$ pellet did decrease with the addition of aerobic boiled CFE then the result could be due to a non-proteinaceous co-factor re-associating with the enzyme since all proteins should be denatured in the boiled CFE. If the activity in the 65-90% $(NH_4)_2SO_4$ pellet did not decrease from addition of the boiled CFE, but did decrease with the addition of CFE, then it would be assumed that a regulator may be a protein that has been denatured in the boiled CFE.

Four Liters of cells were grown and harvested as above; i.e. aerobically. They were placed in a serum bottle and capped with a 22 gauge needle inserted in the cap to allow pressure to escape from the heating. The serum bottle was placed in a water bath and heated to boiling for 4 hours. The bottle was then allowed to cool overnight at 4°C. The assay was carried out as specified above using 2.0 ml assay buffer pH 6.0, 0.023 mM F_{420}, 0.27 mM NADPH, and 50 µl sample. The experiment was designed so that controls were first performed using CFE alone, boiled CFE alone, and 65-90% $(NH_4)_2SO_4$ pellet alone. Then 50 µl of 65-90% $(NH_4)_2SO_4$ pellet was added with 50 µl of CFE, and 50 µl 65-90% $(NH_4)_2SO_4$ pellet with 50 µl boiled CFE.

II.2.13. Stabilization in the 65-90% $(NH_4)_2SO_4$ pellet

Attempts to prevent loss of $NADP^+$:F_{420} oxidoreductase activity in the 65-90% $(NH_4)_2SO_4$ pellet were made with a variety of widely used stabilizing agents. The first of these attempts was using solutions containing 20% glycerol, 20% ethylene glycol, and 50 mM sucrose. As well, as the reducing agents 1 mM Dithiothreitol (DTT), 1 mM 2-Mercaptoethanol were tested to check if oxidation of the protein was the reason for a loss of activity in the 65-90% $(NH_4)_2SO_4$ pellet. Finally a salt solution of 1 M KCl was also tested.

Storage of the 65-90% $(NH_4)_2SO_4$ pellet at temperatures of 4, 23 and 37°C in harvesting buffer (described above) were tested. The temperature experiments were performed because previous work had shown that the CFE showed a greater increase in activity at 23°C than at 4°C upon incubation, and that this activity increased over 5 days, whereas the 65-90% $(NH_4)_2SO_4$ pellet lost all activity with incubation at 4°C by 48 hours,

but maintained a greater degree of its activity at 4°C than at other temperatures. Finally, the harvesting buffer was tried at different pH's, and different buffers were tried to determine if a buffer other than the harvesting buffer could be used to stabilize the enzyme. The harvesting buffer was tried at pH's of 5.6, 6.1, 7.0, 7.5, 8.0, 8.5, and 9.0. Each of the different buffers was used at their respective pKa's at a strength of 20 mM. The buffers tried were MES, pH 6.1; Citrate, 6.4; Bis-Tris Propane, 6.8; MOPS, 7.2, HEPES, 7.5; TEA, 7.8; Tris 8.1; TAPS, 8.55; and Tricine, 8.1, and all pH adjustments to the buffers were done with concentrated HCl or KOH. The Tris-HCl pH 8.1 buffer was then tried at different strengths of 20 mM, 30 mM, and 50 mM. Each of the stabilization experiments was performed over the course of 5 days with the 65-90% $(NH_4)_2SO_4$ pellet incubated at 4°C. In each of the stabilization experiments, the 0-65% $(NH_4)_2SO_4$ supernatant was aliquoted into Eppendorf tubes to a volume of 1.5 ml. These were then ultracentrifuged for 10 minutes. The resulting supernatant was then pipetted off with a Pasteur pipette, and the pellet resuspended in the appropriate buffer.

II.2.14. Testing for Allostery

The possibility of the $NADP^+$:F_{420} oxidoreductase possessing an allosteric property was considered and examined. Freshly pressed cells were divided into two pools; one consisting of CFE and the other of 65-90% $(NH_4)_2SO_4$ fractionation. The 65-90% $(NH_4)_2SO_4$ pellet was collected and resuspended in 5 ml of harvesting buffer, and the two assays were then conducted. The first consisted of using a constant volume of CFE (50 µl) and a varied volume of the resuspended 65-90% $(NH_4)_2SO_4$ pellet. The resuspended pellet volume ranged from 0 to 50 µl in steps of 10 µl. To keep the total volume constant,

an appropriate volume of anaerobic harvesting buffer was added to the system to maintain a final volume of 2.3 ml. For example, a tube for testing 25 µl of 65-90% $(NH_4)_2SO_4$ pellet contained 2.0 ml of assay buffer, 25 µl of 65-90% $(NH_4)_2SO_4$ pellet, 50 µl of CFE, 100 µl (0.023 mM) F_{420}, 100 µl (0.27 mM) NADPH, and 25 µl of anaerobic harvesting buffer. The second assay consisted of the same procedure, except that the volume of the CFE was varied, and a constant 65-90% $(NH_4)_2SO_4$ pellet volume of 50 µl was used. As a control, both the CFE and the resuspended 65-90% $(NH_4)_2SO_4$ pellet were assayed alone using as system with a total volume of 2.3 ml. Each of the controls used volumes of 0, 10, 25, 40, and 50 µl of sample to derive a straight line.

II.3. Results

II.3.1. Peak Enzymatic Activity Determination

In order to select the growth phase for cell harvesting that would maximize enzyme activity for further purification, the peak activity was determined over the growth curve. Because of the previously observed large increase in activity in the 65-90% $(NH_4)_2SO_4$ pellet, cells were harvested in late log phase and the enzyme activities were checked for both CFE and the 65-90% $(NH_4)_2SO_4$ pellet. Total activity is the activity found in the assay tube with all components added, and corrected for the total volume of the CFE. The same experiments were carried out in both the CFE and the 65-90% $(NH_4)_2SO_4$ pellet in order to observe any differences in location or intensity of peak activity along the growth curve between the two conditions. If the placement of the peak activity was different for the 65-90% $(NH_4)_2SO_4$ pellet from the CFE this could imply either the presence of more

than one enzyme capable of catalysing this reaction, or that the enzymes' properties were being altered by the $(NH_4)_2SO_4$ treatment.

The CFE and 65-90% $(NH_4)_2SO_4$ pellet peaks of total activity were reached at the same time in the growth curve and were similar, but had differently shaped and sized peaks in the total and specific activity. In the CFE, total and specific activity jumped at 16, then decreased and rose again from 24 to 32 hours (Figure II.1.). The peak activity roughly correlated between the total and specific activity at 34 hours for the total activity, and 40 hours for the specific activity. For the total activity, this corresponded with A_{660}= 0.4, and the specific activity at A_{660}= 0.56. In the CFE there is a peak of synthesis of the enzyme early in the exponential phase at 18-30 hours, with little or no synthesis thereafter. This can be concluded since at 30 and 60 hours the total activity is stable while the cell numbers continue to increase. At 80 hours, there is a constant total activity while the drop in specific activity corresponds to the drop in absorbance.

For the 65-90% $(NH_4)_2SO_4$ pellet, the total activity increased from 14 to 78 µmol/min over an 8 hour period (32 to 40 hrs; A_{660} = 0.37 - 0.67), and declined very sharply to 10 µmol/min at 46 hrs; A_{660} = 0.88 (Figure II.2.). In comparison, the specific activity increased from 0.6 to 2.6 µmol/min/mg protein from 32 to 36 hrs (A_{660} = 0.37 - 0.50), then decreased to 0.2 µmol/min/mg protein by 46 hrs (A_{660} = 0.88).

Once the peak activity was determined, it was then possible to observe and compare the enzymes stability from different points of growth over time during incubation. As well, at the peak activity there was maximal enrichment for enzyme purification. It was found that both the total and specific activity peaks in the CFE and 65-90% $(NH_4)_2SO_4$ pellet occurred at approximately A_{660} = 0.50.

Figure II.1.: Identification of the Peak of Total ( ) and Specific (▲) Activity of the $NADP^+$:F_{420} oxidoreductase in CFE during growth in *Methanosphaera stadtmanae*. Growth was measured at 660 nm. (●). Peak enzymatic activity occurred at A_{660}= 0.5, corresponding to approximately 32 hours post-inoculation. Experiment was performed in twice in triplicate, with the average value used.

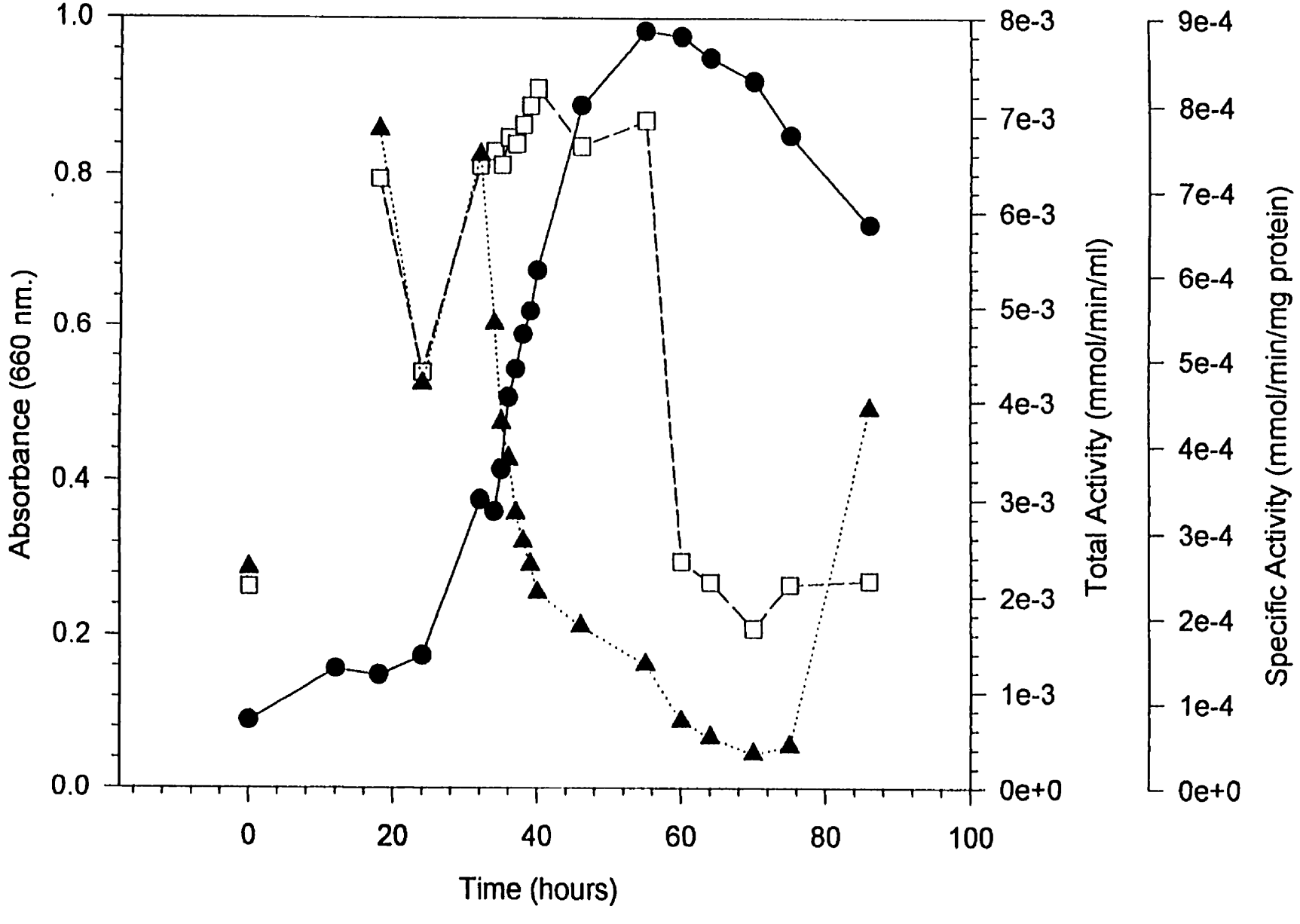
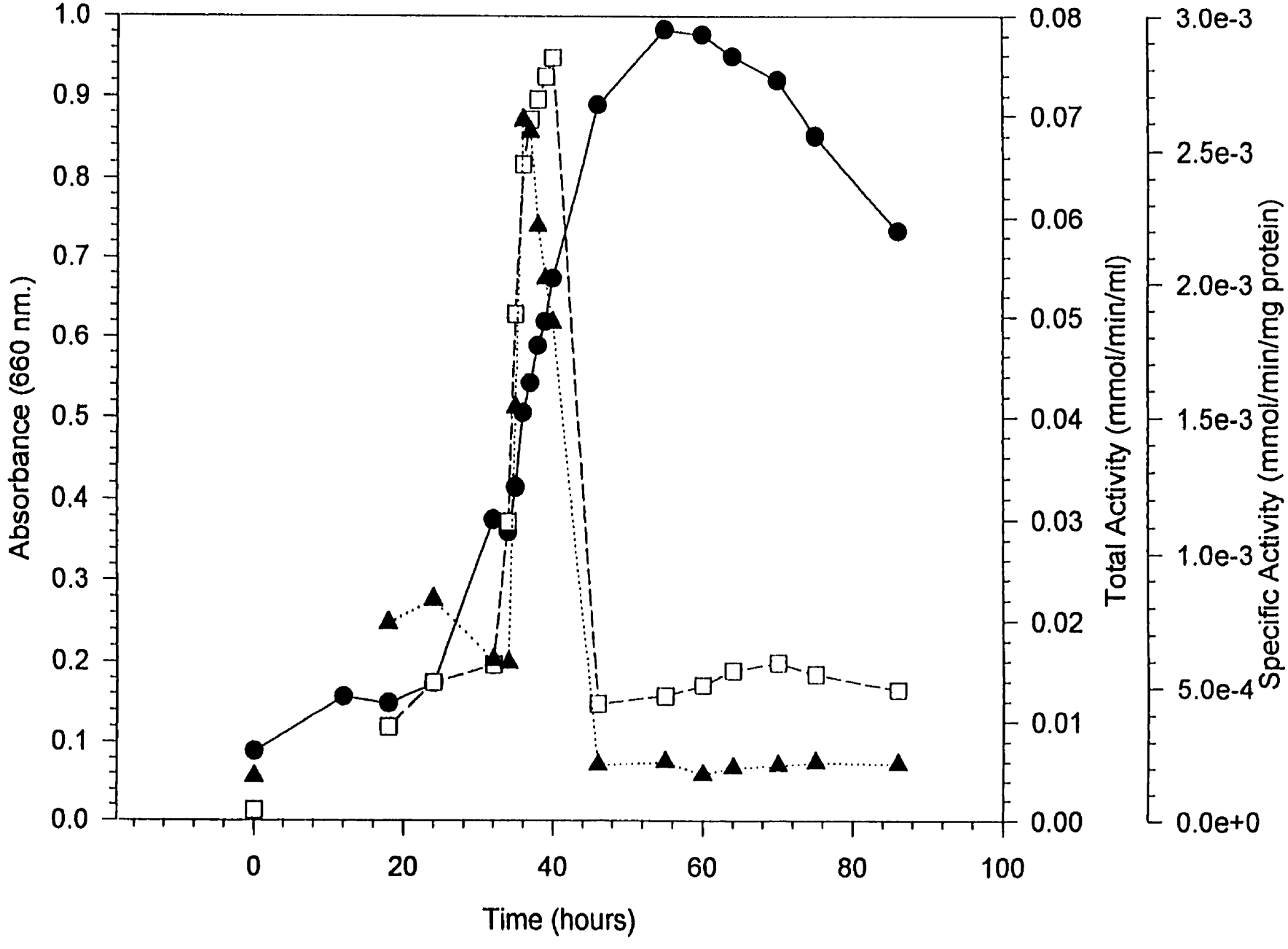

Absorbance (660 nm.)
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Time (hours)
8e-3
7e-3
6e-3
5e-3
4e-3
3e-3
2e-3
1e-3
0e+0
Total Activity (mmol/min/ml)
9e-4
8e-4
7e-4
6e-4
5e-4
4e-4
3e-4
2e-4
1e-4
0e+0
Specific Activity (mmol/min/mg protein)

Figure II.2.: Total ( ) and Specific (▲) Activity of the 65-90% $(NH_4)_2SO_4$ pellet of the $NADP^+$:F_{420} oxidoreductase of *Methanosphaera stadtmanae* during growth. Growth was measured at 660 nm. (●). Peak activity occurred at A660= 0.55, corresponding to approximately 36 hours post-inoculation. Experiment was performed in twice in triplicate, with the average value used.


Absorbance (660 nm.)
Total Activity (mmol/min/ml)
Specific Activity (mmol/min/mg protein)
Time (hours)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
3.0e-3
2.5e-3
2.0e-3
1.5e-3
1.0e-3
5.0e-4
0.0e+0
0
20
40
60
80
100

II.3.2. Determinations of K_m and V_{max} in the CFE and the 65-90% $(NH_4)_2SO_4$ Pellet

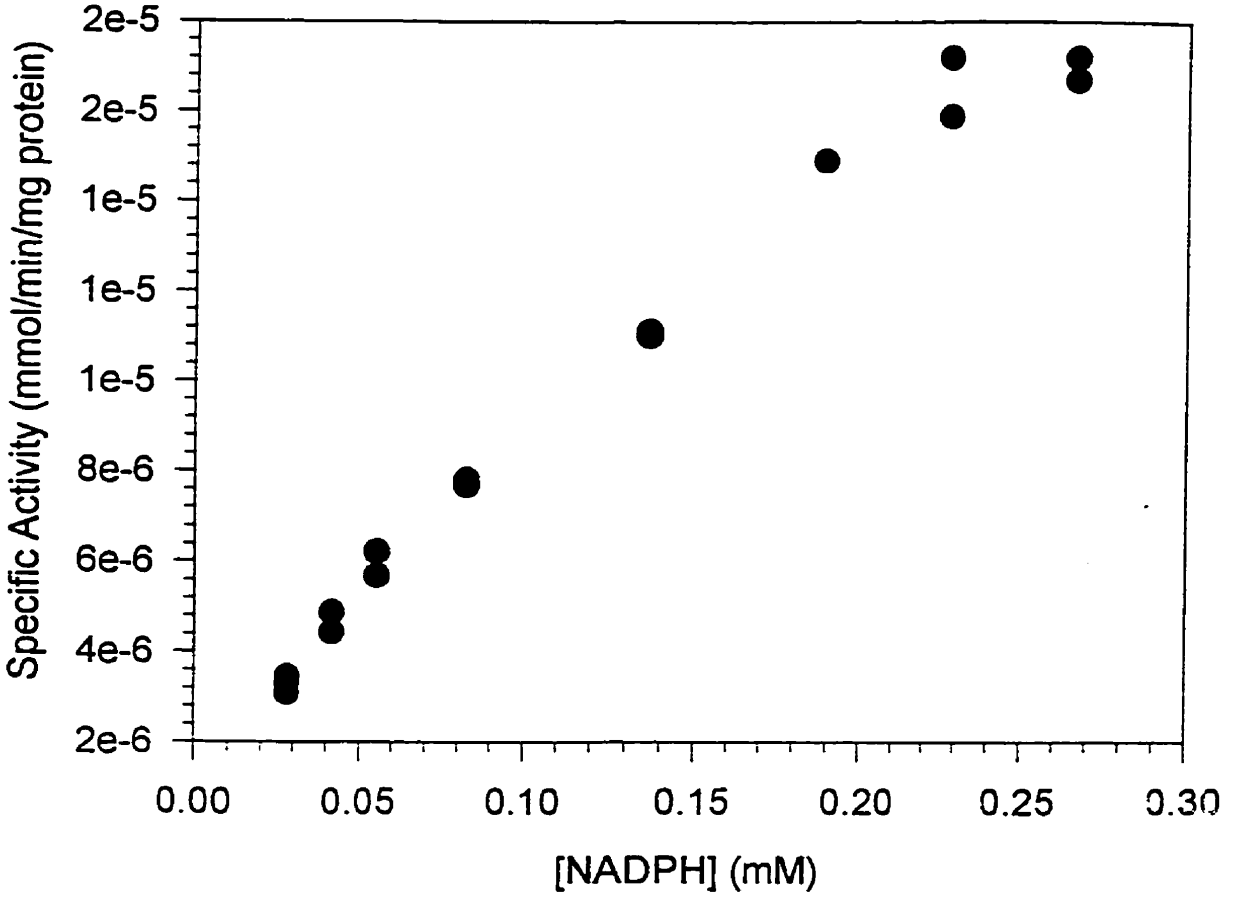
Because of the increase in total activity seen previously (Figures I.1 and I.2.), kinetic studies of the oxidoreductase were performed to determine the K_m in both the CFE and 65-90% $(NH_4)_2SO_4$ to see whether there was a change in the affinity of the enzyme during enrichment. This may indicate the loss of a regulatory factor which may be the reason for the 10-fold increase in total activity after 65-90% $(NH_4)_2SO_4$ treatment.

For NADPH oxidation, the kinetics in the CFE appear as first order looking at regressions from Michealis-Menton, Lineweaver-Burke (Lineweaver and Burke, 1934), and Eadie-Hofstee plots (Hofstee, 1959). The K_m for NADPH were determined from Lineweaver-Burke plots to be 256 μM (Figure II.3.), and by the Eadie-Hofstee plots 286 μM (Figure II.4.). For F_{420} in the CFE, the kinetics also appear to be first order. The Michealis-Menton plot showed a typical hyperbolic curve. A K_m of 16.1 μM was determined using Lineweaver-Burke (Figure II.5.), and a similar K_m of 9.8 μM was found with Eadie-Hofstee (Figure II.6.).

In the 65-90% $(NH_4)_2SO_4$ pellet, NADPH showed consistent non-first order kinetics. The Michealis-Menton plot was a straight line, while the Lineweaver-Burke plot showed a slightly hyperbolic curve (Figure II.7.). The Eadie-Hofstee plot (Figure II.8.) could have been interpreted as a straight line, but the displacement of points made it difficult to decide. If it was taken as a straight line, the K_m would have been 225 μM, which was close to the CFE values.

F_{420} in the 65-90% $(NH_4)_2SO_4$ pellet showed first-order kinetics in both Michealis-Menton and Lineweaver-Burke (Figure II.9.), and Eadie-Hofstee (Figure II.10.) plots. The Michealis-Menton plot showed the hyperbolic curve, while the Lineweaver-Burke gave a

Figure II.3. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for NADPH in the CFE. Lineweaver-Burke Plot indicates a K_m of 256 μM. Experiment was performed in twice in triplicate.


Specific Activity (mmol/min/mg protein)
2e-5
2e-5
1e-5
1e-5
1e-5
8e-6
6e-6
4e-6
2e-6
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADPH] (mM)

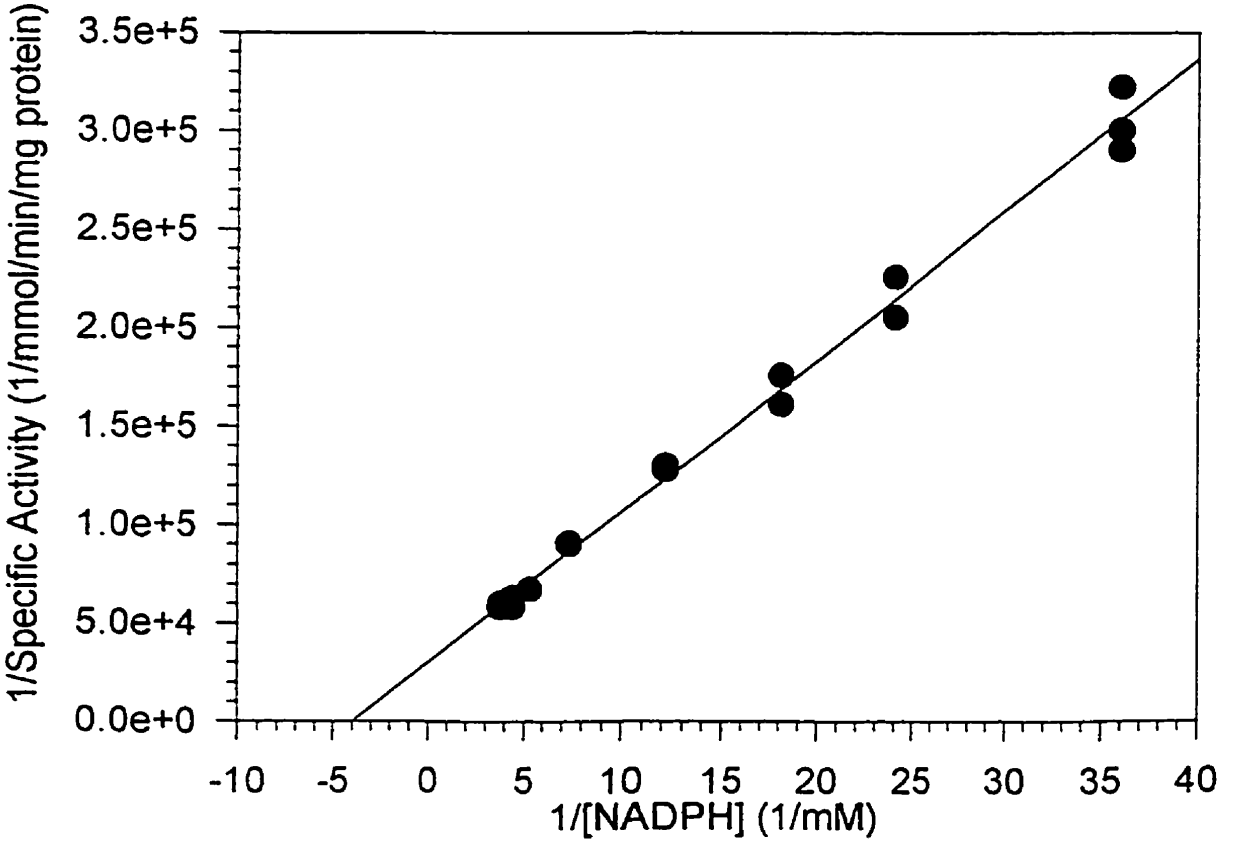

1/Specific Activity (1/mmol/min/mg protein)
3.5e+5
3.0e+5
2.5e+5
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0.0e+0
-10
-5
0
5
10
15
20
25
30
35
40
1/[NADPH] (1/mM)

Figure II.4. Eadie-Hofstee Plot for NADPH in the CFE. The plot indicates a K_m value of 286 μM. Experiment was performed in twice in triplicate.

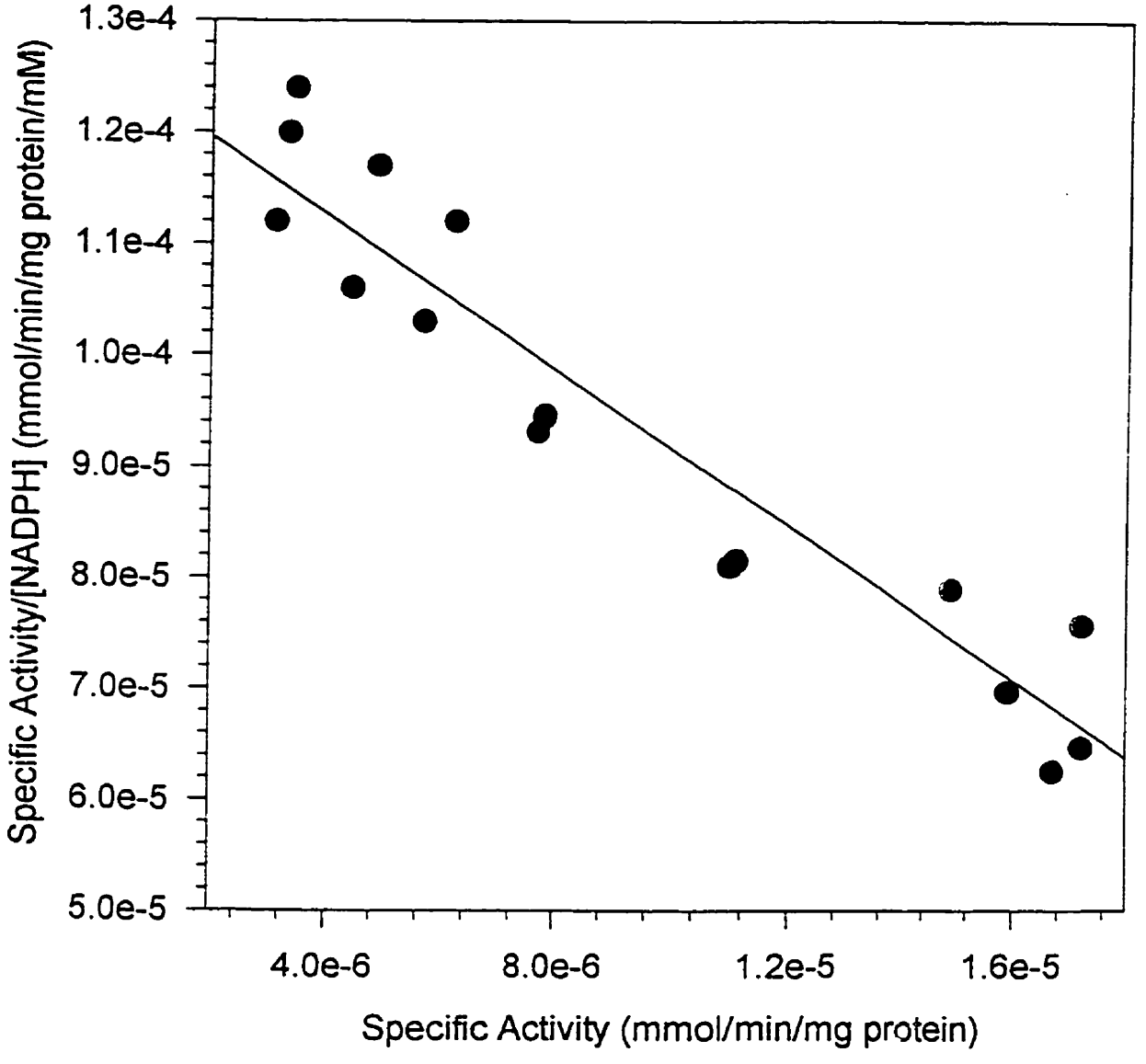
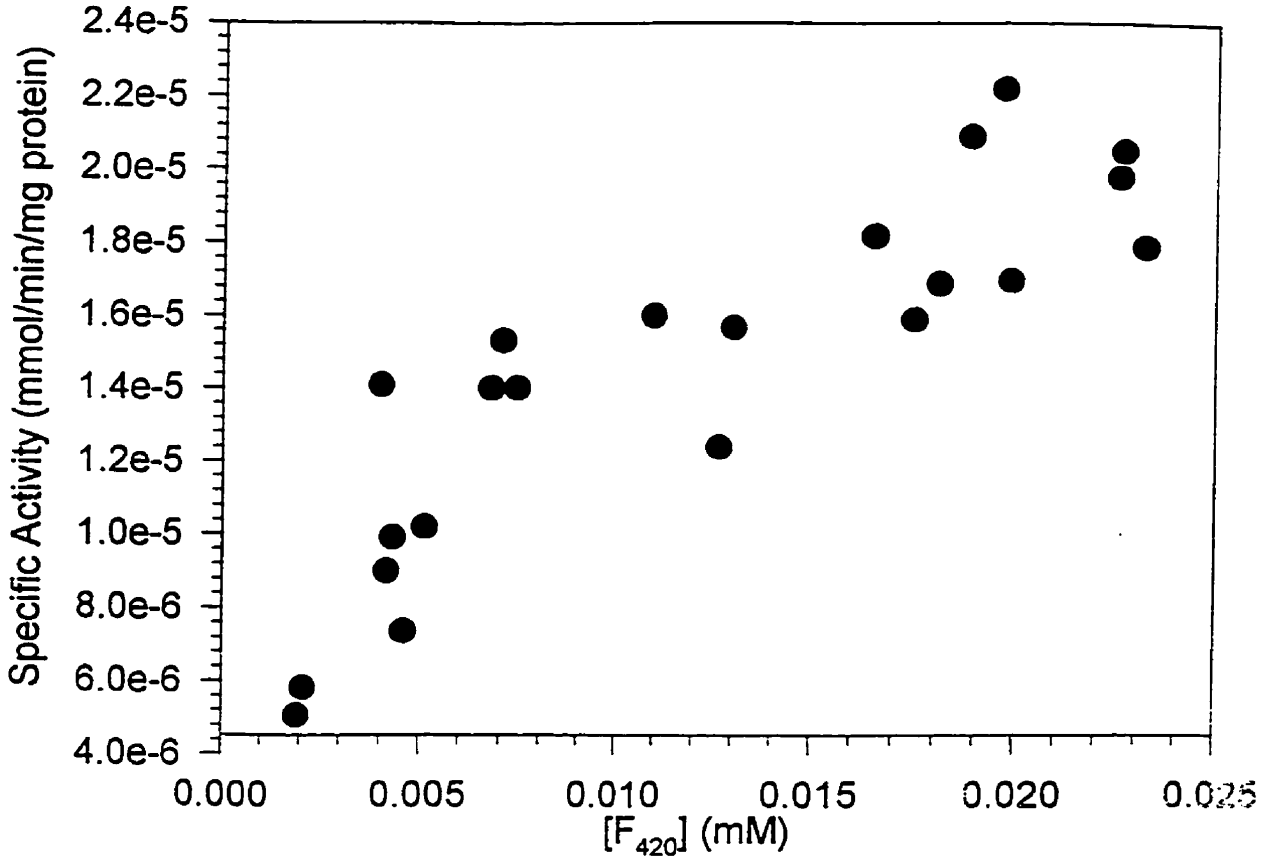

1.3e-4
1.2e-4
1.1e-4
1.0e-4
9.0e-5
8.0e-5
7.0e-5
6.0e-5
5.0e-5
Specific Activity/[NADPH] (mmol/min/mg protein/mM)
4.0e-6
8.0e-6
1.2e-5
1.6e-5
Specific Activity (mmol/min/mg protein)

Figure II.5. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for F_{420} in the CFE. The Lineweaver-Burke Plot shows a Km value of 16.1 μM. The experiment was performed in twice in triplicate.


Specific Activity (mmol/min/mg protein)
2.4e-5
2.2e-5
2.0e-5
1.8e-5
1.6e-5
1.4e-5
1.2e-5
1.0e-5
8.0e-6
6.0e-6
4.0e-6
0.000
0.005
0.010
0.015
0.020
0.025
[F420] (mM)

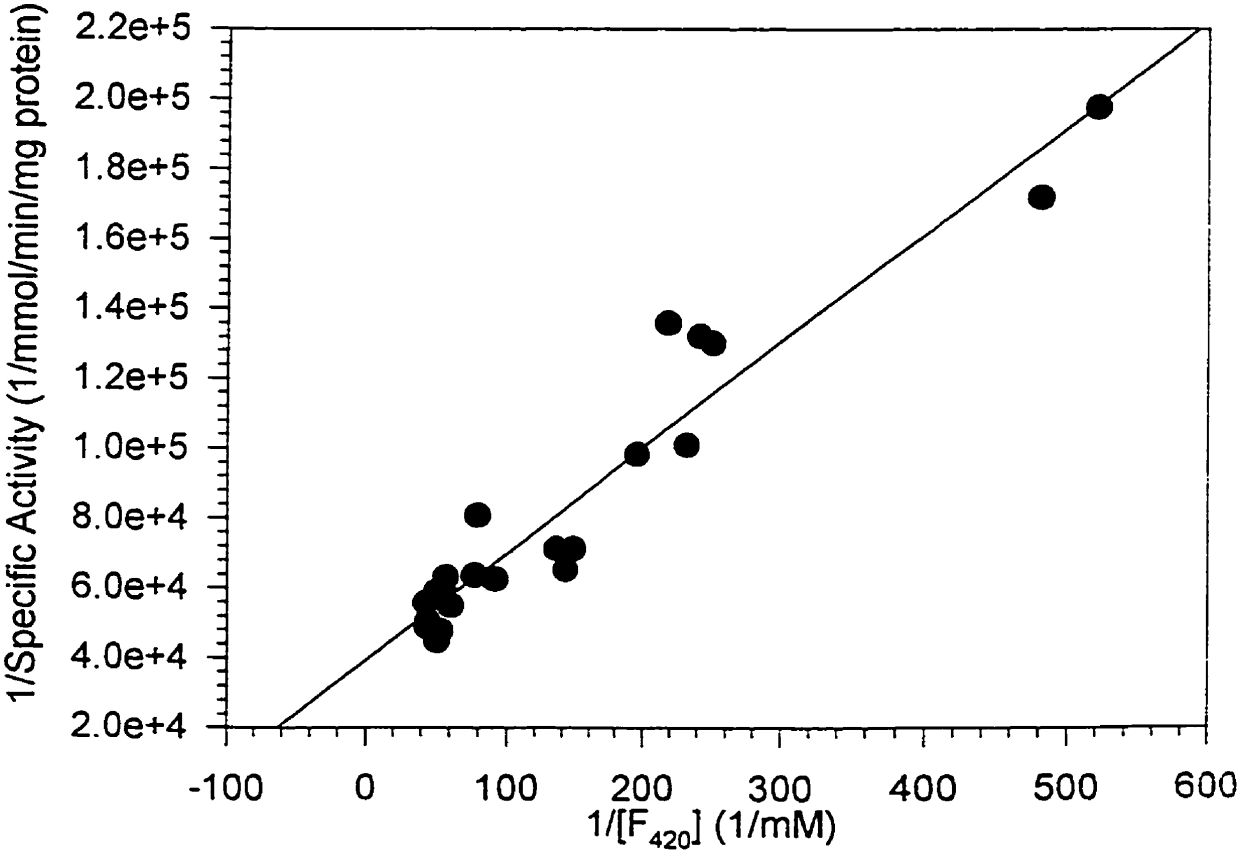

1/Specific Activity (1/mmol/min/mg protein)
2.2e+5
2.0e+5
1.8e+5
1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
-100
0
100
200
300
400
500
600
1/[F420] (1/mM)

Figure II.6. Eadie-Hofstee Plot for F_{420} in the CFE. The plot gives a K_m value of 9.8 μM. The experiment was performed in twice in triplicate.

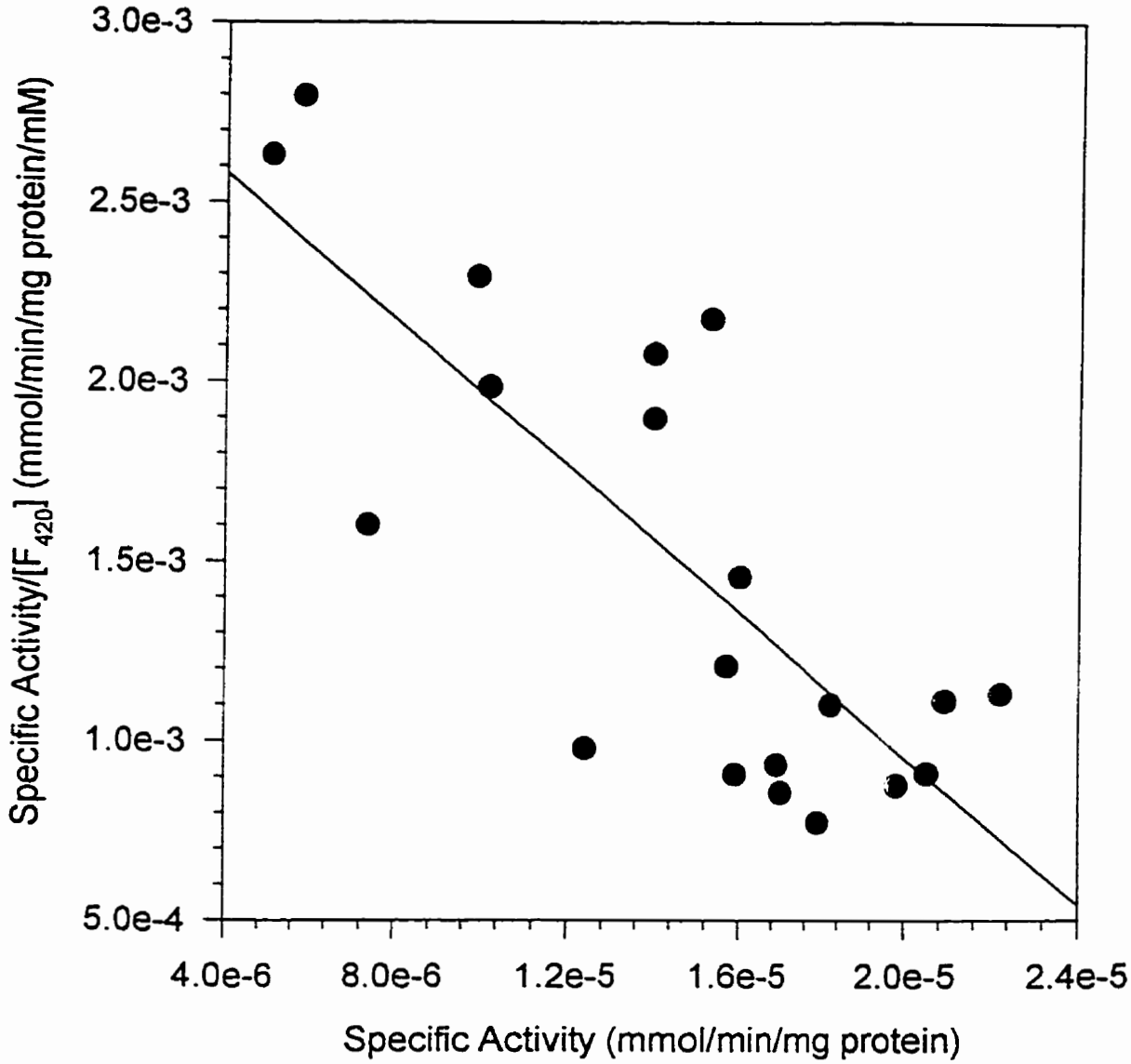
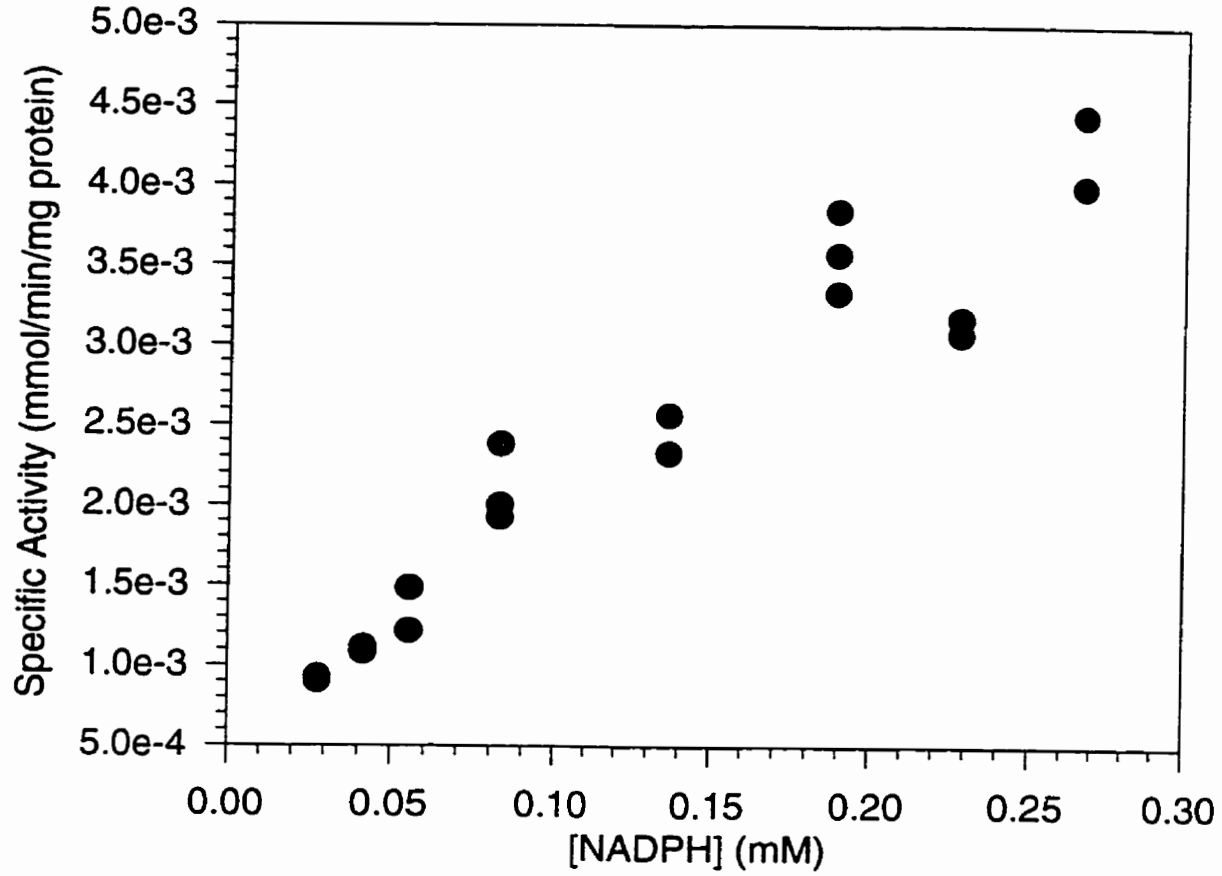

3.0e-3
2.5e-3
2.0e-3
1.5e-3
1.0e-3
5.0e-4
Specific Activity/[F420] (mmol/min/mg protein/mM)
4.0e-6
8.0e-6
1.2e-5
1.6e-5
2.0e-5
2.4e-5
Specific Activity (mmol/min/mg protein)

Figure II.7. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for NADPH in the 65-90% $(NH_4)_2SO_4$ pellet. The Lineweaver-Burke Plot showed a hyperbolic curve indicating a non-first order reaction. Experiment was performed in twice in triplicate.


Specific Activity (mmol/min/mg protein)
5.0e-3
4.5e-3
4.0e-3
3.5e-3
3.0e-3
2.5e-3
2.0e-3
1.5e-3
1.0e-3
5.0e-4
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADPH] (mM)

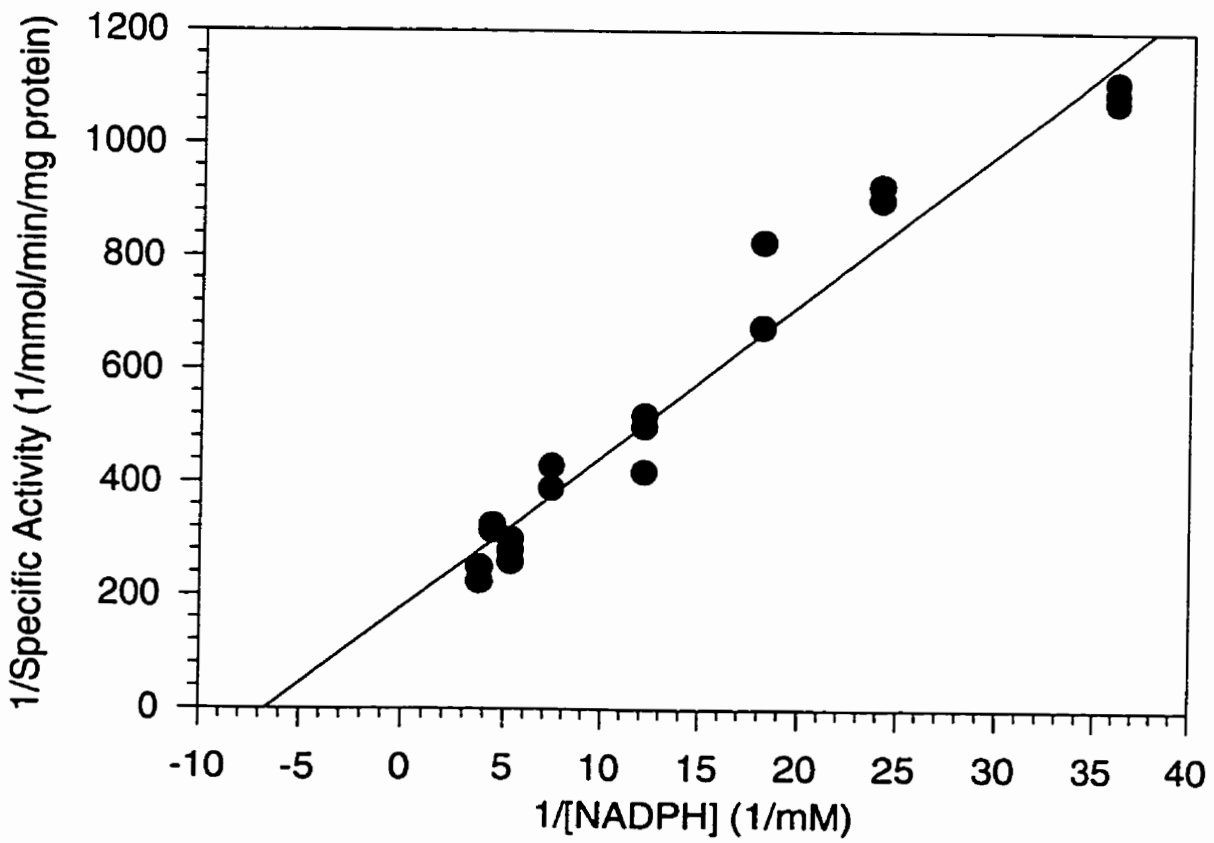

1/Specific Activity (1/mmol/min/mg protein)
1200
1000
800
600
400
200
0
-10
-5
0
5
10
15
20
25
30
35
40
1/[NADPH] (1/mM)

Figure II.8. Eadie-Hofstee Plot for NADPH in the 65-90% $(NH_4)_2SO_4$ pellet. The plot shows a K_m value of 225 μM, but r^2 value was poor. Experiment was performed in twice in triplicate.

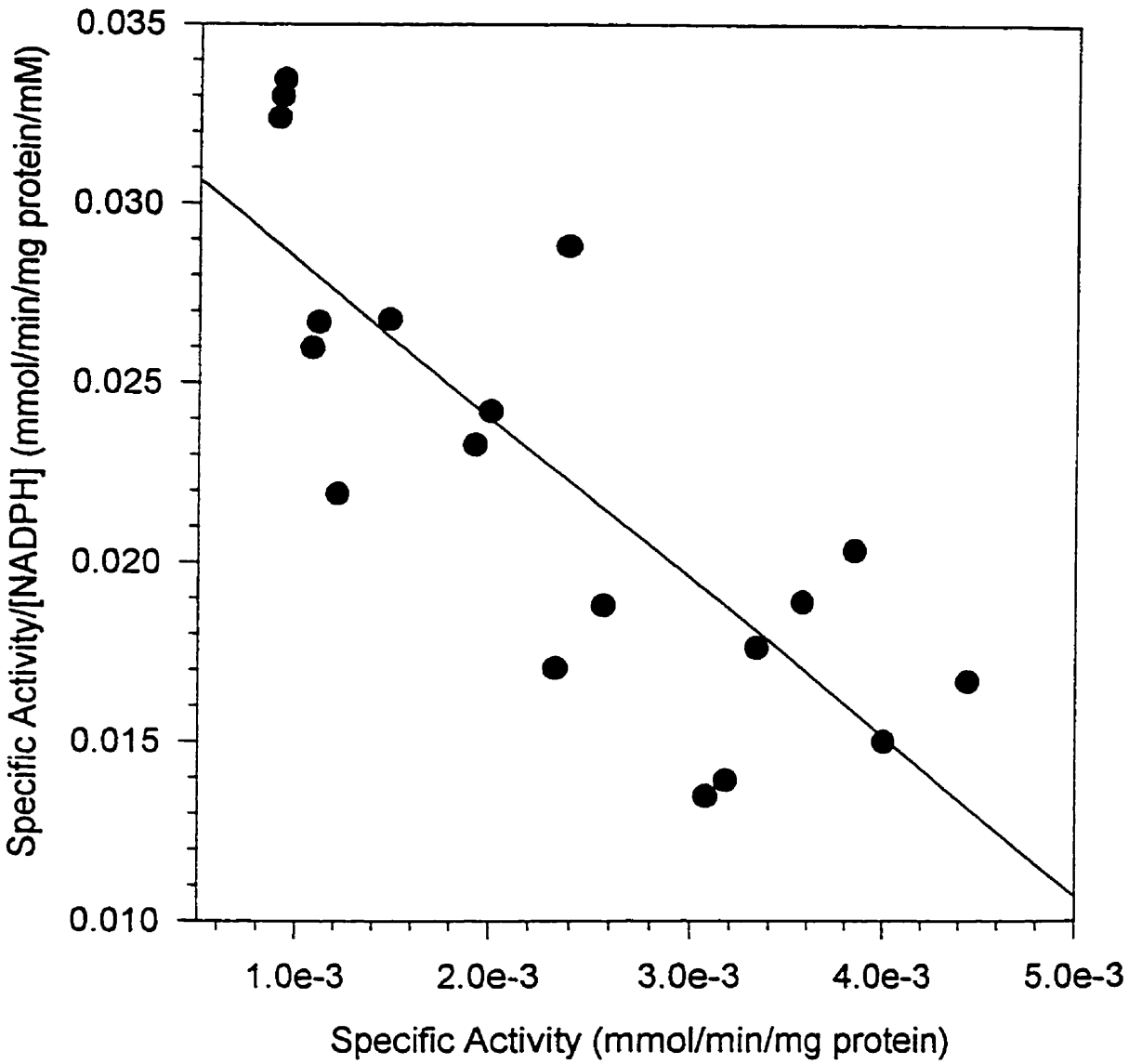
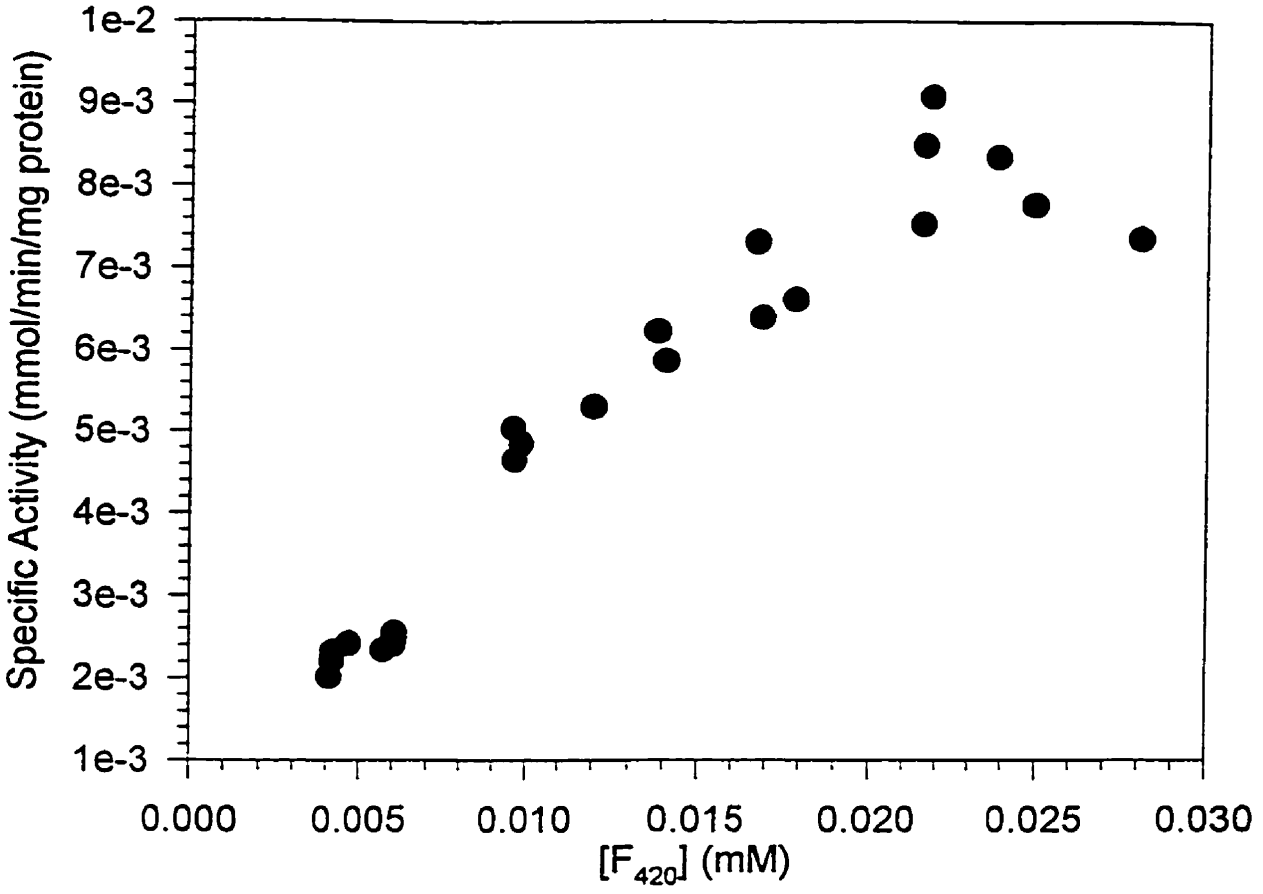

0.035
0.030
0.025
0.020
0.015
0.010
Specific Activity/[NADPH] (mmol/min/mg protein/mM)
1.0e-3
2.0e-3
3.0e-3
4.0e-3
5.0e-3
Specific Activity (mmol/min/mg protein)

Figure II.9. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for F_{420} in the 65-90% $(NH_4)_2SO_4$ pellet. Lineweaver-Burke Plot showed a K_m value of 37 μM. Experiment was performed in twice in triplicate.


Specific Activity (mmol/min/mg protein)
1e-2
9e-3
8e-3
7e-3
6e-3
5e-3
4e-3
3e-3
2e-3
1e-3
0.000
0.005
0.010
0.015
0.020
0.025
0.030
[F420] (mM)

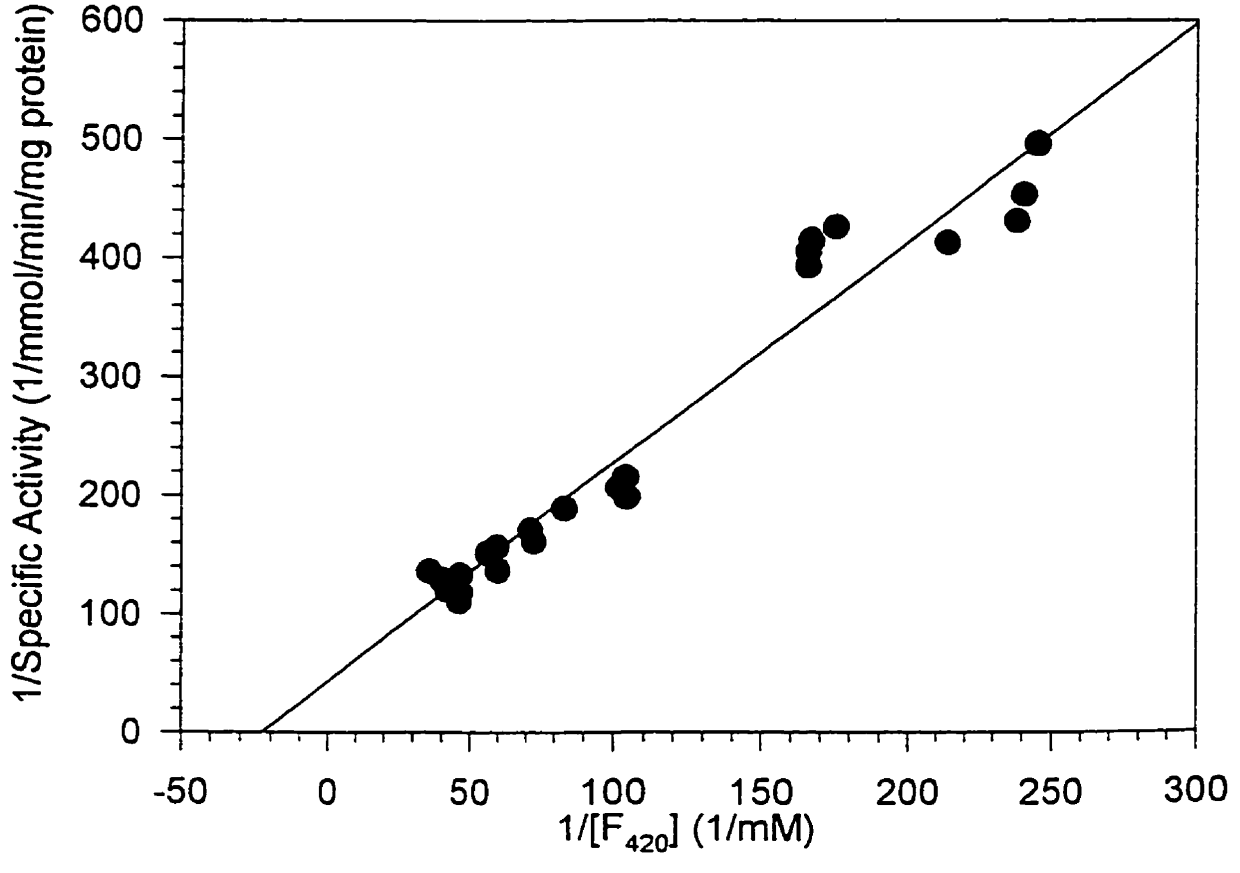
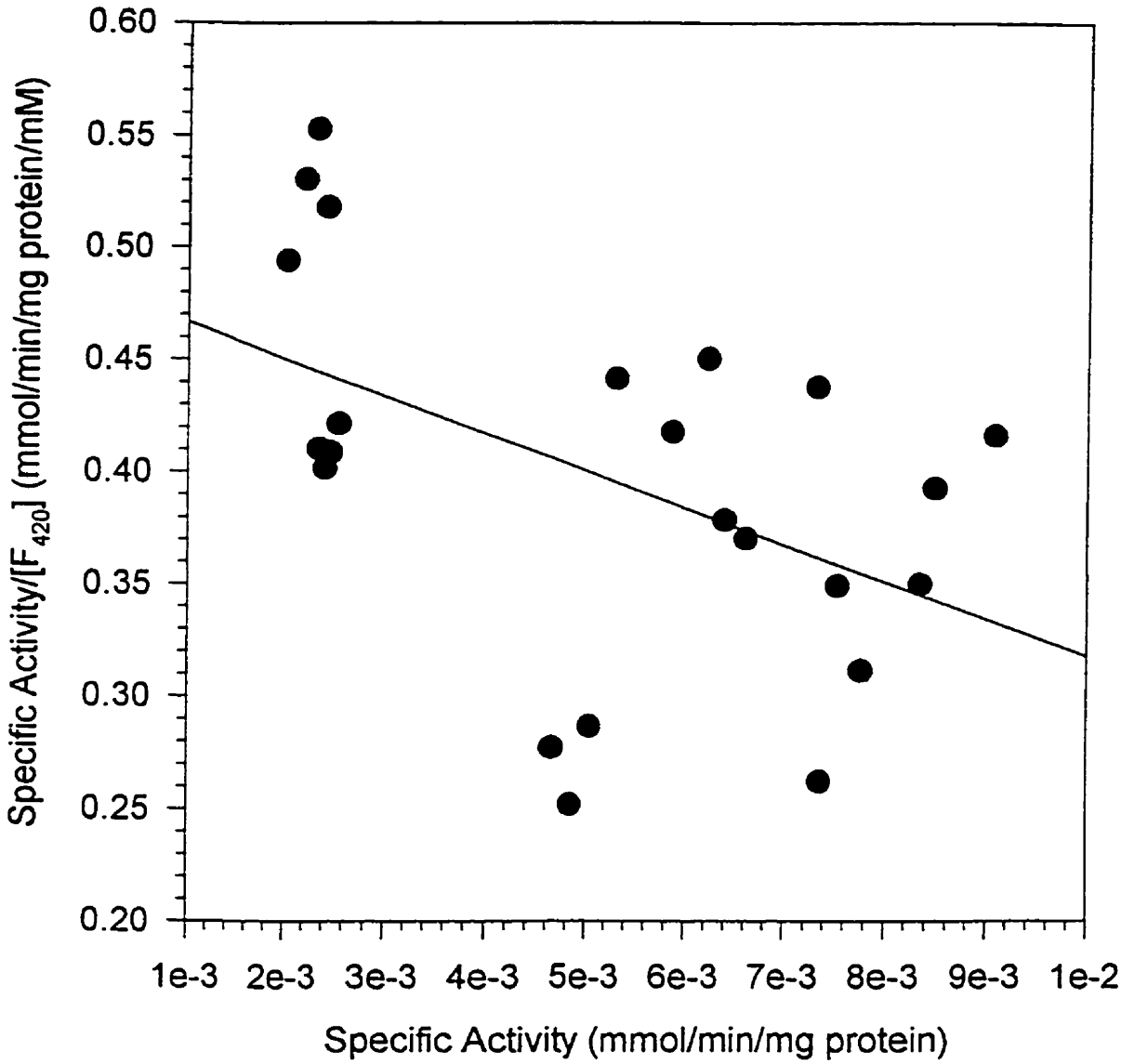

1/Specific Activity (1/mmol/min/mg protein)
600
500
400
300
200
100
0
-50
0
50
100
150
200
250
300
1/[F420] (1/mM)

Figure II.10. Eadie-Hofstee Plot for F_{420} in the 65-90% $(NH_4)_2SO_4$ pellet. The plot gave a K_m value of 60.3 μM. Experiment was performed in twice in triplicate.

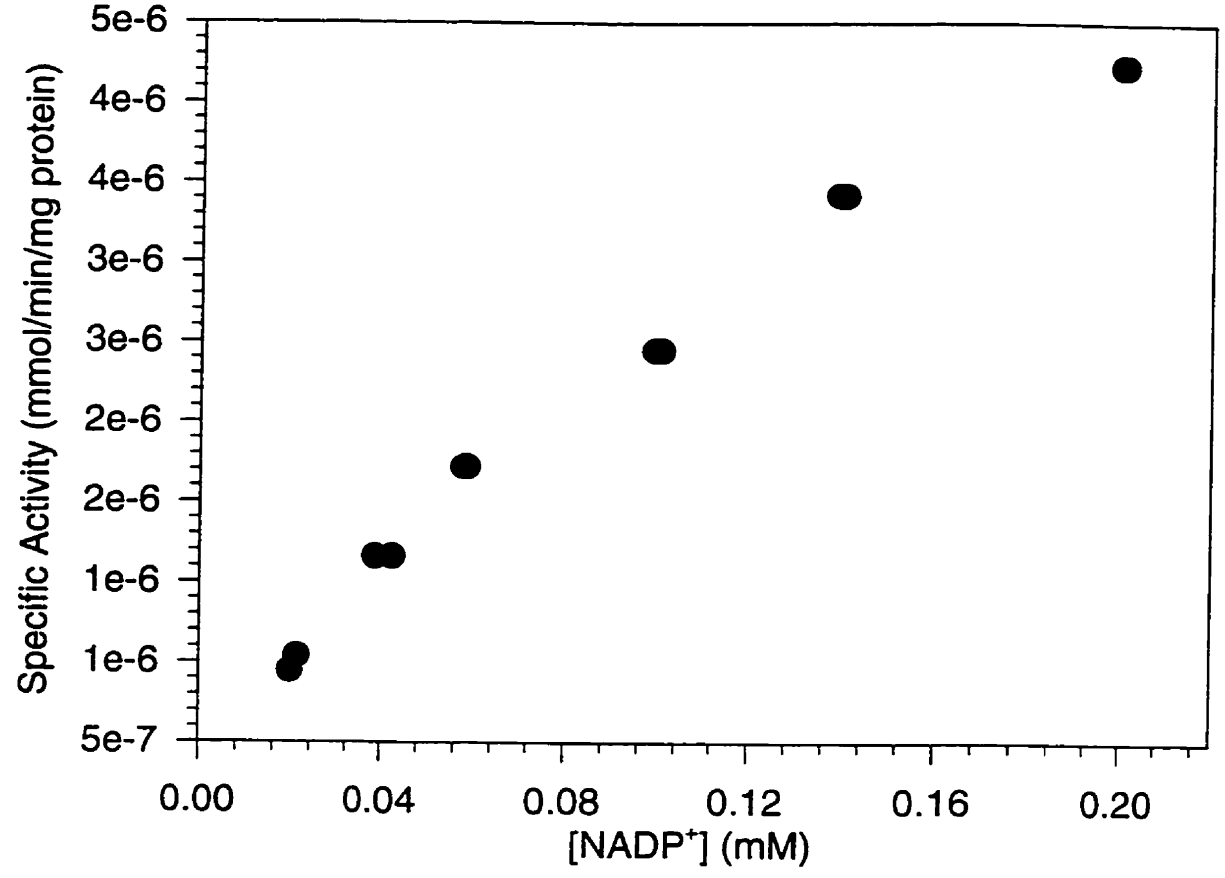

0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
Specific Activity/[F420] (mmol/min/mg protein/mM)
1e-3
2e-3
3e-3
4e-3
5e-3
6e-3
7e-3
8e-3
9e-3
1e-2
Specific Activity (mmol/min/mg protein)

K_m of 37 μM. The Eadie-Hofstee plot also appeared first-order with a K_m of 60.3 μM, but was again difficult to interpret.

Looking at the direction of $NADP^+$ reduction, both $NADP^+$ and $F_{420}H_2$ were tested for K_m's in the CFE and 65-90% $(NH_4)_2SO_4$ pellet. In the CFE, $NADP^+$ showed a hyperbolic curve in the Michealis-Menton plot (Figure II.11.), and gave a straight line in the Lineweaver-Burke plot, giving a K_m value of 13 μM. The Eadie-Hofstee plot also showed a straight line with a K_m value of 15.5 (Figure II.12.), which agreed well with the Lineweaver-Burke plot. For $F_{420}H_2$, the Michealis-Menton showed a typical curve and Lineweaver-Burke plots showed a K_m of 40 μM (Figure II.13.). The Eadie-Hofstee plot showed a K_m of 96.3 μM (Figure II.14.).

In the 65-90% $(NH_4)_2SO_4$ pellet, $NADP^+$ kinetics appeared to be non-first order. Although the Michealis-Menton plot showed as typical hyperbolic curve, the Lineweaver-Burke plot showed a curve (Figure II.15.). If the Lineweaver-Burke plot was taken as first-order, the K_m would have been 222 μM, while the Eadie-Hofstee plot would have given a K_m value of 23.6 μM (Figure II.16.); a 10-fold difference. Therefore, $NADP^+$ in the 65-90% $(NH_4)_2SO_4$ pellet was taken as non-first order. The kinetics for $F_{420}H_2$ in the 65-90% $(NH_4)_2SO_4$ pellet were taken as non-first order. Michealis-Menton and Lineweaver-Burke plots in the 65-90% $(NH_4)_2SO_4$ pellet showed sigmoidal curves (Figure II.17.). The Eadie-Hofstee plot in the 65-90% $(NH_4)_2SO_4$ pellet showed a hyperbolic curve in the reverse direction to what was expected (Figure II.18.). Table V.1. summarizes the kinetics of the enzyme in the CFE, 65-90% $(NH_4)_2SO_4$ pellet, and in the purified form.

Figure II.11. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for $NADP^+$ in the CFE. The Lineweaver-Burke plot gave a K_m of 130 μM. Experiment was performed in twice in duplicate.


5e-6
4e-6
4e-6
3e-6
3e-6
2e-6
2e-6
1e-6
1e-6
5e-7
Specific Activity (mmol/min/mg protein)
0.00
0.04
0.08
0.12
0.16
0.20
[NADP+] (mM)

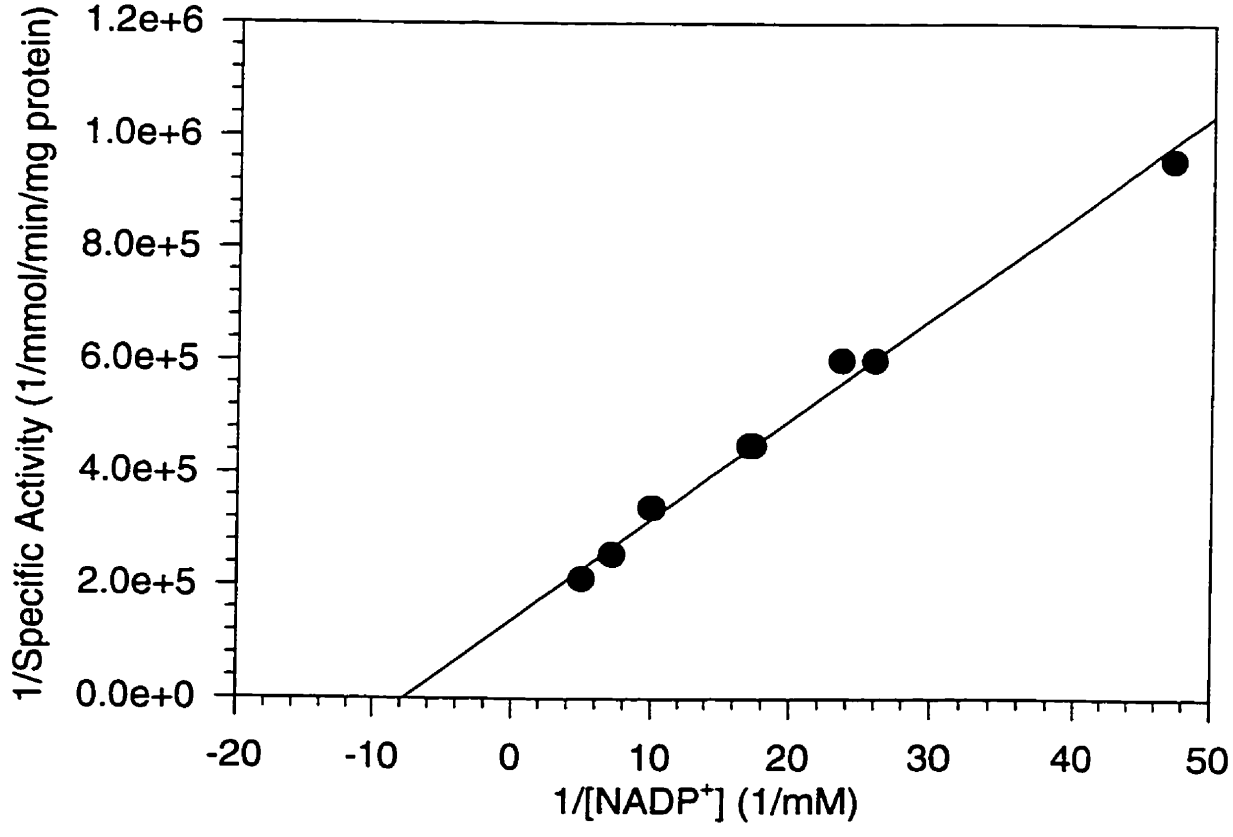

1.2e+6
1.0e+6
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0e+0
1/Specific Activity (1/mmol/min/mg protein)
-20
-10
0
10
20
30
40
50
1/[NADP+] (1/mM)

Figure II.12. Eadie-Hofstee Plot for $NADP^+$ in the CFE. The plot showed a K_m value of 15.5 μM. Experiment was performed in twice in duplicate.

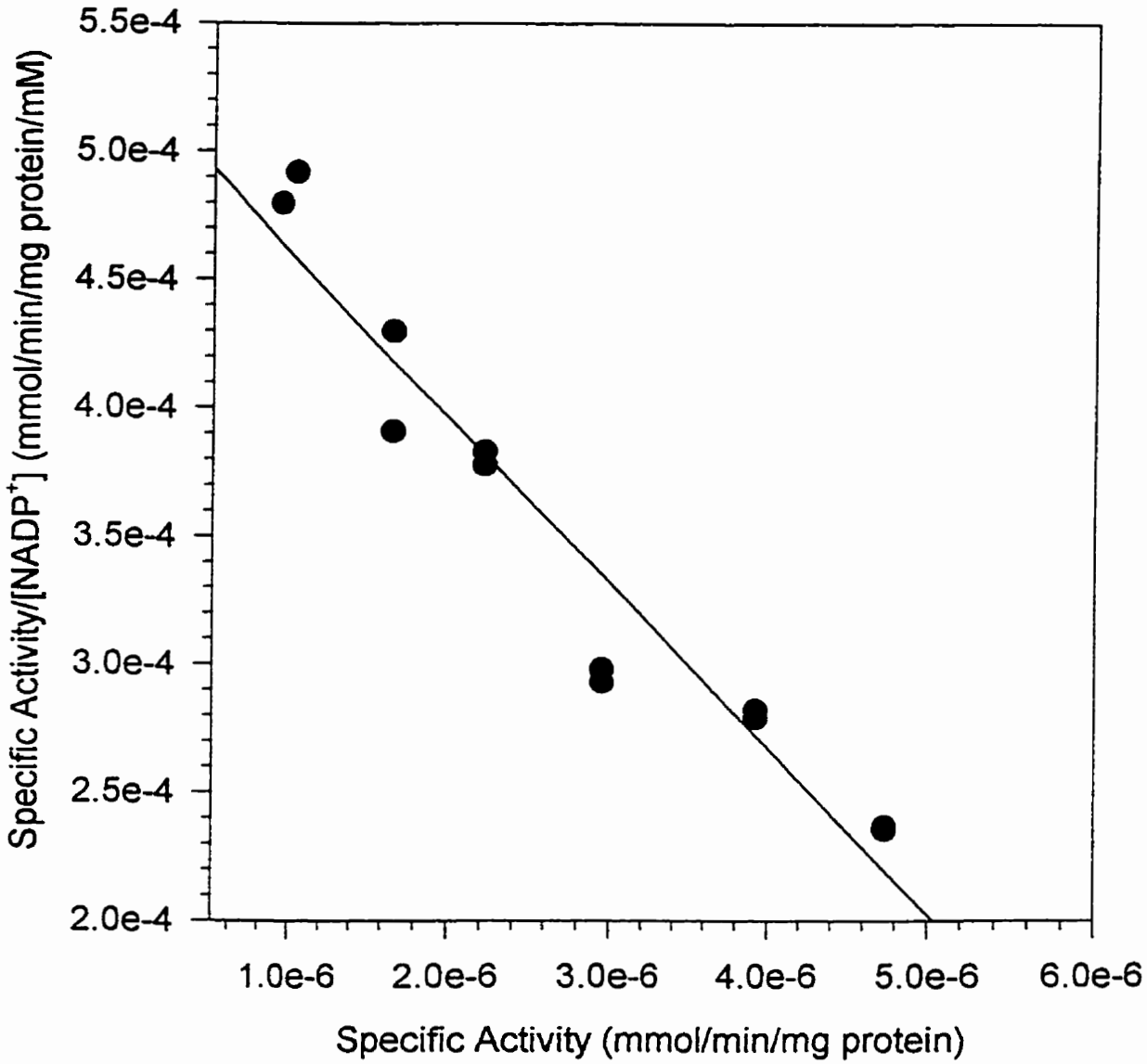
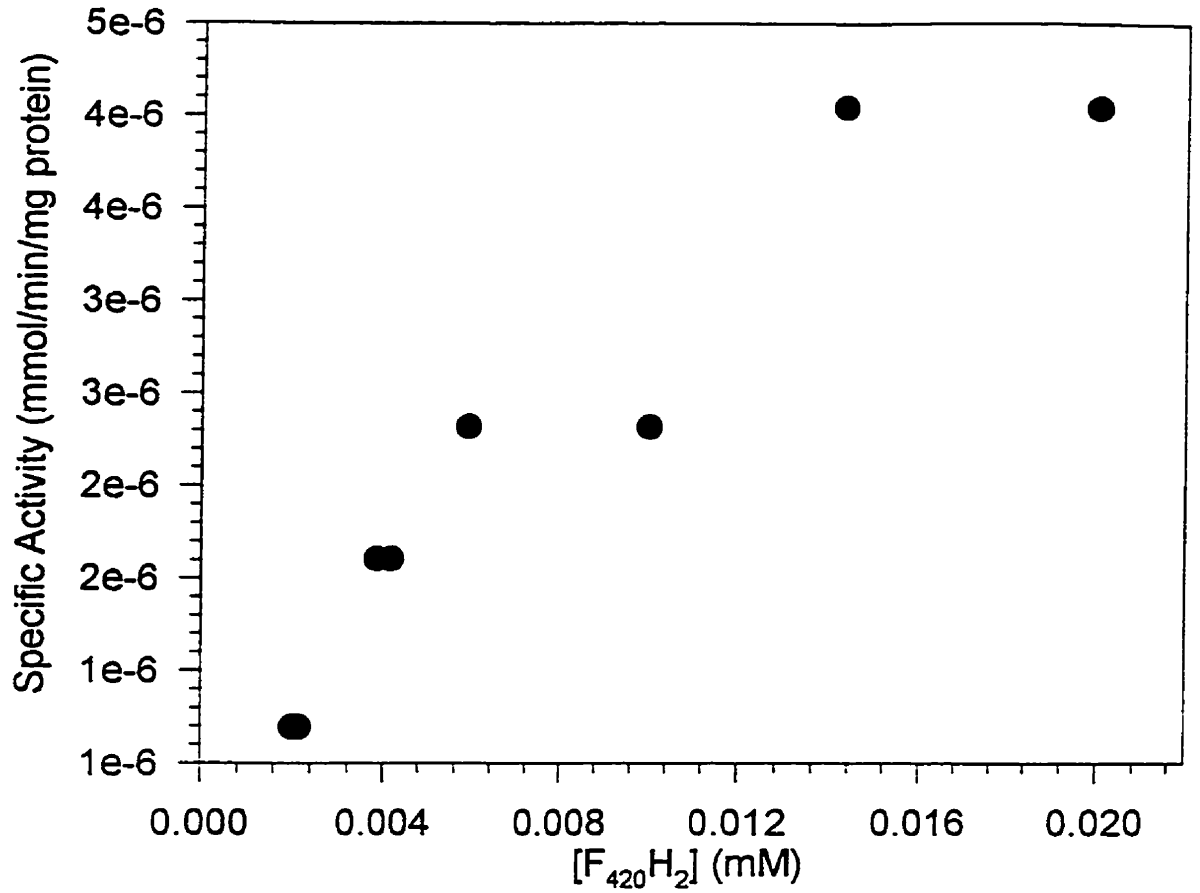

5.5e-4
5.0e-4
4.5e-4
4.0e-4
3.5e-4
3.0e-4
2.5e-4
2.0e-4
Specific Activity/[NADP+] (mmol/min/mg protein/mM)
1.0e-6
2.0e-6
3.0e-6
4.0e-6
5.0e-6
6.0e-6
Specific Activity (mmol/min/mg protein)

Figure II.13. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for $F_{420}H_2$ in the CFE. The Lineweaver-Burke plot gave a K_m of 40 μM. Experiment was performed in twice in duplicate.


5e-6
4e-6
4e-6
3e-6
3e-6
2e-6
2e-6
1e-6
1e-6
Specific Activity (mmol/min/mg protein)
0.000
0.004
0.008
0.012
0.016
0.020
[F420H2] (mM)

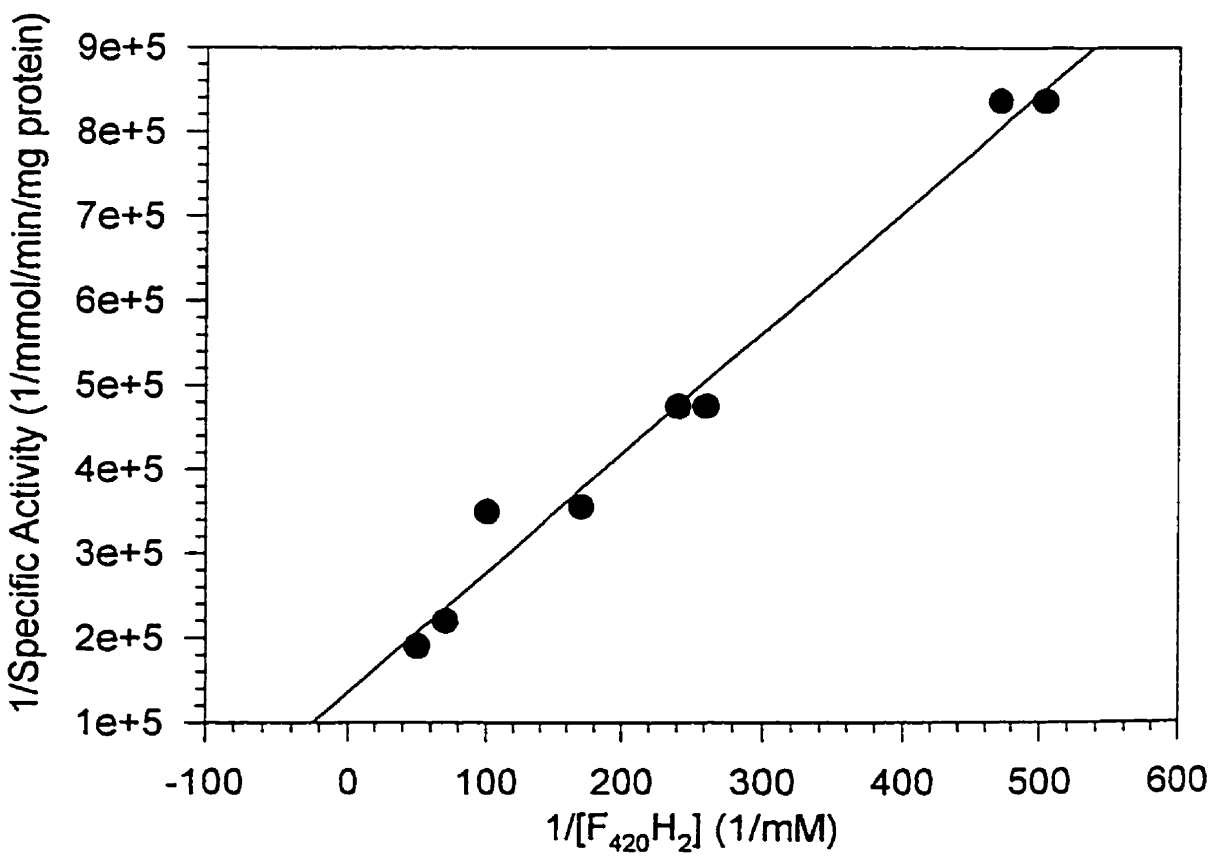

9e+5
8e+5
7e+5
6e+5
5e+5
4e+5
3e+5
2e+5
1e+5
1/Specific Activity (1/mmol/min/mg protein)
-100
0
100
200
300
400
500
600
1/[F420H2] (1/mM)

Figure II.14. Eadie-Hofstee Plot for $F_{420}H_2$ oxidation in the CFE. the plot gave a K_m value of 96.3 μM. Experiment was performed in twice in duplicate.

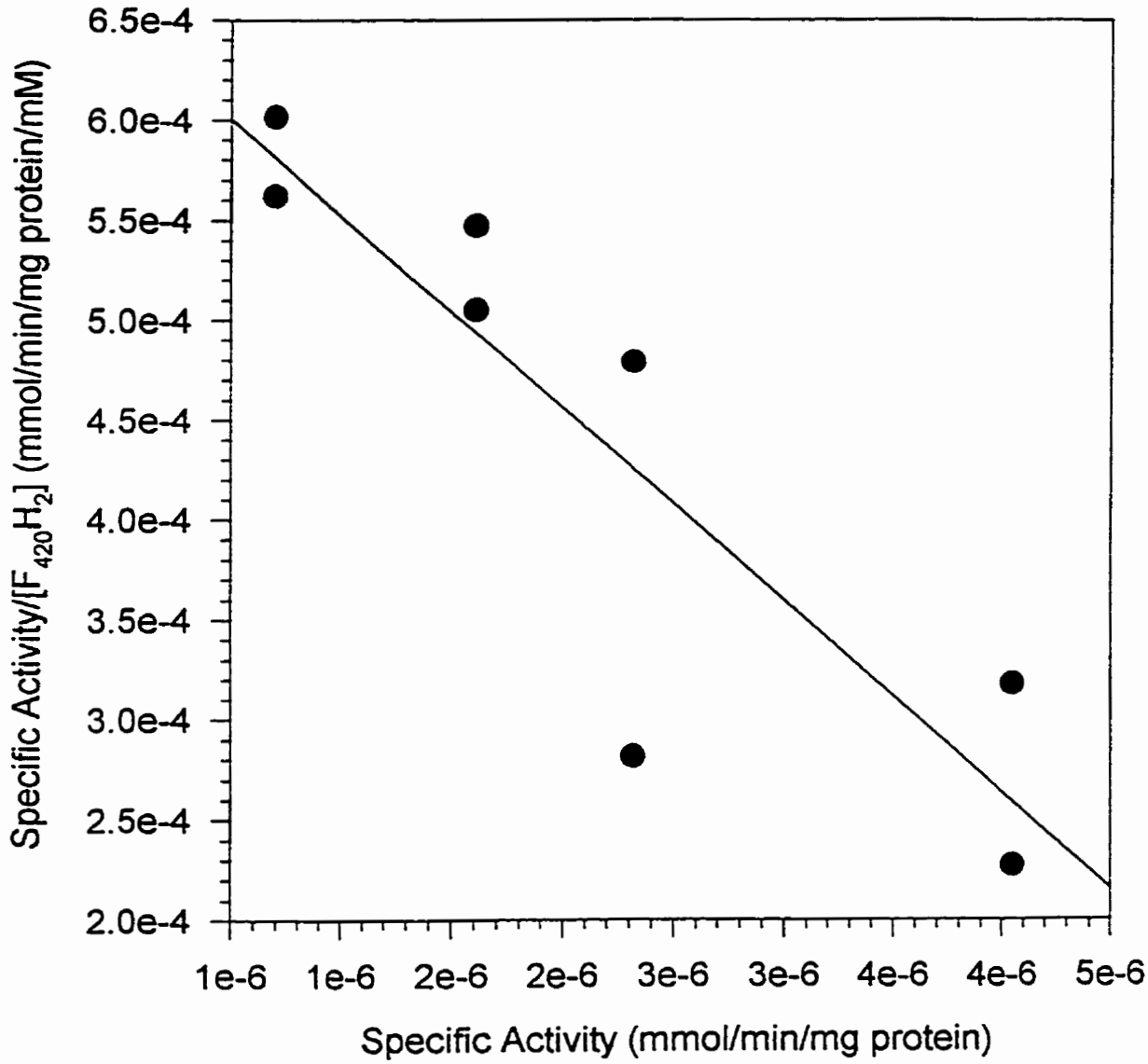
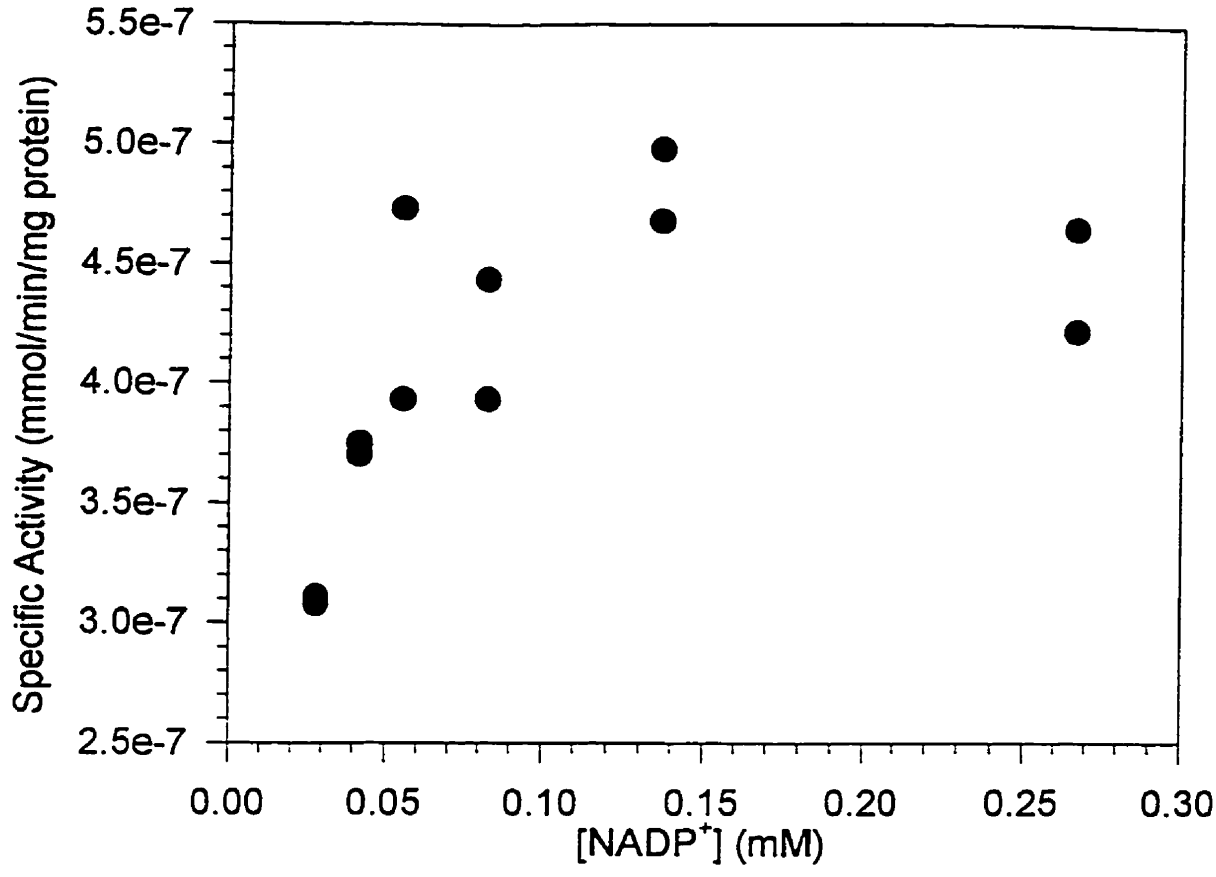

6.5e-4
6.0e-4
5.5e-4
5.0e-4
4.5e-4
4.0e-4
3.5e-4
3.0e-4
2.5e-4
2.0e-4
Specific Activity/[F420H2] (mmol/min/mg protein/mM)
1e-6
1e-6
2e-6
2e-6
3e-6
3e-6
4e-6
4e-6
5e-6
Specific Activity (mmol/min/mg protein)

Figure II.15. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for $NADP^+$ in the 65-90% $(NH_4)_2SO_4$ pellet. The Lineweaver-Burke plot showed a non-linear regression and was taken as non-first order. Experiment was performed in twice in duplicate. Results were highly similar in both experiments.


5.5e-7
5.0e-7
4.5e-7
4.0e-7
3.5e-7
3.0e-7
2.5e-7
Specific Activity (mmol/min/mg protein)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADP+] (mM)

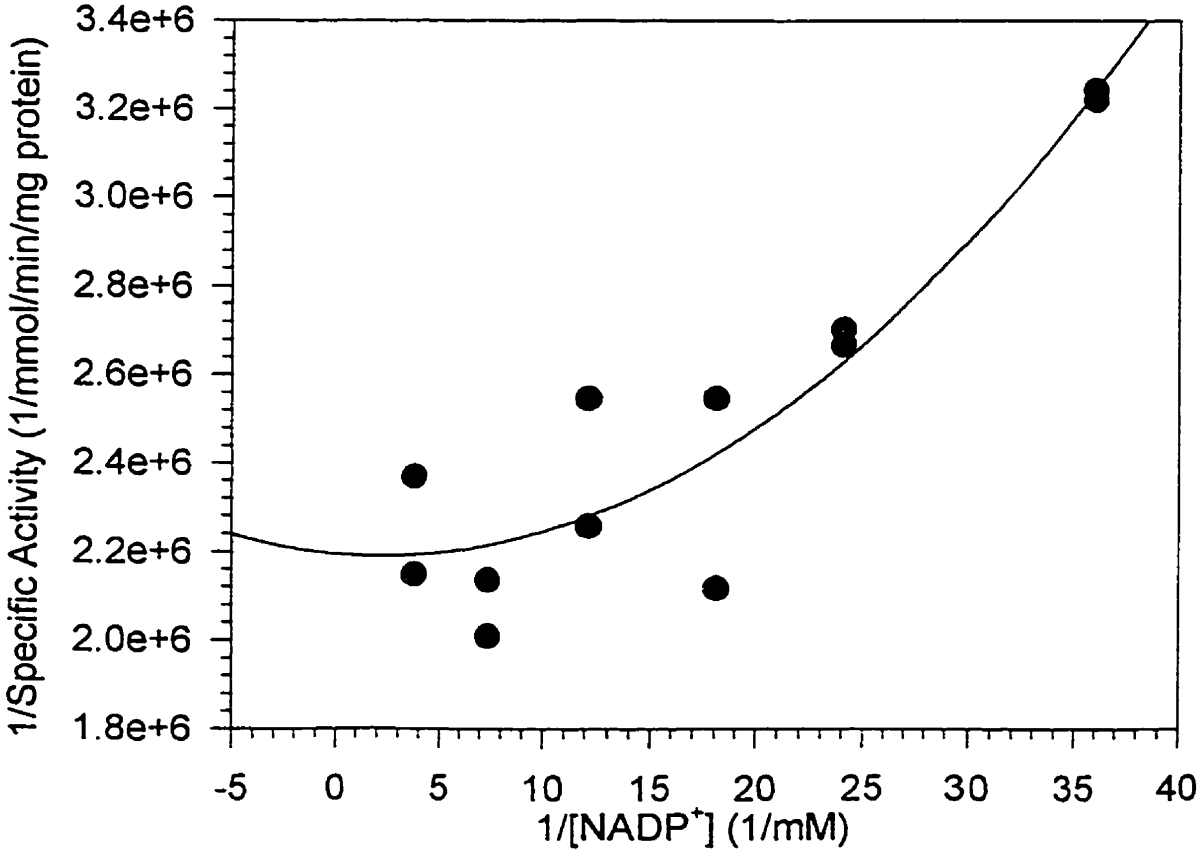

3.4e+6
3.2e+6
3.0e+6
2.8e+6
2.6e+6
2.4e+6
2.2e+6
2.0e+6
1.8e+6
1/Specific Activity (1/mmol/min/mg protein)
-5
0
5
10
15
20
25
30
35
40
1/[NADP+] (1/mM)

Figure II.16. Eadie-Hofstee Plot for $NADP^+$ in the 65-90% $(NH_4)_2SO_4$ pellet. The plot showed a very poor regression and was taken as non-first order. Experiment was performed in twice in duplicate.

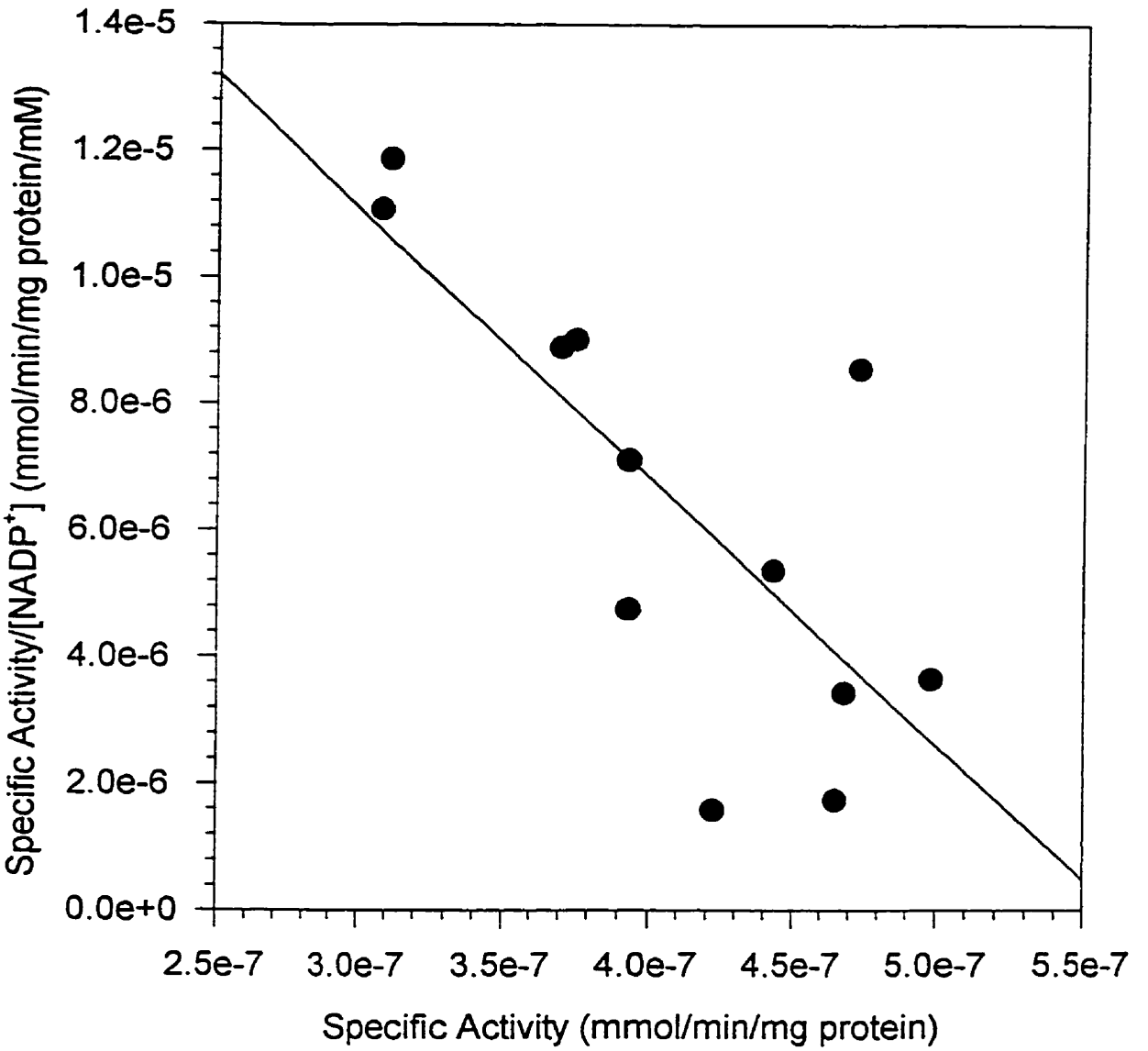
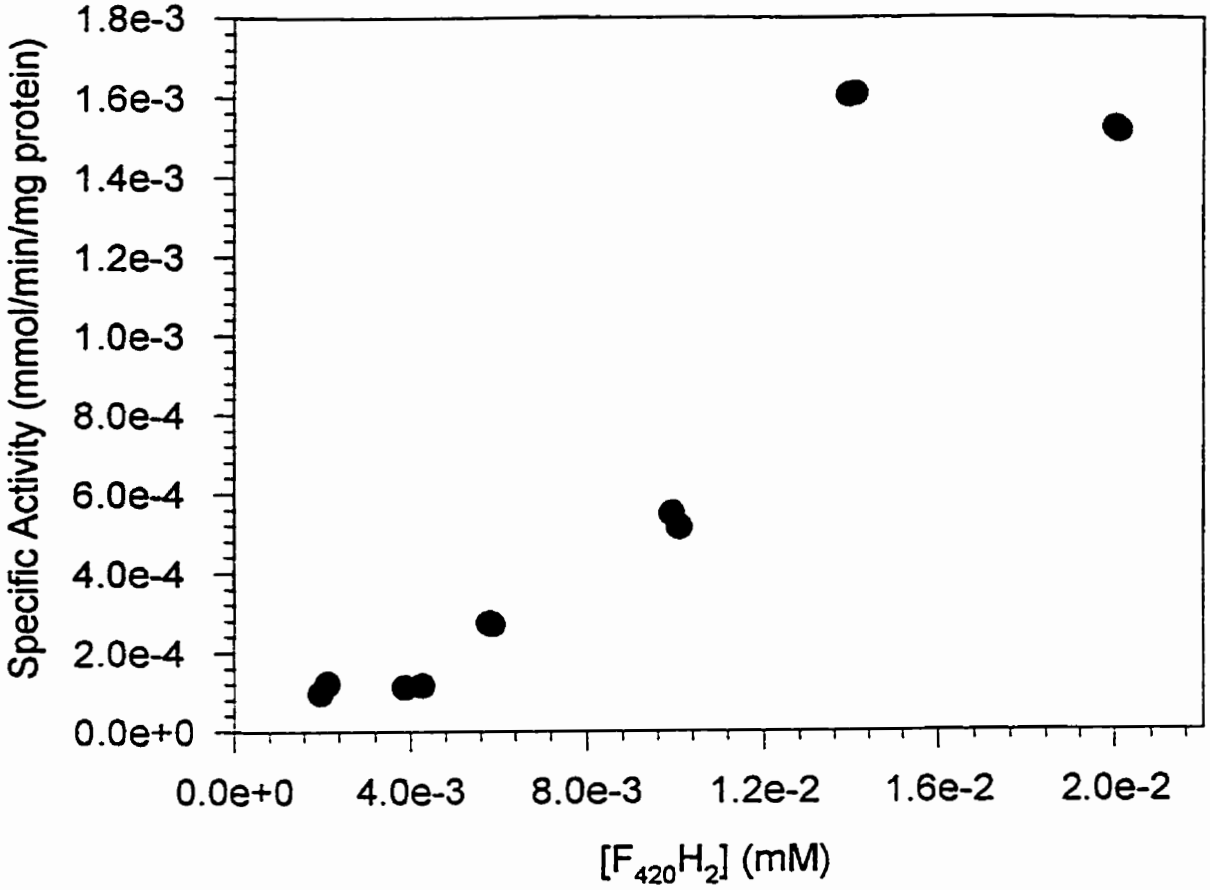

1.4e-5
1.2e-5
1.0e-5
8.0e-6
6.0e-6
4.0e-6
2.0e-6
0.0e+0
Specific Activity/[NADP+] (mmol/min/mg protein/mM)
2.5e-7
3.0e-7
3.5e-7
4.0e-7
4.5e-7
5.0e-7
5.5e-7
Specific Activity (mmol/min/mg protein)

Figure II.17. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for $F_{420}H_2$ in the 65-90% $(NH_4)_2SO_4$ pellet. Both the Michealis-Menton and Lineweaver-Burke plots gave sigmoidal curves indicating a non-first order reaction. Experiment was performed in twice in duplicate.


Specific Activity (mmol/min/mg protein)
1.8e-3
1.6e-3
1.4e-3
1.2e-3
1.0e-3
8.0e-4
6.0e-4
4.0e-4
2.0e-4
0.0e+0
0.0e+0
4.0e-3
8.0e-3
1.2e-2
1.6e-2
2.0e-2
[F420H2] (mM)

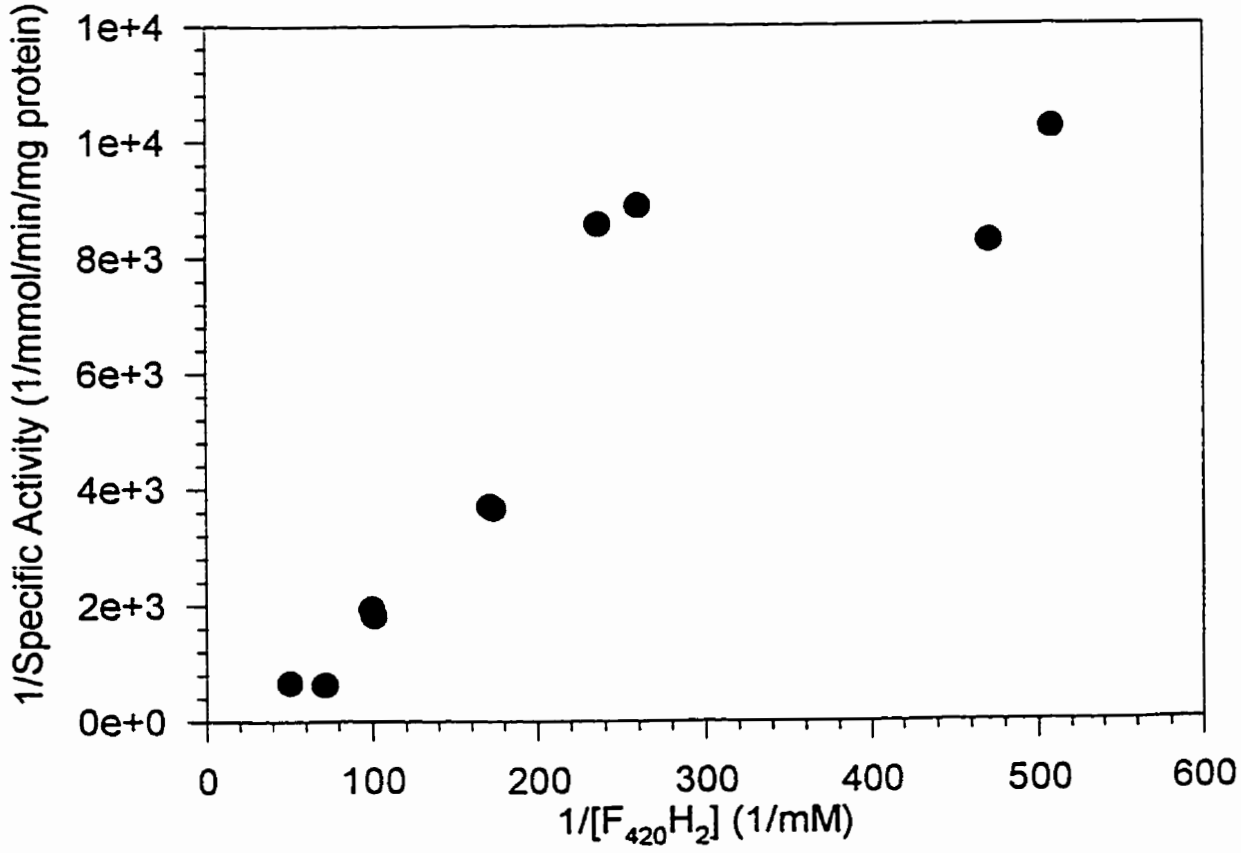
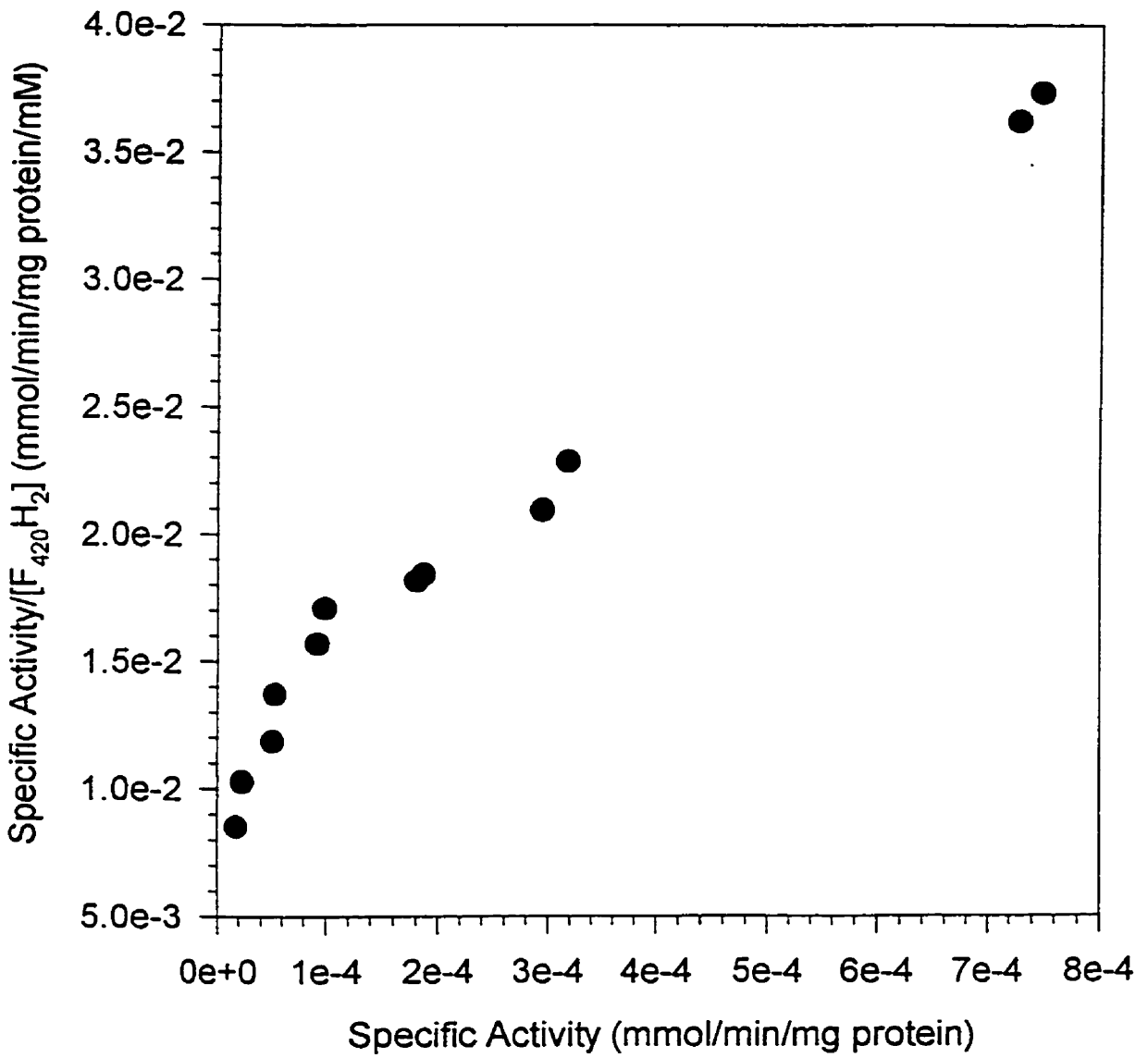

1/Specific Activity (1/mmol/min/mg protein)
1e+4
1e+4
8e+3
6e+3
4e+3
2e+3
0e+0
0
100
200
300
400
500
600
1/[F420H2] (1/mM)

Figure II.18. Eadie-Hofstee Plot for $F_{420}H_2$ in the 65-90% $(NH_4)_2SO_4$ pellet. The plot gave a hyperbolic curve indicating a non-first order reaction. Experiment was performed in twice in duplicate.


4.0e-2
3.5e-2
3.0e-2
2.5e-2
2.0e-2
1.5e-2
1.0e-2
5.0e-3
Specific Activity/[F420H2] (mmol/min/mg protein/mM)
0e+0
1e-4
2e-4
3e-4
4e-4
5e-4
6e-4
7e-4
8e-4
Specific Activity (mmol/min/mg protein)

II.3.3. Protease Inhibitor Studies

Protease inhibitor studies were also performed in the CFE and the 65-90% $(NH_4)_2SO_4$ pellet to determine whether the loss of a specific protein may account for the differences observed in the CFE and the 65-90% $(NH_4)_2SO_4$ pellet was due to proteinase activity. The proteinase inhibitors used were 1 mM PMSF, 5 mM EDTA, and a combination of both against a control; EDTA is a metal chelator, and PMSF is a serine protease inhibitor. These inhibitors were added immediately upon lysis of the cells and were maintained throughout further processes. This was done for both the total and specific activity, and performed at 0, 48, 72, and 96 hours post-incubation.

Until 96 hrs post-incubation, there was no difference with any of the inhibitors (5mM EDTA, 1 mM PMSF, or using both) in the CFE or 65-90% $(NH_4)_2SO_4$ pellet compared to controls. At 96 hrs post-incubation, there was an increase in both total and specific activity (Figure II.19.) in the CFE using PMSF (or both), and in the 65-90% $(NH_4)_2SO_4$ pellet total activity and specific activity (Figure II.20.) with EDTA (or both). After 96 hours, the CFE and CFE+EDTA samples continued to decrease, but the samples containing CFE+PMSF or CFE+PMSF+EDTA increased to near 48 hour post-incubation levels. The specific activity showed similar results, except that after 96 hours the specific activity returned to time 0 levels in the CFE+PMSF and CFE+PMSF+EDTA samples.

In the 65-90% $(NH_4)_2SO_4$ pellet, the total activity increased from 0 to 48 hours, then drastically dropped by 72 hours, and continued by 96 hours in 65-90% $(NH_4)_2SO_4$ pellet and 65-90% $(NH_4)_2SO_4$ pellet+PMSF, but increased to ~2/3 of time 0 values in 65-90% $(NH_4)_2SO_4$ pellet+EDTA and 65-90% $(NH_4)_2SO_4$ pellet+EDTA+PMSF samples. The

Figure II.19.: Effect of Protease Inhibitors PMSF and EDTA on the Specific Activity in CFE. Assays were performed twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.

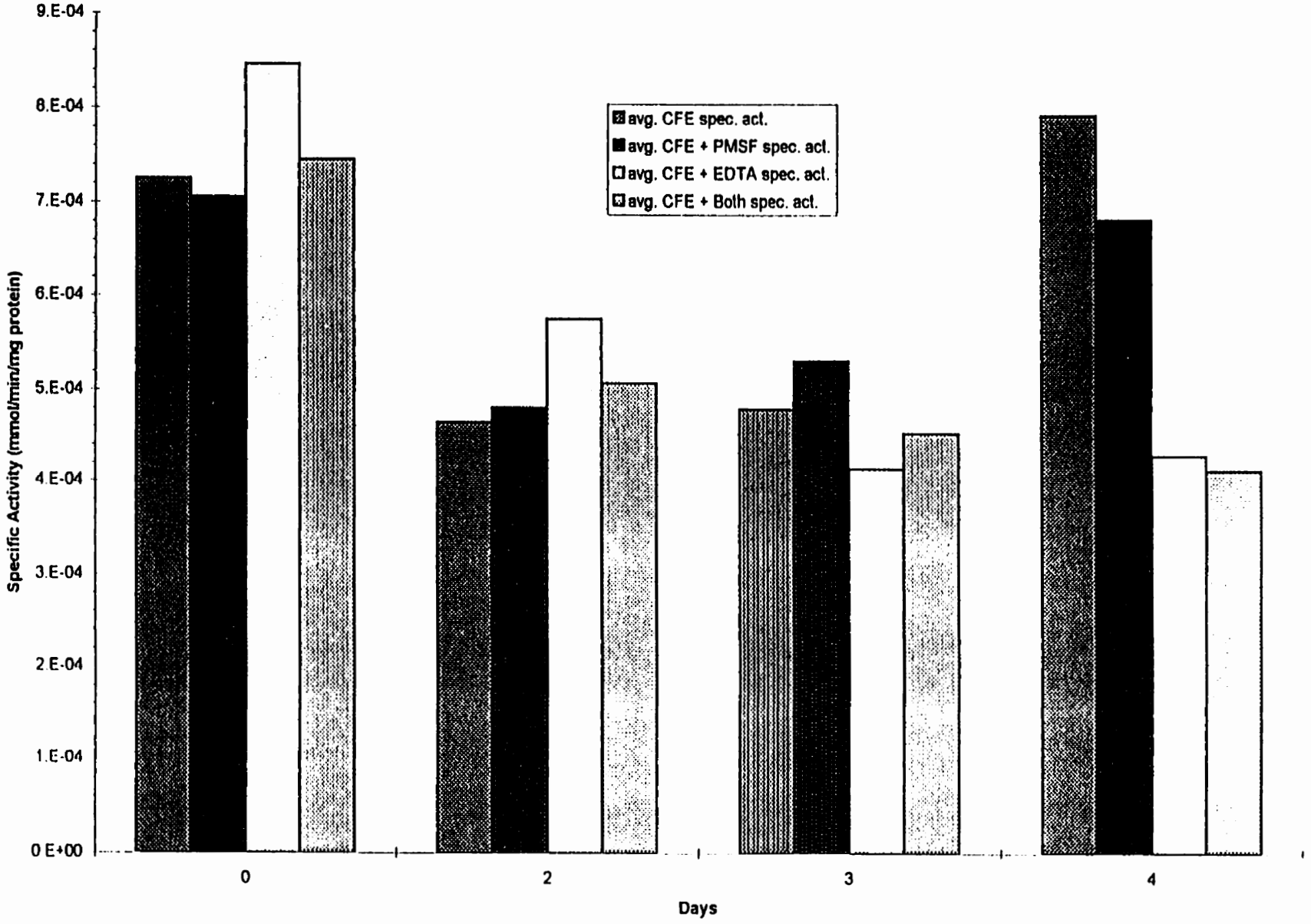
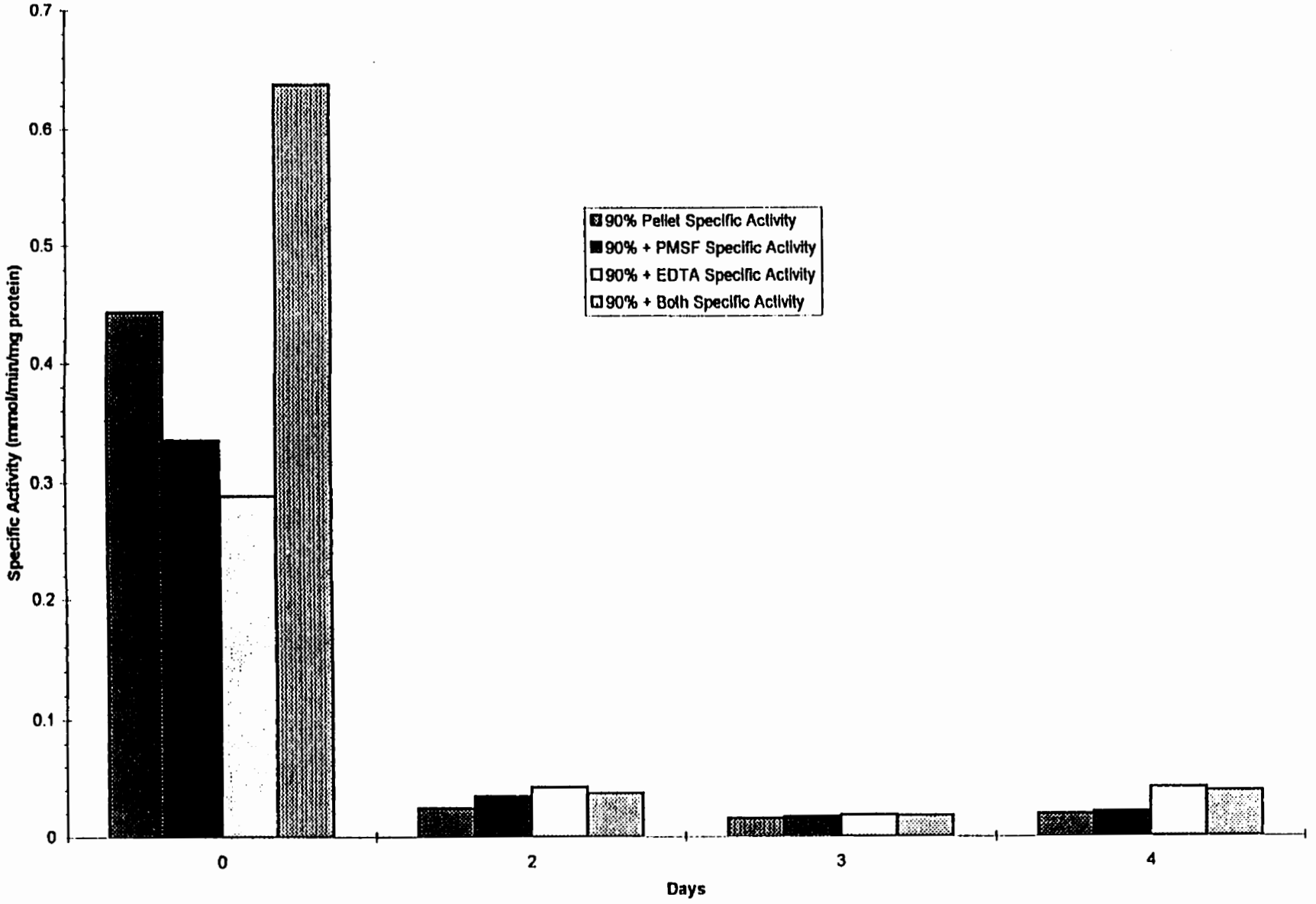

9.E-04
8.E-04
7.E-04
6.E-04
5.E-04
4.E-04
3.E-04
2.E-04
1.E-04
0 E+00
Specific Activity (mmol/min/mg protein)
avg. CFE spec. act.
avg. CFE + PMSF spec. act.
avg. CFE + EDTA spec. act.
avg. CFE + Both spec. act.
0
2
3
4
Days

Figure II.20.: Effect of Protease Inhibitors PMSF and EDTA on the Specific Activity in 65-90% $(NH_4)_2SO_4$ pellet. Assays were performed twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Specific Activity (mmol/min/mg protein)
90% Pellet Specific Activity
90% + PMSF Specific Activity
90% + EDTA Specific Activity
90% + Both Specific Activity
0
2
3
4
Days

specific activity of the 65-90% $(NH_4)_2SO_4$ pellet decreased drastically to near zero values by 48 hours, and remains at these levels at 72 and 96 hours.

II.3.4. NH_4^+ or High Salt Activation in the 65-90% $(NH_4)_2SO_4$ pellet

The experiment to test whether high salt or NH_4^+ activated the $NADP^+$:F_{420} oxidoreductase showed that neither was responsible for the activation of the enzyme during the $(NH_4)_2SO_4$ treatment. Solutions of CFE, CFE + 500 mM NH_4Cl, CFE + 500 mM KCl, and CFE + 500 mM $(NH_4)_2SO_4$ were tested immediately after preparation (as in methods) and after 48 hours incubation at 4°C. The specific activity of the 65-90% $(NH_4)_2SO_4$ pellet was 1.05 μmol/min/mg protein on day 0 (immediately after preparation) while the CFE alone displayed a specific activity of 0.0037 μmol/min/mg protein (Figure II.21.). Not only did NH_4^+ nor high salt fail to increase the specific activity of the enzyme to near 65-90% $(NH_4)_2SO_4$ pellet levels, but in fact the specific activity decreased on day 0. Only the $(NH_4)_2SO_4$ solution remained near the CFE values, while the NH_4Cl and the KCl systems retained only ~72% of the specific activity of the CFE alone. Similar effects were seen in the total activity (Figure II.22.).

At 48 hours post-incubation, the NH_4Cl and KCl systems in the CFE possessed specific activities of approximately 0.0033 μmol/min/mg protein as compared to approximately 0.0027 μmol/min/mg protein in both the CFE and CFE + $(NH_4)_2SO_4$ systems. Although this increase represents an approximate 20% increase in specific activity as compared to the CFE alone, it is still far from the specific activity seen in the 65-90% $(NH_4)_2SO_4$ pellet.

Figure II.21. Effect of NH_4^+, high salt, and NH_4Cl vs. $(NH_4)_2SO_4$ in the activation of the specific activity in the CFE for the $NADP^+$:F_{420} oxidoreductase. Assays were performed twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.

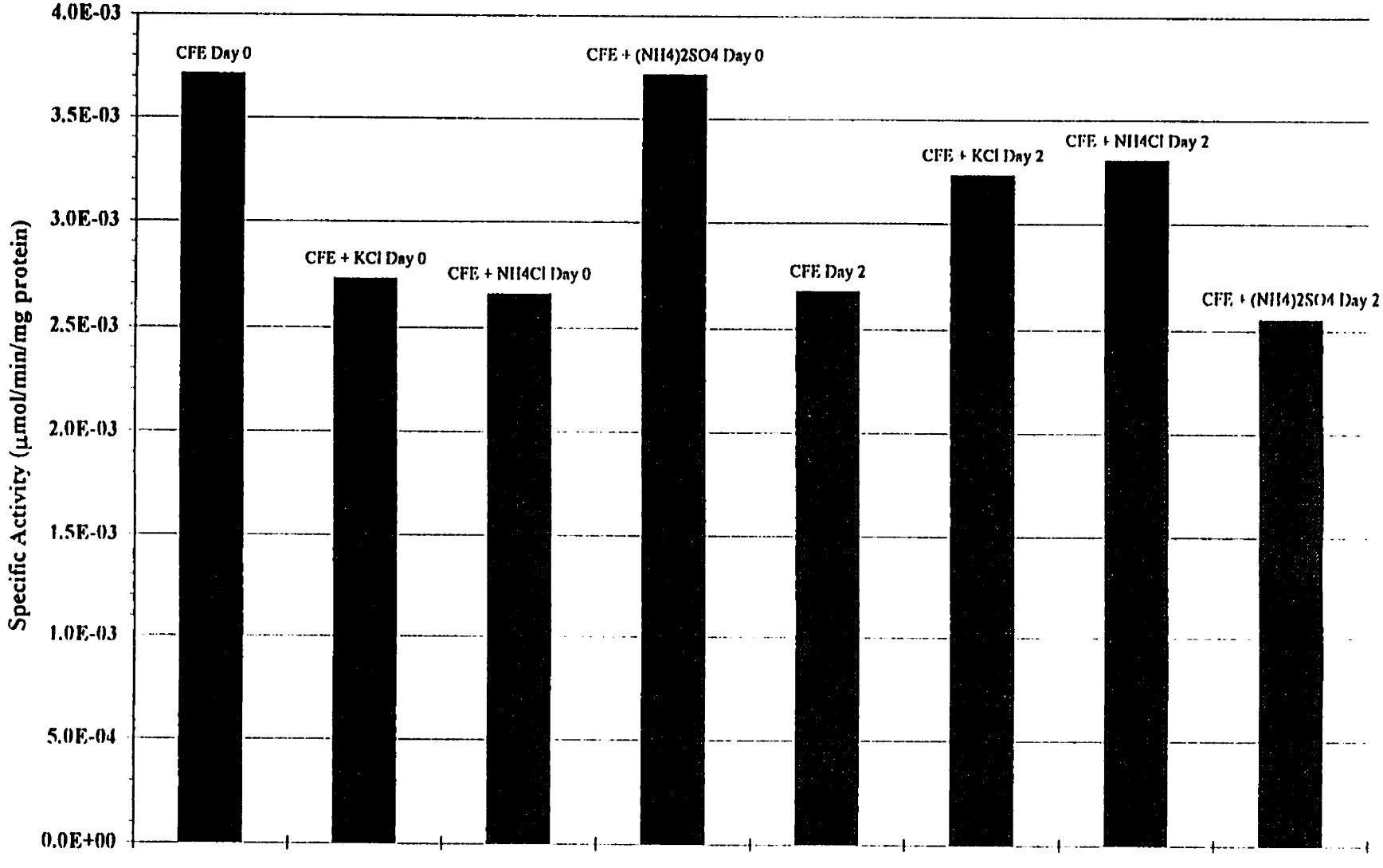
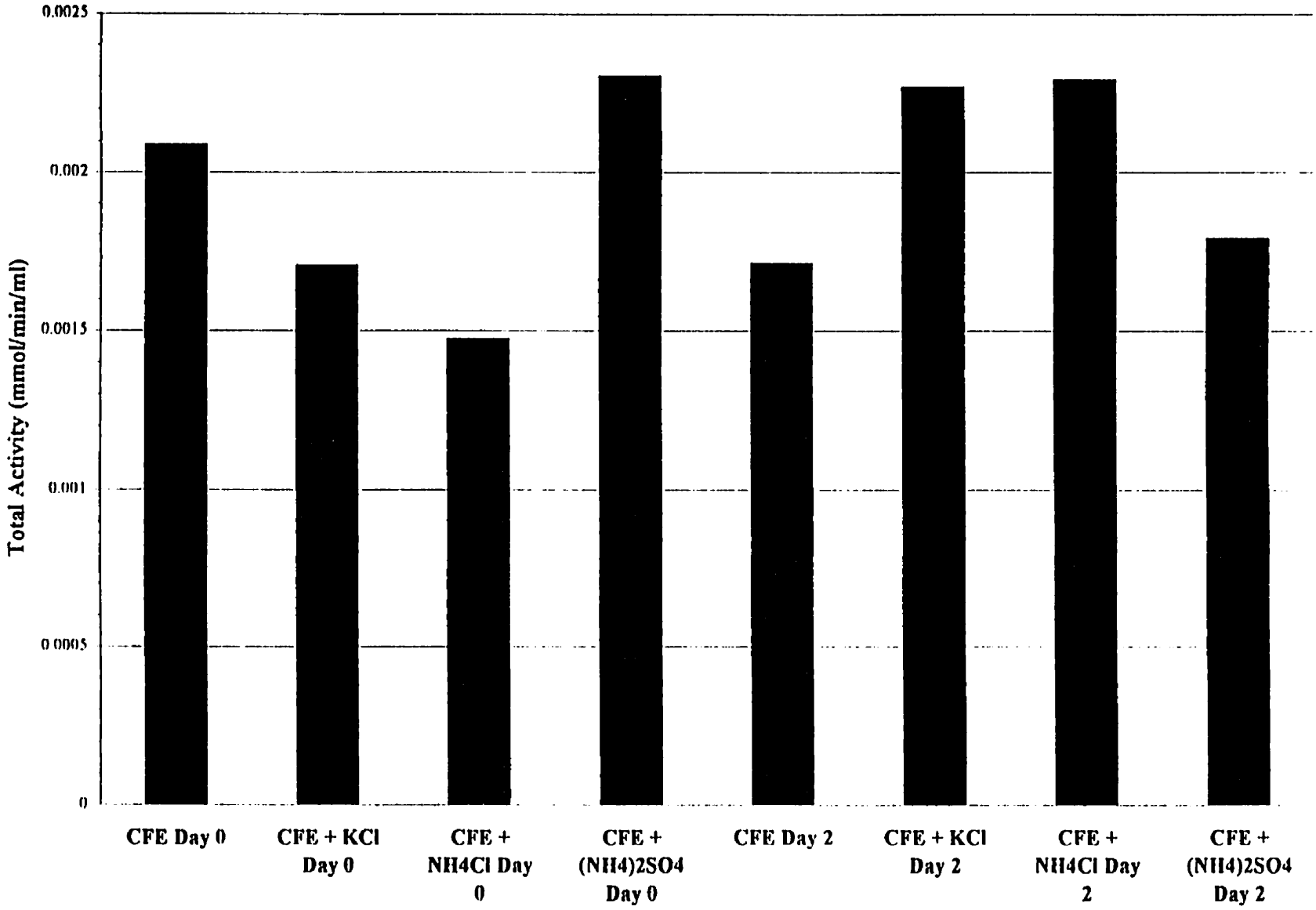

4.0E-03
3.5E-03
3.0E-03
2.5E-03
2.0E-03
1.5E-03
1.0E-03
5.0E-04
0.0E+00
Specific Activity (µmol/min/mg protein)
CFE Day 0
CFE + KCl Day 0
CFE + NH4Cl Day 0
CFE + (NH4)2SO4 Day 0
CFE Day 2
CFE + KCl Day 2
CFE + NH4Cl Day 2
CFE + (NH4)2SO4 Day 2

Figure II.22. Effect of NH_4^+, high salt, and NH_4Cl vs. $(NH_4)_2SO_4$ in the activation of total activity in the $NADP^+$:F_{420} oxidoreductase. Assays were performed twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.


0.0025
0.002
0.0015
0.001
0.0005
0
Total Activity (mmol/min/ml)
CFE Day 0
CFE + KCl Day 0
CFE + NH4Cl Day 0
CFE + (NH4)2SO4 Day 0
CFE Day 2
CFE + KCl Day 2
CFE + NH4Cl Day 2
CFE + (NH4)2SO4 Day 2

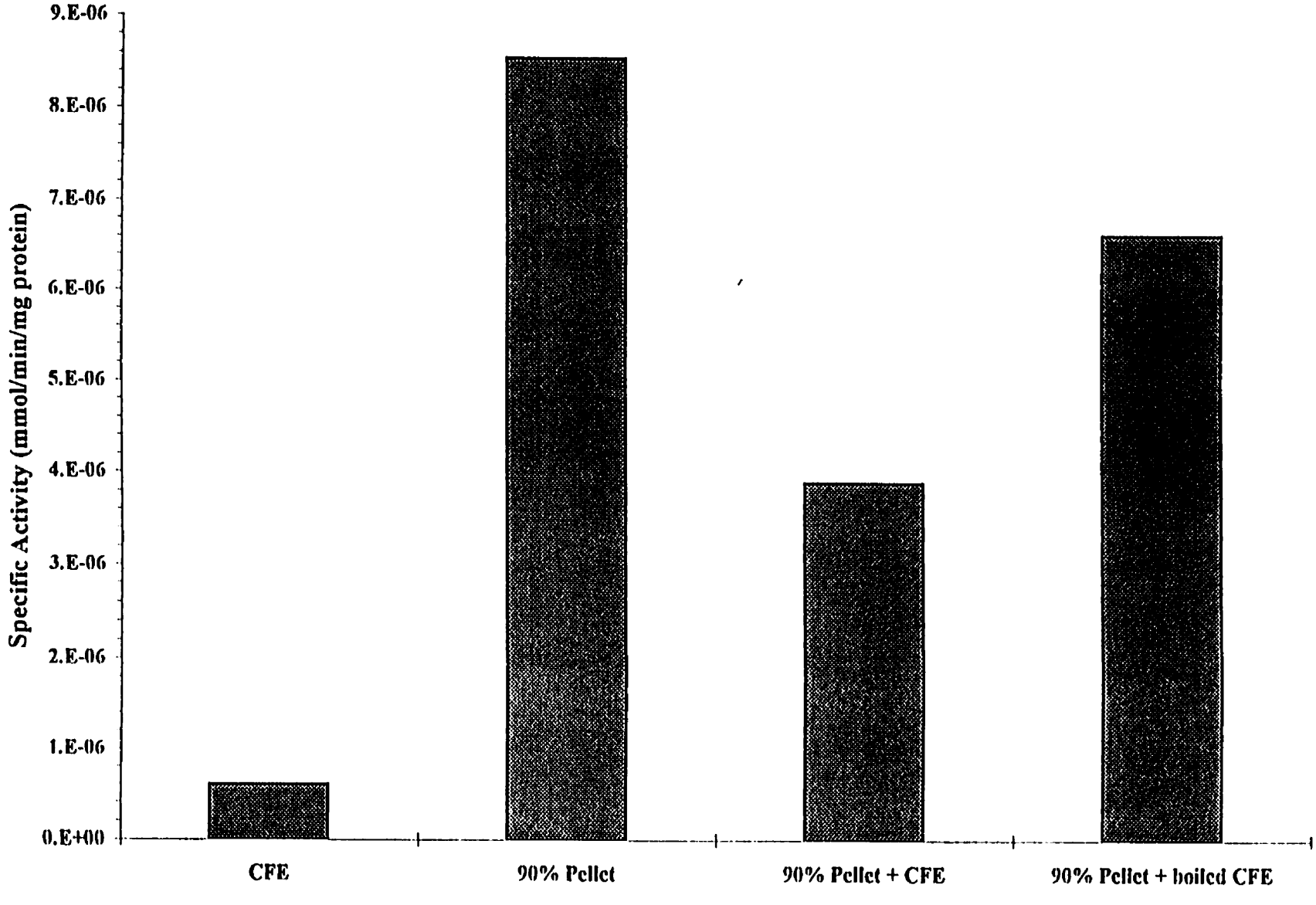
II.3.5. Testing for a Regulatory Co-factor or Co-enzyme

Since the reason for the up to 30-fold increase in specific activity between the CFE and 65-90% $(NH_4)_2SO_4$ pellet proved not to be the high concentration of salt or NH_4^+, the next assumption to test was for the presence of a "regulatory" co-factor or co-enzyme. The experiment consisted of two parts; one testing for the presence of a proteinaceous co-enzyme and the other for a non-proteinaceous co-factor. This was accomplished using CFE to test for the proteinaceous component and boiled CFE (bCFE) to test for the non-proteinaceous co-factor (Figure II.23).

The CFE alone showed a specific activity of 0.0006 μmol/min/mg protein, while the 65-90% $(NH_4)_2SO_4$ pellet showed a specific activity of 0.0085 μmol/min/mg protein. Although this increase was only ~ 15-fold from the CFE to the 65-90% $(NH_4)_2SO_4$ pellet with the batch of cells tested, the effect of the 65-90% $(NH_4)_2SO_4$ fractionation is still clearly visible. When the 65-90% $(NH_4)_2SO_4$ pellet was combined with the CFE, the activity decreased to 0.0039 μmol/min/mg protein, and when the 65-90% $(NH_4)_2SO_4$ pellet was combined with the bCFE, the activity decreased to only 0.0066 μmol/min/mg protein.

This could be explained this way. The CFE showed 0.0006 μmol/min/mg protein, while the 65-90% $(NH_4)_2SO_4$ pellet showed an activity of 0.0085 μmol/min/mg protein. The 65-90% $(NH_4)_2SO_4$ pellet contained approximately the same concentration of protein as the CFE (CFE was 41.75 μg/ml and the 65-90% $(NH_4)_2SO_4$ pellet had 39.55 μg/ml). So, 50 ml of CFE had 2.09 mg of protein and 2 ml of the 65-90% $(NH_4)_2SO_4$ pellet had 1.98 mg of protein in the 65-90% $(NH_4)_2SO_4$ pellet. Thus the CFE contained 0.001254 mmol/min/ 2.09 mg protein and the 65-90% $(NH_4)_2SO_4$ pellet had 0.017424 μmol/min

Figure II.23. Effect on the specific activity of the 65-90% $(NH_4)_2SO_4$ pellet when CFE or bCFE is added to the 65-90% $(NH_4)_2SO_4$ pellet. Assays were performed twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.


9.E-06
8.E-06
7.E-06
6.E-06
5.E-06
4.E-06
3.E-06
2.E-06
1.E-06
0.E+00
Specific Activity (mmol/min/mg protein)
CFE
90% Pellet
90% Pellet + CFE
90% Pellet + boiled CFE

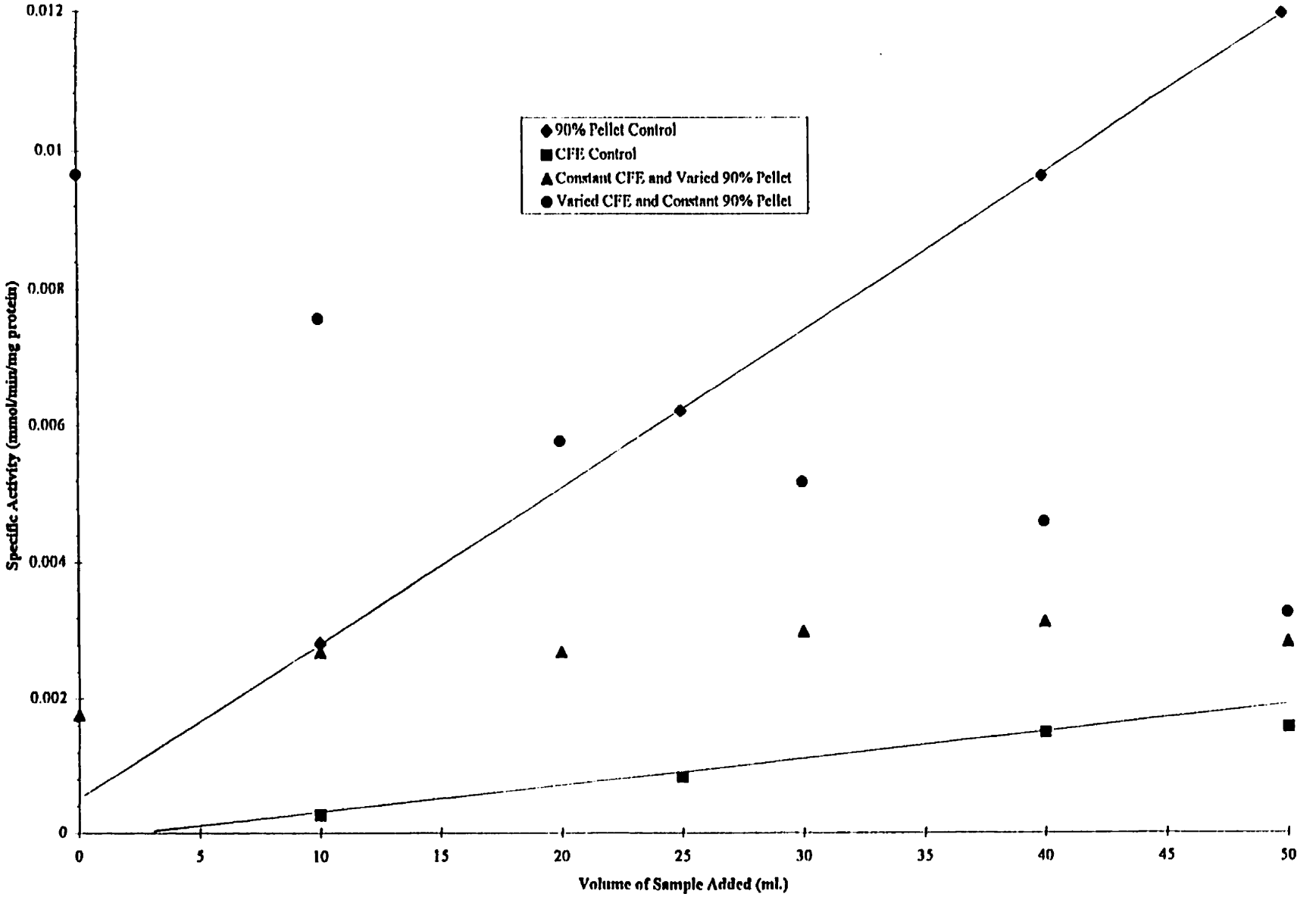
1.98 mg protein. If these values are added together, it gives a value of 0.018678 µmol/min/ 4.07 mg protein or 0.0046 µmol/min/mg protein. This is reasonably close to the value found of 0.0039 µmol/min/mg protein. The addition of the bCFE to the 65-90% $(NH_4)_2SO_4$ pellet showed much less of a change, indicating that there is an effect of some proteinaceous unit on the activity of the enzyme.

II.3.6. Testing for Allosteric Modifiers

As previously stated, in order to explain the large increase seen in specific activity after 65-90% $(NH_4)_2SO_4$ precipitation, it was suggested that there was a loss of some sort of regulatory factor. To test this idea, varying volumes of 65-90% $(NH_4)_2SO_4$ pellet were added to a constant volume of CFE, and vice versa and compared to controls for both the CFE and the 65-90% $(NH_4)_2SO_4$ pellet.

Looking at the specific activities, both the CFE control and the 65-90% $(NH_4)_2SO_4$ pellet controls yielded straight lines, indicating that there is a linear relationship between the amount of sample and the activity per milligram protein (Figure II.24.). That is, there are no synergistic effects seen when increasing concentrations of either the CFE or the 65-90% $(NH_4)_2SO_4$ pellet are used alone in a system. When a constant volume of CFE (50 µl) was added to a system with varying volumes of the 65-90% $(NH_4)_2SO_4$ pellet (5 to 50 µl in increments of 10µl) however, there was almost no difference in specific activity between the system containing 50 µl of CFE and no 65-90% $(NH_4)_2SO_4$ pellet, and that containing 50 µl of each. The system with only CFE had a specific activity of 1.735 µmol/min/mg protein while the system with 50 µl of each sample showed a specific activity of 2.818 µmol/min/mg protein; an increase of 1.05 fold as opposed to the CFE

Figure II.24. Testing for an allosteric effect in the $NADP^+$:F_{420} oxidoreductase activity of the CFE and the 65-90% $(NH_4)_2SO_4$ pellet. Assays were performed twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.


90% Pellet Control
CFE Control
Constant CFE and Varied 90% Pellet
Varied CFE and Constant 90% Pellet
Specific Activity (mmol/min/mg protein)
0.012
0.01
0.008
0.006
0.004
0.002
0
0
5
10
15
20
25
30
35
40
45
50
Volume of Sample Added (ml.)

control which increased by 6.14 fold over a range of 40 µl (10 µl to 50 µl). In the system with a constant volume of 65-90% $(NH_4)_2SO_4$ pellet (50 µl) with increasing volumes of CFE, the specific activity rapidly decreased from 9.646 µmol/min/mg protein, with only 50 µl of 65-90% $(NH_4)_2SO_4$ pellet, to 3.251 µmol/min/mg protein (50 µl CFE + 50 µl 65-90% $(NH_4)_2SO_4$ pellet). This represents a decrease in specific activity of three fold. It is worthy to note that in both systems, when each contained 50 µl of CFE and 50 µl of 65-90% $(NH_4)_2SO_4$ pellet, the specific activity was ~ 3.0 µmol/min/mg protein. This indicates that the results obtained in the two experiments are reliable, in that both systems' final specific activity were highly similar. Also, in the constant CFE system, the assay with only 50 µl of CFE had a specific activity of 1.735 µmol/min mg protein, and the assay for the CFE control with 50 µl of CFE had a specific activity of 1.554 µmol/min/mg protein. These values again are highly similar and lend credence to the validity of the results obtained. A similar situation is seen for the 50 µl assay of the 65-0% $(NH_4)_2SO_4$ pellet; 11.945 µmol/min/mg protein and 9.646 µmol/min/mg protein, respectively.

With respect to total activity, the system with a constant volume of 65-90% $(NH_4)_2SO_4$ pellet and increasing CFE showed no significant increase in total activity when either no CFE was present, or when there were equal volumes of CFE and 65-90% $(NH_4)_2SO_4$ pellet (50 µl). With no CFE present, the total activity was 16.7 µmol/min/ml, while in volumes of 50 µl of CFE and 50 µl 65-90% $(NH_4)_2SO_4$ pellet there was a total activity of 17.18 µmol/min/ml. At 50 µl of each, the total activity dropped to 13.8

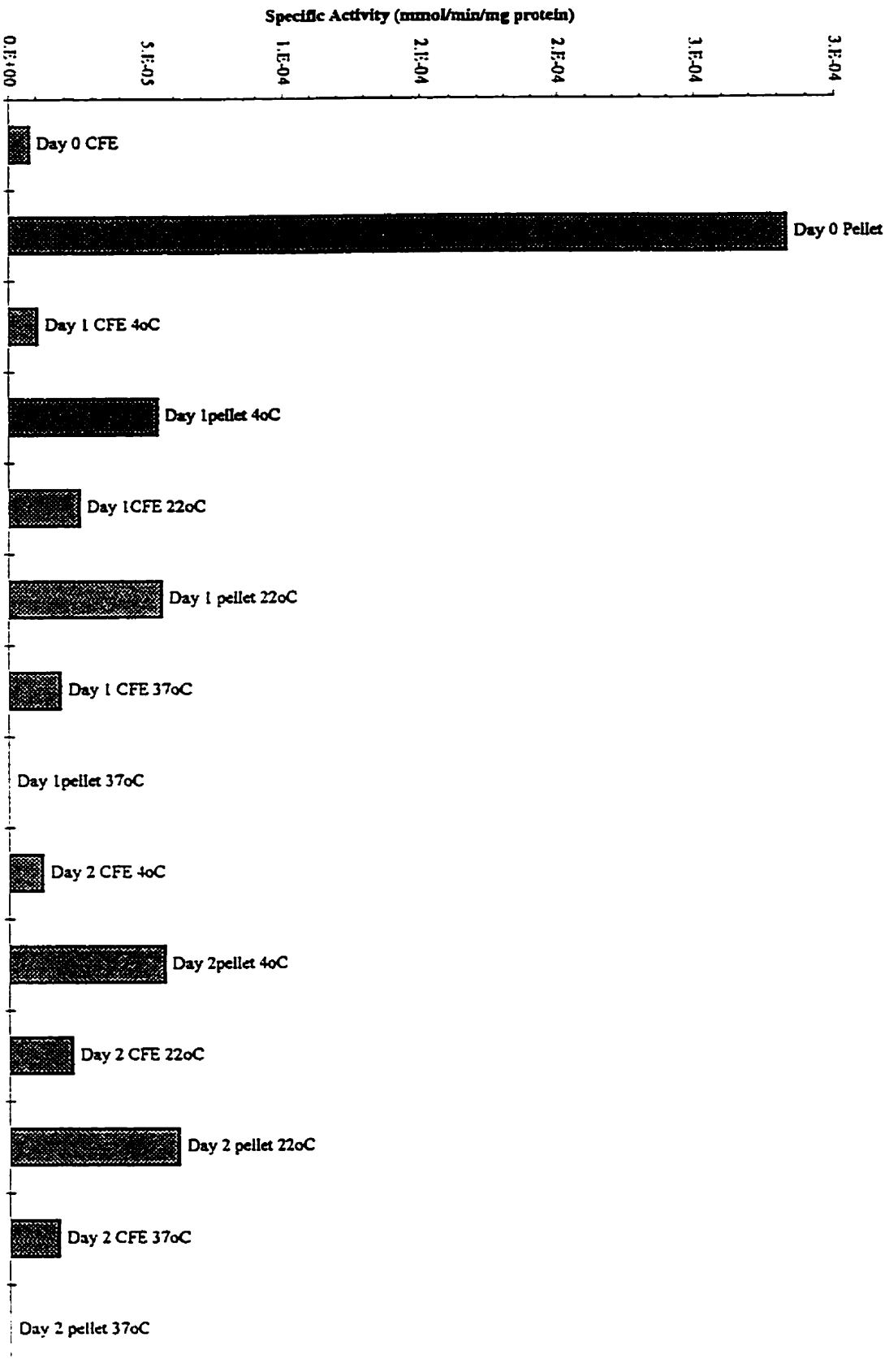
μmol/min/ml, but it is assumed that this was due to experimental error when the trend for the rest of the experiment is considered.

In the system with a constant volume of CFE and increasing volumes of 65-90% $(NH_4)_2SO_4$ pellet, there was a fairly linear increase seen as increasing volumes of the 65-90% $(NH_4)_2SO_4$ pellet were added to the system. At first this appears strange in that if one considers that the possibility of a non-covalently bound co-enzyme was correct, then the smaller volumes of 65-90% $(NH_4)_2SO_4$ pellet shouldn't have an effect on the total activity as the apoenzyme (65-90% $(NH_4)_2SO_4$ pellet) would be overwhelmed by the holoenzyme (CFE) thereby reducing the activity to roughly the levels of the CFE alone. This is in fact the case, further demonstrating the possibility of a non-covalently bound, proteinaceous unit being lost in the 65-90% $(NH_4)_2SO_4$ pellet.

II.3.7. Effect of Temperature on Stability

Both the CFE and the 65-90% $(NH_4)_2SO_4$ pellet were incubated at temperatures of 4°C, 22°C, and 37°C to a). reconfirm previous workers results (mainly in the CFE), and b). To observe whether storage of the 65-90% $(NH_4)_2SO_4$ pellet at a certain temperature could aid in stabilization. In the case of the CFE, the specific activity increased by 40.8% at 4°C, 241.8% at 22°C, and 151.5% at 37°C after 24 hours post-incubation (Figure II.25.). After 24 hours in the 65-90% $(NH_4)_2SO_4$ pellet however, the specific activity decreased by 81.0% at 4°C, 80.4% at 22°C, and at 37°C all activity was lost. By 48 hours post-incubation, the specific activity of the CFE had increased by 62.7% at 4°C, 204.2% at 22°C, and 134.9% at 37°C as compared to time 0 values, whereas the specific activity of the 65-90% $(NH_4)_2SO_4$ pellet had remained roughly constant from 24 hours values.

Figure II.25. Effect of temperature on the stabilization of the specific activity of the 65-90% $(NH_4)_2SO_4$ pellet. Each temperature was tested twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.


Specific Activity (mmol/min/mg protein)
0.E+00
5.E-05
1.E-04
2.E-04
2.E-04
3.E-04
3.E-04
Day 0 CFE
Day 0 Pellet
Day 1 CFE 4oC
Day 1pellet 4oC
Day 1CFE 22oC
Day 1 pellet 22oC
Day 1 CFE 37oC
Day 1pellet 37oC
Day 2 CFE 4oC
Day 2pellet 4oC
Day 2 CFE 22oC
Day 2 pellet 22oC
Day 2 CFE 37oC
Day 2 pellet 37oC

II.3.8. Stabilization of the 65-90% $(NH_4)_2SO_4$ Pellet Activity

Various attempts were made to stabilize the 65-90% $(NH_4)_2SO_4$ pellet. The 65-90% $(NH_4)_2SO_4$ pellet was resuspended in different solutions to determine what, if any, chemical composition could prevent the loss of activity previously seen when the 65-90% $(NH_4)_2SO_4$ pellet was resuspended in harvesting buffer pH 7.5 (as described above). All assays were performed at room temperature with the 65-90% $(NH_4)_2SO_4$ resuspended pellet being stored at 4°C, except for temperature experiments.

The first attempts were made using solutions of 20% glycerol in harvesting buffer at pH 7.5, 20% ethylene glycol in harvesting buffer at pH 7.5, and 500mM sucrose in harvesting buffer at pH 7.5. With respect to total activity, the control sample (harvesting buffer pH 7.5 alone) lost 62.5% of its total activity within 52 hours post-incubation, decreasing from 12.6 µmol/min/ml to 4.70 µmol/min/ml, and remained constant thereafter (Figure II.26.). Similar results were seen for the 20% glycerol solution, with a loss of 56.9% from 13.5 µmol/min/ml to 5.90 µmol/min/ml within 52 hours post-incubation. However, the 20% ethylene glycol and 500mM sucrose solutions decreased slightly after 29 hours post-incubation from time 0 values, but by 52 hours post-incubation had rebounded to slightly above time 0 values. At 98 hours post-incubation, both the 20% ethylene glycol and 500 mM sucrose solutions remained very close to time 0 values.

With respect to the specific activity, the control sample decreased from 0.77 µmol/min/mg protein to 0.22 µmol/min/mg protein by 52 hours post-incubation, a loss of 62.5% of the initial specific activity, and to 0.4 µmol/min/mg protein by 98 hours post-incubation, a loss of 71.5% of the initial specific activity (Figure II.27.). The 20%

Figure II.26. Attempts to stabilize the total activity of the 65-90% $(NH_4)_2SO_4$ pellet of the $NADP^+$:F_{420} oxidoreductase with ethylene glycol, glycerol, and sucrose. All samples were kept at 4°C and tested at room temperature. Assays were performed twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.

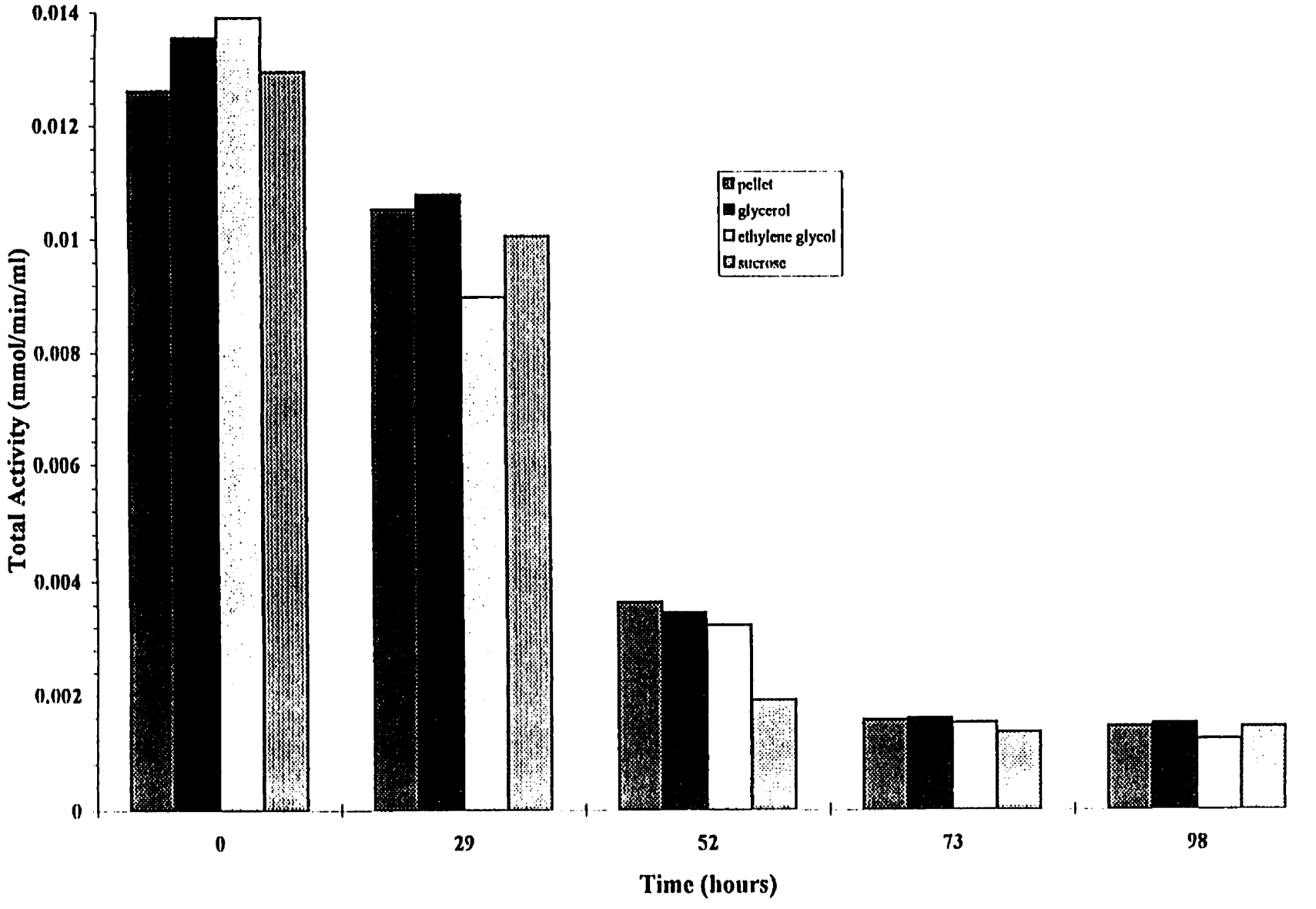
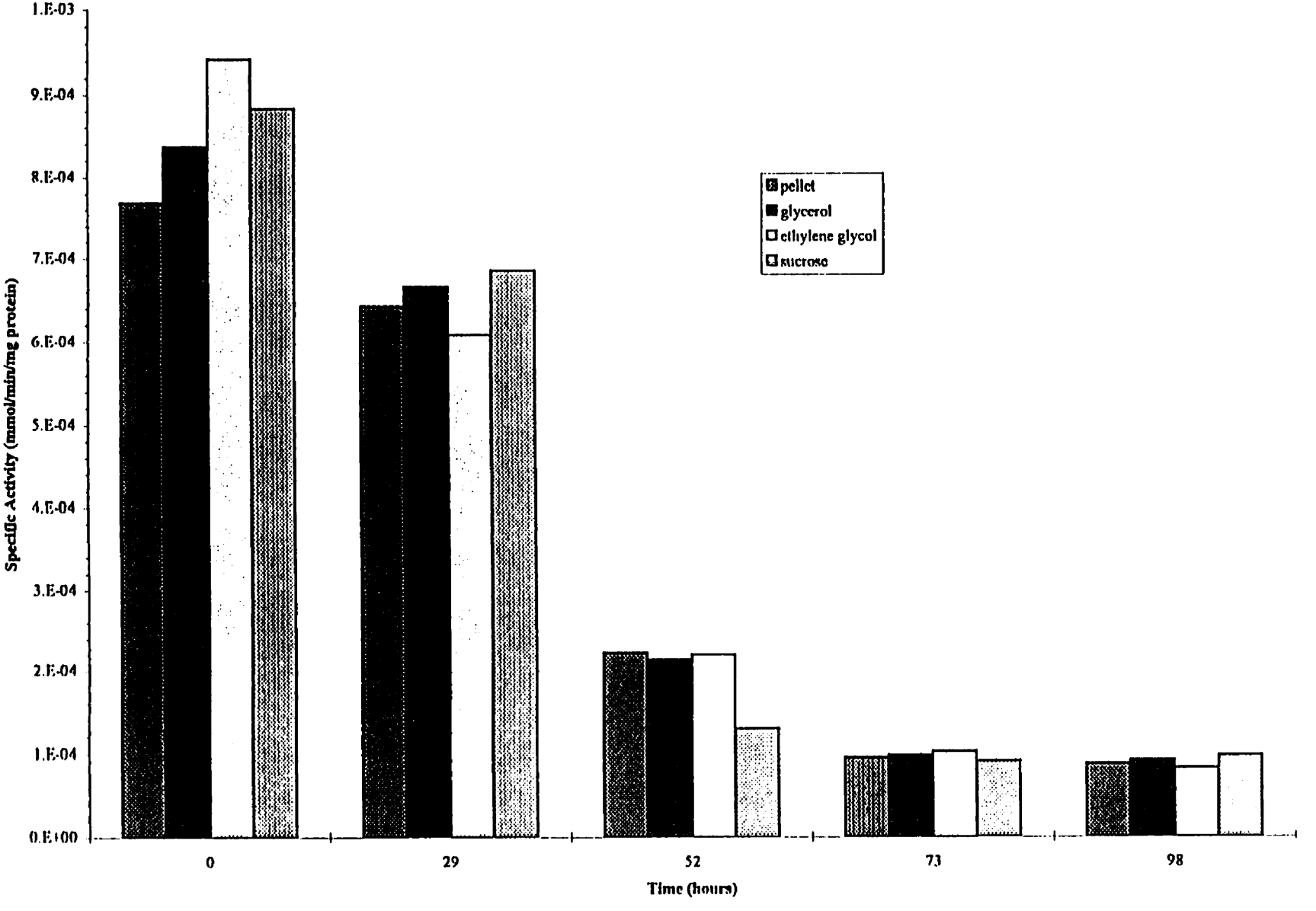

0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Total Activity (mmol/min/ml)
pellet
glycerol
ethylene glycol
sucrose
0
29
52
73
98
Time (hours)

Figure II.27. Attempts to stabilize the specific activity of the 65-90% $(NH_4)_2SO_4$ pellet of the $NADP^+$:F_{420} oxidoreductase with ethylene glycol, glycerol, and sucrose. All samples were kept at 4°C and tested at room temperature. Assays were performed twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.


1.E-03
9.E-04
8.E-04
7.E-04
6.E-04
5.E-04
4.E-04
3.E-04
2.E-04
1.E-04
0.E+00
Specific Activity (mmol/min/mg protein)
pellet
glycerol
ethylene glycol
sucrose
0
29
52
73
98
Time (hours)

glycerol solution followed a similar pattern with a loss of 56.5% and 72.5% of the initial specific activity after 52 and 98 hours of incubation, respectively. Both the 20% ethylene glycol and 500mM sucrose solutions decreased at 29 hours post-incubation, but inexplicably increased to above time 0 values at 52 hours post-incubation. By 73 hours post-incubation however, both had significantly decreased, with the 20% ethylene glycol solution showing a loss of 72.9% of its initial specific activity, and the 500mM sucrose solution losing 72.4% of its initial specific activity. Both remained relatively constant from 73 to 98 hours post-incubation.

Solutions with a final concentration of 1mM dithiothreitol (DTT), 1 mM 2-mercaptoethanol, and 1 M KCl were also used to attempt to stabilize the 65-90% $(NH_4)_2SO_4$ pellet. The most interesting result was that of 1mM 2-mercaptoethanol. Immediately upon addition of the 2-mercaptoethanol solution to the 65-90% $(NH_4)_2SO_4$ pellet, all activity, both total (Figure II.28.) and specific (Figure II.29.), was lost and remained at 0 for the length of the 96 hour experiment.

The total activity was decreased to almost 0 in all cases by 48 hours p.i. The specific activity remained constant for the first 24 hours of incubation, but by 48 hours post-incubation, there was a loss of 49.6% of initial specific activity, and remained constant for the remainder of the experiment with a similar loss in all others similar to that seen in the total activity.

The final attempt made at stabilizing the 65-90% $(NH_4)_2SO_4$ pellet was to use the harvesting buffer at different pH's to determine if a change in the [H^+] would stabilize the enzyme, since H-bonding and van der Waals forces may have played a role in stabilizing the quaternary structure of the enzyme. Also, different buffers are routinely

Figure II.28. Effect of DTT, 2-mercaptoethanol, and KCl on the total activity of the 65-90% $(NH_4)_2SO_4$ pellet. All samples were kept at 4°C and tested at room temperature. Assays were performed twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.

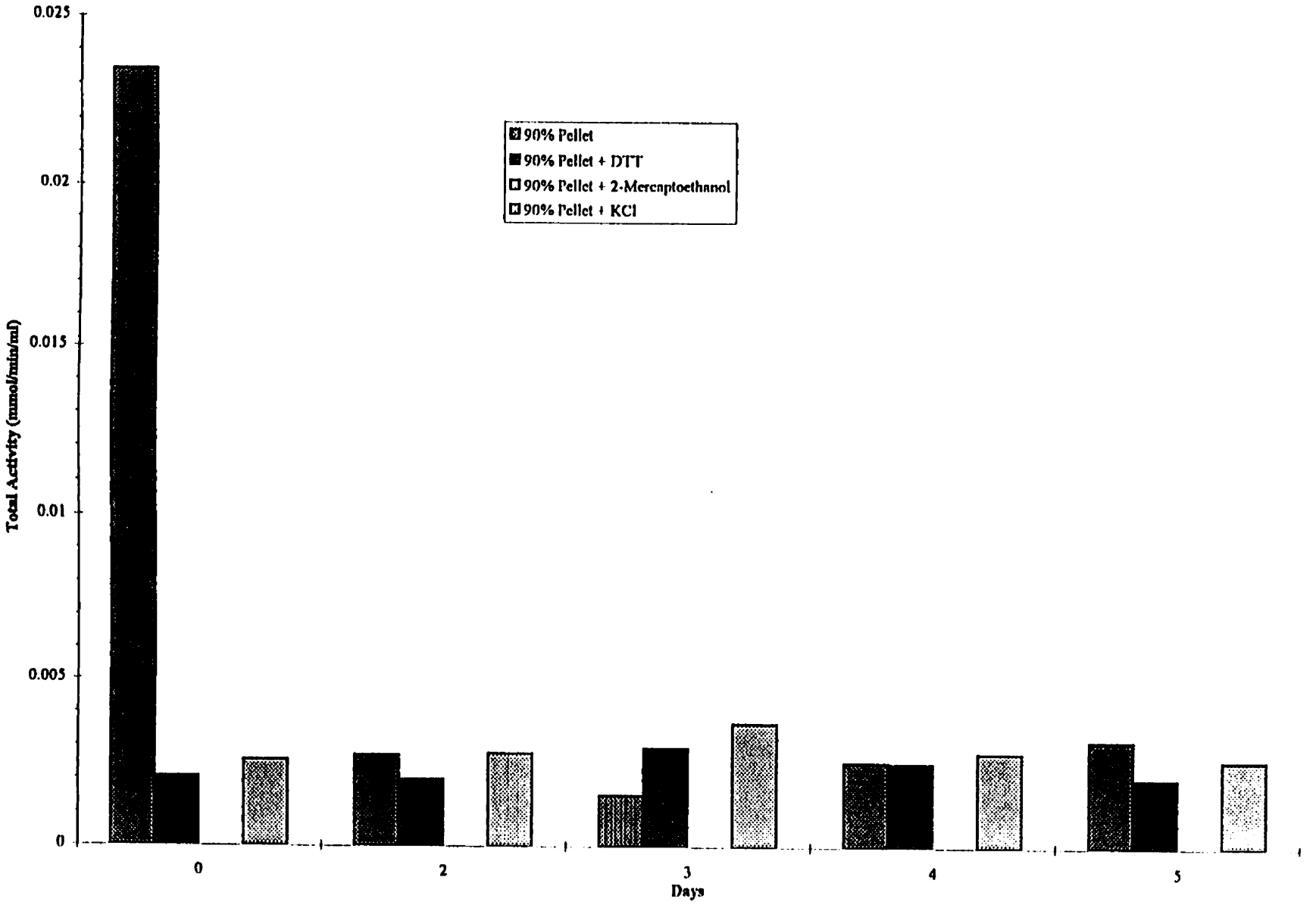
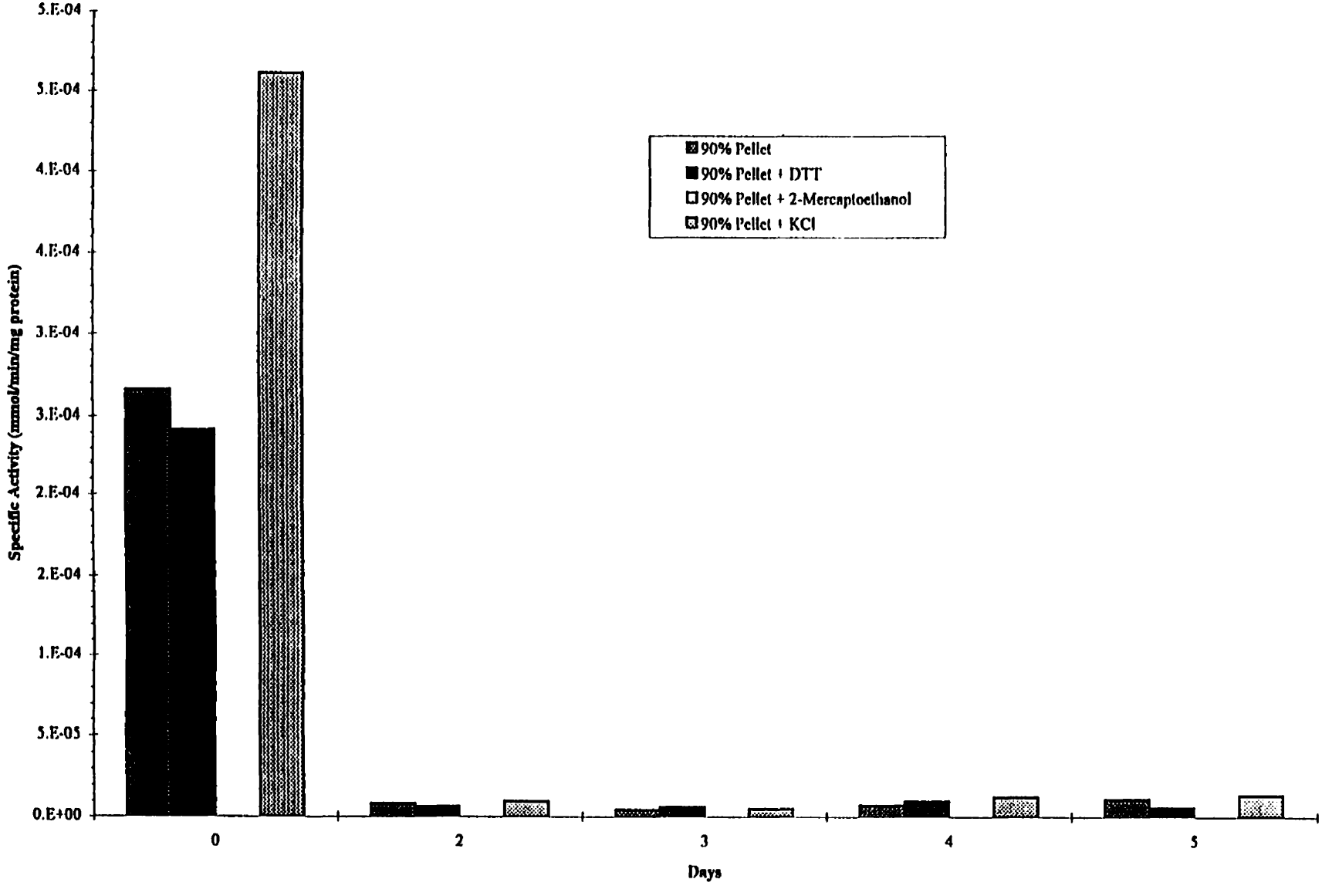

0.025
0.02
0.015
0.01
0.005
0
Total Activity (nmol/min/ml)
90% Pellet
90% Pellet + DTT
90% Pellet + 2-Mercaptoethanol
90% Pellet + KCl
0
2
3
4
5
Days

Figure II.29. Effect of DTT, 2-mercaptoethanol, and KCl on the specific activity of the 65-90% $(NH_4)_2SO_4$ pellet. All samples were kept at 4°C and tested at room temperature. Assays were performed twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.


5.E-04
5.E-04
4.E-04
4.E-04
3.E-04
3.E-04
2.E-04
2.E-04
1.E-04
5.E-05
0.E+00
Specific Activity (mmol/min/mg protein)
90% Pellet
90% Pellet + DTT
90% Pellet + 2-Mercaptoethanol
90% Pellet + KCl
0
2
3
4
5
Days

used to stabilize and maintain the activity of different enzymes were used at their respective pK_a's for the same reason as above.

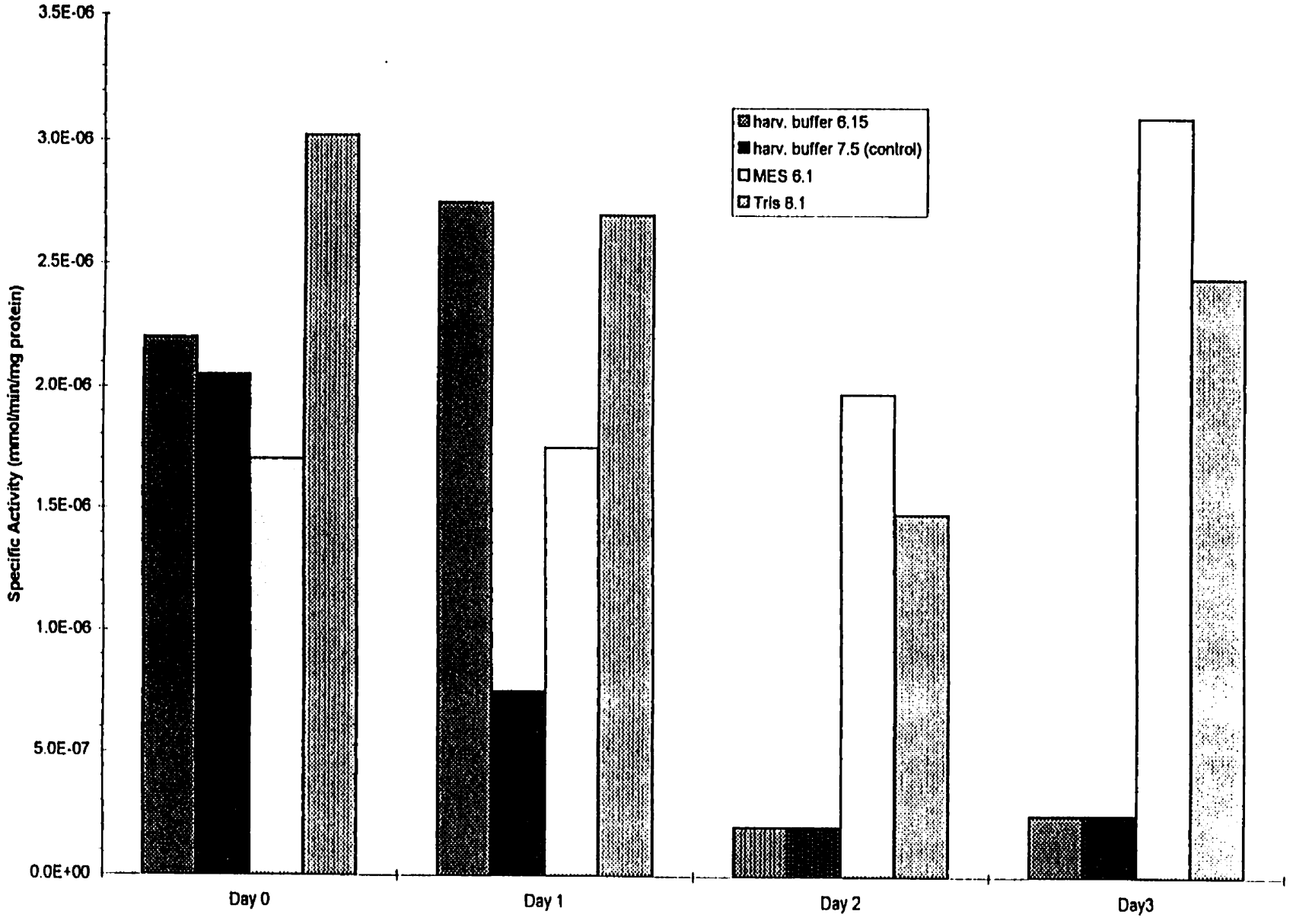
With respect to specific activity, at time zero all buffers were at least as high in activity as the control sample (harvesting buffer pH 7.5) with the exception of MES pH 6.1. By 48 hours p.i., the control had lost 20.3% of its initial activity, while the harvesting buffer pH 6.1 had increased by 20.5% (but had lost 35% of its initial activity by 96 hours). Tris-HCl pH 8.1 however had only lost 9.7% of initial activity by 48 hours p.i., and by 96 hours p.i. had lost 17.4%, and after 120 hours p.i., the enzyme had only lost 19.5% of its initial activity. In comparison to the control, the control had lost 31.2% by 96 hours p.i., and 46.0% by 120 hours p.i. Although TEA pH 7.8 had the highest activity at time zero, by 48 hours p.i. the activity had decreased by 77.8%. (Figure II.30.).

Once it was confirmed that 20 mM Tris-HCl pH 8.1 was the best solution for stabilization of the 65-90% $(NH_4)_2SO_4$ pellet at 4°C, the buffer was tried at strengths of 20 mM, 30 mM, and 50 mM. This was done to see if there could be further retention of the initial activity with a higher strength of the buffer. There was negligible difference between the three strengths of buffer, so it was decided that 20 mM would be the buffer system used for the purification of the enzyme.

II.4. Discussion

Some interesting observations were made with respect to the kinetics of the partially purified enzyme. First, the total and specific activity were found to possess a peak activity at mid to late log of the growth curve. This makes sense in that a biosynthetic enzyme should be working at a maximum rate when cells are growing and dividing at an

Figure II.30. The effect of pH and Different Buffers on Stabilization of the Specific Activity in the 65-90% $(NH_4)_2SO_4$ pellet. All samples were kept at 4°C and tested at room temperature. Assays were performed twice in triplicate, with each point representing the average value with less than 10% variation. When higher variability was observed error bars are shown.


3.5E-06
3.0E-06
2.5E-06
2.0E-06
1.5E-06
1.0E-06
5.0E-07
0.0E+00
Specific Activity (mmol/min/mg protein)
harv. buffer 6.15
harv. buffer 7.5 (control)
MES 6.1
Tris 8.1
Day 0
Day 1
Day 2
Day3

exponential rate. The $(NH_4)_2SO_4$ treatments over the growth curve indicate that at any point in the growth of the organism, the enzyme will consistently precipitate in the 65-90% $(NH_4)_2SO_4$ pellet.

The decreased stability of the enzyme after $(NH_4)_2SO_4$ treatment is an interesting observation in that it could indicate an alteration in the enzyme in some way, or indicate a decrease in stability when diluted. The fact that this instability was corrected with Tris-Cl at pH 8.1 and MES at 6.1 is curious in that the maximal activity for NADPH oxidation is 8.5 and 6.0 for $NADP^+$ reduction.

This reduction in activity did not appear to be due to the actions of a proteinase as tests with various proteinase inhibitors had no effect on the stability of the enzyme. The fact that the large increase in both total and specific activity was due only to $(NH_4)_2SO_4$, and not NH_4^+ or a high salt content further indicates that it is not a preference for high ionic strength or high salt that stimulates the enzyme, but that some alteration in the enzyme itself may be occurring.

The tests performed for an allosteric effect indicate even further that there was an alteration in the activity of the enzyme after $(NH_4)_2SO_4$. The linear relationship between activity and protein additions for both the CFE and the 65-90% $(NH_4)_2SO_4$ pellet were straight lines, but a mixing of the two samples gave curves heading towards the highest amount of sample on each calibration line. Even more convincing than this was the observation that when CFE was mixed with 65-90% $(NH_4)_2SO_4$ pellet, there was an immediate decline in activity.

The K_m's of the CFE and 65-90% $(NH_4)_2SO_4$ pellet indicate that the enzyme catalyzes a first order reaction when in the CFE for $NADP^+$, NADPH, F_{420} and $F_{420}H_2$. However,

the only substrate to remain first order was F_{420}, while all others showed non-first order kinetics. This indicates a change in the affinity of the enzyme for the substrates. If there is a non-covalently bound proteinaceous subunit being cleaved, then it is also possible that this subunit is being lost over the course of time after cell lysis.

Therefore, evidence points towards the possible loss of a non-covalently bound proteinaceous subunit by $(NH_4)_2SO_4$ treatment which performs some regulatory function *in vivo*. This may be the method by which *Msph. stadtmanae* controls the $NADP^+$/NADPH ratio *in vivo*. In preparation for the purification of the enzyme (see chapter V), it was found that preservation of activity over time was best using Tris-Cl at pH 8.1 and MES at 6.1.

Chapter III

Investigation of the $NADP^+$:F_{420} Oxidoreductase in Methanogens

III.1. Introduction

NADPH is the major source of electrons for anabolic reactions in most organisms, including methanogens (Harris, 1995). In hydrogenotrophic methanogens the major source of electrons for $NADP^+$ reduction is F_{420} (8-hydroxy-5-deazaflavin), which receives its electrons from hydrogen oxidation via the F_{420}-dependent hydrogenase. In the case of anabolism, these electrons are used for the reduction of $NADP^+$, and NADPH is then used in a variety of anabolic pathways. The enzyme mainly responsible for controlling the size of the $NADP^+$/NADPH intracellular pool is the $NADP^+$:F_{420} oxidoreductase. Since the $NADP^+$:F_{420} oxidoreductase plays such a crucial role in anabolic metabolism in methanogens, it is interesting that this enzyme has not been widely studied, even in otherwise well-studied organisms as *Methanosarcina barkeri*. $NADP^+$:F_{420} oxidoreductase have been purified and characterized from *Methanococcus vannielii* (Yamazaki and Tsai, 1980) and *Streptomyces griseus* (Eker et. al., (1989), along with an $NADP^+$ reductase from *Methanobacterium thermoautotrophicum* ΔH (Eirich and Dugger, 1984). On this basis, this enzyme has been expected to be present in methanogens in general.

However, due to the scarcity of literature on this particular enzyme, a survey was conducted on a variety of methanogenic organisms including *Methanobacterium thermoautotrophicum* strain Marburg, *Methanobacterium* strain G2R, *Methanococcus*

thermolithotrophicus, *Methanococcus voltae*, *Methanosphaera stadtmanae* (grown both on H_2:CO_2/methanol and H_2:CO_2/methanol/2-propanol), *Methanosphaera caniculi*, *Methanospirillum hungatei*, *Methanolobus tindarius*, *Methanosarcina barkeri* strain Fusaro, and *Methanosaeta concilli.*

The objectives of the survey were: 1) to determine the presence of either an $NADP^+$:F_{420} oxidoreductase or an $NADP^+$ reductase/NADPH oxidase in each of the respective organisms, 2) to determine the dominant direction of each enzyme under the in vitro testing conditions, and 3) determine if large increases in both total and specific activity seen previously in *Methanosphaera stadtmanae* (H_2:CO_2/methanol) (Chapter II) could be observed in other extracts after $(NH_4)_2SO_4$ treatment.

Methanosphaera stadtmanae was tested in the presence and absence of 2-propanol because 2-propanol oxidation provides a direct source of NADPH, (Wong et. al., 1994). When grown in the absence of 2-propanol, $NADP^+$ should only be reduced by the $NADP^+$:F_{420} oxidoreductase via electrons from $F_{420}H_2$. When 2-propanol is present, it is oxidized to acetone via the $NADP^+$-dependent secondary alcohol dehydrogenase (2-ADH), thereby reducing $NADP^+$ to NADPH. It is possible that, the amount of activity in the $NADP^+$:F_{420} oxidoreductase may be altered as the $NADP^+$/NADPH pool can be recycled from a different source.

III.2. Materiais and Methods

III.2.1. Chemicals and Materials

All chemicals and buffers were of reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO). The culture bottles used (1 L. volume) were purchased from Corning. Assay tubes and stoppers were purchased from Canlab.

III.2.2. Microorganisms and culturing conditions

In all organisms used, Na_2S-$9H_2O$ was added as described in section II.2.2 of this thesis. The protocols of anaerobic media preparation, based on Daniels et. al., (1986), have been previously described in chapter II.2.2.

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

NaCl	0.3g/L
$MgCl_2$-$6H_2O$	0.064g/L
NH_4Cl	0.40g/L
Na Acetate	0.42g/L
K_2HPO_4	0.82g/L
$CaCl_2$-$2H_2O$	0.064g/L
Resasurin (25mg/100 ml stock solution)	1 ml/L
Na_2CO_3	1.0g/L
Mineral Elixir	10.0 ml/L
Vitamin Supplement	10.0 ml/L

Mst. concilli (DSM 3671) was grown at 35°C, pH 7.0 under N_2:CO_2 (80:20, v/v) as described by Patel (1984b):

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
Resasurin (25mg/100 ml stock solution)	1.0 ml/L
Mineral Elixir	10.0 ml/L
Vitamin Supplement	10.0 ml/L
Na Acetate	6.80g/L

Ms. barkeri Fusaro (DSM 804) was grown at 37°C, pH 7.0 under H_2:CO_2 (80:20, v/v) as described by Daniels et. al., (1986):

K_2HPO_4	0.35g/L
KH_2PO_4	0.23g/L
NH_4Cl	0.50g/L
Yeast Extract	2.00g/L
$MgSO_4$-$7H_2O$	0.50g/L
$CaCl_2$-$2H_2O$	0.25g/L
NaCl	2.25g/L
$NaHCO_3$	0.85g/L

Resasurin (25mg/100 ml stock solution)	1 ml/L
Mineral Elixir	10.0 ml/L
Vitamin Supplement	10.0 ml/L

Ml. tindarius (DSM 2278) was grown at 25°C, pH 6.5 under N_2:CO_2 (80:20, v/v) as described by Konig 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
Resasurin (25mg/100 ml stock solution)	1.00 ml/L
Mineral Elixir	10.0 ml/L
Vitamin Supplement	10.0 ml/L
Methanol (40% sterile stock solution, after autoclaving)	5.0 ml/L

Mb. thermoautotrophicum (DSM 2133) was grown at 65°C under H_2:CO_2 (80:20, v/v) as described by Daniels et. al., (1986):

KH_2PO_4	0.42g/L

K_2HPO_4	0.23g/L
$MgCl_2$-$6H_2O$	0.04g/L
$CaCl_2$-$2H_2O$	0.03g/L
NaCl	0.595g/L
NH_4Cl	0.70g/L
Na_2CO_3	0.16g/L
Resasurin (25mg/100 ml stock solution)	1.00 ml/L
Mineral Elixir	10.0 ml/L

Msph. stadtmanae (DSM 3091) was grown at 37°C, pH 7.0 under H_2:CO_2 (80:20, v/v) as described by Miller and Wolin, (1985), with some modification (Sparling et. al., 1993b) as described in chapter II.

Mc. voltae (DSM 1537) was grown at 35°C, pH 7.0 under H_2:CO_2 (80:20, v/v) as described by 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
Na Acetate	1.00g/L
Resasurin (25mg/100 ml stock solution)	1.00 ml/L
Mineral Elixir	10.0 ml/L
Vitamin Supplement	10.0 ml/L

Mc. thermolithotrophicus (DSM 2095) was grown at 65°C, pH 6.0 with formate, under N_2:CO_2 (80:20, v/v) as described by Sparling and Daniels, (1986); chapter II.2.2.

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

Cultures were grown to approximately A_{660}=0.8. Cells were harvested and pressed using the French Pressure Cell Press immediately after removal from the growing environment. Harvesting and preparation procedures were the same for all organisms as detailed for *Msph. stadtmanae* in Chapter II.2.2.

III.2.4. Preparation of the 65-90% $(NH_4)_2SO_4$ pellet from CFE

All organisms were subjected to $(NH_4)_2SO_4$ fractionation for kinetic analysis. The procedure was the same as detailed for *Msph. stadtmanae*; chapter II.2.5.

III.2.5. Assay Conditions for the $NADP^+$:F_{420} Oxidoreductase

All assay conditions were identical to those used for kinetic assays for *Msph. stadtmanae*; chapter II.2.6. All assays were done in duplicate. Experiments were repeated two times to ensure consistency in results.

III.2.6. K_m Determinations for $NADP^+$:F_{420} Oxidoreductases of Various Methanogens

Assays were conducted for some methanogens. The assay conditions and procedures were as outlined in chapter II.2.9. All assays were done in duplicate.

III.2.7. Reduction of F_{420} to $F_{420}H_2$

The procedure for the reduction of F_{420} for kinetic analysis was the same for all organisms tested as detailed in chapter II.2.3.

III.2.8. Protein Determination

Protein content for all organisms was determined in the same manner as detailed in chapter II.2.4.

III.3. Results

III.3.1. Determination of the Presence of $NADP^+$:F_{420}-dependent Activities

a.). NADPH Oxidation

For this section, the reader is referred to Table III.1. Each of the organisms tested were capable of reducing $NADP^+$ and oxidizing NADPH in the CFE. Under the growth

Table III.1.: Average Total and Specific Activities of Various Methanogens for NADPH Oxidation

Organism	CFE		65% s		0-65% p		90% s		65-90% p	
	TA	SA	TA	SA	TA	SA	TA	SA	TA	SA
Mb. thermoautotrophicum	0.13	0.11	0.31	0.16	0.04	0.08	0.46	4.11	0.02	0.25
Mb. G2R	0.37	0.39	0.48	2.01	0.01	0.06	0.75	4.70	0.01	0.20
Msph. stadtmanae[a]	1.98	0.94	5.00	29.10	0.17	0.26	0.30	11.70	4.50	11.70
Msph. stadtmanae[b]	2.92	0.47	4.96	8.10	0.30	0.15	0.61	3.27	1.47	4.25
Msph. caniculi	0.58	0.12	2.62	20.55	0.05	0.11	0.80	4.44	0.63	13.55
Msp. hungatei	0.19	0.19	0.49	3.24	0.02	0.03	0.52	3.47	0.02	0.29
Ml. tindarius	0.13	0.07	0.17	0.57	0.01	0.01	0.41	1.73	0.01	0.16
Ms. barkeri	0.19	0.06	0.15	0.15	0.02	0.01	0.41	1.72	0.02	0.06
Mst. concilli	0.14	0.08	0.23	1.52	0.04	0.07	0.28	1.59	0.01	0.17
Mc. thermolithotrophicus	0.18	0.11	0.18	0.23	0.03	0.09	0.26	0.80	0.01	0.04
Mc. voltae	0.65	0.03	0.16	0.65	0.01	0.01	0.16	0.49	0.02	0.02

a-grown on H_2/CO_2 + 0.4% Methanol
b-grown on H_2/CO_2 + 0.4% Methanol + 0.4% 2-propanol
TA=Total Activity (nmol/min/ml CFE)
SA=Specific Activity (nmol/min/mg protein)

conditions provided, the total activity for *Msph. stadtmanae* in the presence of 2-propanol was 2.9 nmol/min/ml CFE while *Msph. stadtmanae* without 2-propanol was 2.0 nmol/min/ml CFE. The next highest total activities were for *Msph. caniculi* at 0.6 nmol/min/ml CFE and *Mb.* G2R at 0.4 nmol/min/ml CFE. All other organisms were between 0.06 and 0.2 nmol/min/ml CFE.

The specific activity for *Msph. stadtmanae* with or without 2-propanol and in *Msph. caniculi* were close to 1.0 nmol/min/mg protein. *Mb.* G2R followed at 0.4 nmol/min/mg protein, with all other organisms having less than 0.1 nmol/min/mg protein under the conditions tested.

After 65% saturation with $(NH_4)_2SO_4$, there was up to 3-fold increase in total activity in all organisms tested except for *Msph. caniculi*, which showed a 4.5-fold increase in total activity. With respect to specific activity in the 65% $(NH_4)_2SO_4$ supernatant, most organisms showed an 8 to 20-fold enrichment in specific activity except for *Ms. barkeri*, *Mb.* G2R, and *Mc. thermolithotrophicus* which had far less of an increase. *Msph. stadtmanae* without 2-propanol had the highest specific activity increase of over 30-fold.

In all cases there was little activity seen in the 0-65% $(NH_4)_2SO_4$ pellet. *Msph. stadtmanae* with and without 2-propanol had the highest total and specific activities. Total activity with and without 2-propanol was 0.3 and 0.17 nmol/min/ml CFE, respectively with all others ranging from 0.01-0.05 nmol/min/ml CFE. This represented approximately 7% of the total activity found in the CFE. Specific activity in *Msph. stadtmanae* with and without 2-propanol was 0.15 and 0.26 nmol/min/mg protein, respectively with all others ranging from 0.01-0.11 nmol/min/mg protein.

The 90% $(NH_4)_2SO_4$ supernatant yielded some interesting results. For all organisms tested except for those of genus *Methanosphaera*, this is the $(NH_4)_2SO_4$ fraction which had the highest specific activity. As for total activity, Methanococcales ranged from 0.16-0.25 nmol/min/ml CFE while Methanomicrobiales ranged from approximately 0.4-0.5 nmol/min/ml CFE except for *Mst. concilli* which had a total activity of 0.27 nmol/min/ml CFE. Methanobacteriales was somewhat variable. *Mb. thermoautotrophicum* had a total activity of 0.46 nmol/min/ml CFE while *Mb.* G2R showed 0.75 nmol/min/ml CFE. *Msph. caniculi* had 0.8 nmol/min/ml CFE while *Msph. stadtmanae* with 2-propanol had 0.62 nmol/min/ml CFE. *Msph. stadtmanae* without 2-propanol had 0.30 nmol/min/ml CFE. This indicates that there may be more than one isoenzyme type present with different properties. These different types may be induced under the availability of different electron donors.

Although *Msph. stadtmanae* without 2-propanol showed low total activity in the 90% $(NH_4)_2SO_4$ supernatant, it had the highest specific activity of 11.7 nmol/min/mg protein. *Msph. stadtmanae* with 2-propanol showed only 3.3 nmol/min/mg protein in contrast. The rest of Methanobacteriales (*Msph. caniculi*, *Mb.* G2R, *Mb. thermoautotrophicum*) all had specific activities in the range of 4.1-4.7 nmol/min/mg protein. For Methanomicrobiales, *Msp. hungatei* showed the highest activity of 3.47 nmol/min/mg protein while *Mst. concilli*, *Ms. barkeri*, and *Ml. tindarius* all had specific activities of 1.6-1.7 nmol/min/mg protein. Methanococcales showed by far the lowest specific activities ranging from 0.49-0.8 nmol/min/mg protein. However, such differences in specific activities are probably not significant considering batch to batch variations and the possibility of a sharp peak of activity in the growth curve as seen in *Msph. stadtmanae*

without 2-propanol. For the most part, batch to batch variations were on the order of 4-5-fold with respect to specific activity.

The 65-90% $(NH_4)_2SO_4$ pellet also showed differences. *Msph. stadtmanae* without 2-propanol showed a total activity of 4.5 nmol/min/ml CFE as opposed to without 2-propanol having a total activity of 1.47 nmol/min/ml CFE. *Msph. caniculi* was 2.5 times lower having a total activity of only 0.63 nmol/min/ml CFE. All other organisms ranged from 0.01-0.02 nmol/min/ml CFE with the exception of *Mc. thermolithotrophicus* which showed no activity.

Specific activity in this fraction showed a similar trend. Genus *Methanosphaera* had by far the highest activities, but *Msph. caniculi* had the highest at 13.6 nmol/min/mg protein, followed closely by *Msph. stadtmanae* without 2-propanol at 11.7 nmol/min/mg protein. Again the addition of 2-propanol to the growth medium appears to have altered the enzyme for NADPH oxidation as it only showed a specific activity of 4.25 nmol/min/mg protein. As in the total activity, all others showed far lower specific activities ranging from 0.04-0.3 nmol/min/mg protein, with the exception of *Mc. thermolithotrophicus* which again had no activity. Genus *Methanosphaera* appears to be unique in that *Msph. stadtmanae* and *Msph. caniculi* were the only organisms to have the bulk of their activity precipitate in the 65-90% $(NH_4)_2SO_4$ pellet

b.). $NADP^+$ Reduction

For this section, the reader is referred to Table III.2. For $NADP^+$ reduction, *Msph. stadtmanae* without 2-propanol showed the highest total activity of 32.5 nmol/min/ml CFE with *Mst. concilli* (an acetate user) having 12.8 nmol/min/ml CFE and *Mb.*

Table III.2.: Average Total and Specific Activities of Various Methanogens for $NADP^+$ Reduction

Organism	CFE		0-65% s		0-65% p		65-90% s		65-90% p	
	TA	SA	TA	SA	TA	SA	TA	SA	TA	SA
Mb. thermoautotrophicum	9.88	8.24	1.69	8.50	6.03	12.45	0.23	2.10	1.16	14.60
Mb. G2R	0.51	0.53	1.32	6.26	0.06	0.11	1.52	11.45	0.08	0.88
Msph. stadtmanae[a]	32.55	15.60	38.40	222.50	2.93	3.48	13.00	36.95	17.80	546.00
Msph. stadtmanae[b]	2.01	0.64	1.25	1.99	0.08	0.05	0.74	3.80	0.55	1.55
Msph. caniculi	8.69	18.15	11.08	87.30	0.44	1.04	0.84	47.05	5.00	107.50
Msp. hungatei	1.34	1.33	1.35	8.78	0.10	0.15	1.42	9.48	0.07	1.30
Ml. tindarius	0.27	0.16	ND	ND	0.86	0.71	0.70	2.96	0.04	0.41
Ms. barkeri	4.90	1.47	0.93	0.87	0.17	0.17	0.81	3.29	0.60	2.13
Mst. concilli	12.85	6.92	54.09	355.50	1.62	3.00	43.13	249.00	0.65	13.05
Mc. thermolithotrophicus	0.36	0.22	0.05	0.06	0.06	0.21	ND	ND	0.01	0.07
Mc. voltae	3.96	1.69	1.20	0.90	2.58	4.39	ND	ND	0.16	1.06

a-grown on H_2/CO_2 + 0.4% Methanol
b-grown on H_2/CO_2 + 0.4% Methanol + 0.4% 2-propanol
TA=Total Activity (nmol/min/ml CFE)
SA=Specific Activity (nmol/min/mg protein)

thermoautotrophicum having 9.9 nmol/min/ml CFE. *Msph. caniculi* was next at 8.7 nmol/min/ml CFE, and *Ms. barkeri* and *Mc. voltae* followed at 4.9 and 3.96 nmol/min/ml CFE, respectively. All other organisms ranged anywhere from 0.27-2.0 nmol/min/ml CFE.

The specific activity of *Msph. caniculi* was the highest found in the CFE at 18.2 nmol/min/mg protein. This was followed closely by *Msph. stadtmanae* without 2-propanol at 15.6 nmol/min/mg protein. So then although *Msph. stadtmanae* without 2-propanol had the highest total activity, *Msph. caniculi* had a slightly higher specific activity. *Mb. thermoautotrophicum* and *Mst. concilli* were again next with similar specific activities of 8.24 and 7.92 nmol/min/mg protein. All other organisms were far lower at 0.22-1.69 nmol/min/mg protein.

The 65% $(NH_4)_2SO_4$ supernatant in *Mst. concilli*, *Msph. stadtmanae* without 2-propanol, and *Msph. caniculi* had the highest total activities of 54.1, 38.4, and 11.1 nmol/min/ml CFE. *Mb. thermoautotrophicum*, *Mb.* G2R, *Msph. stadtmanae* with 2-propanol, *Msp. hungatei*, and *Ms. barkeri* yielded many times less activity ranging from 0.94-1.69 nmol/min/ml CFE. *Mc. thermolithotrophicus* was far lower at 0.05 nmol/min/mg protein, and interestingly, *Mc. voltae* and *Ml. tindarius* showed no detectable activity for $NADP^+$ reduction in this $(NH_4)_2SO_4$ fraction.

As in the findings for total activity, the specific activity data showed the same trend. *Mst. concilli* and *Msph. stadtmanae* without 2-propanol had enormous activities of 355.5 and 222.5 nmol/min/mg protein with *Msph. caniculi* possessing 87.3 nmol/min/mg protein. *Mb. thermoautotrophicum*, *Mb.* G2R, *Msph. stadtmanae* with 2-propanol had about 10-fold less specific activities of 8.5, 6.26, and 8.77 nmol/min/mg protein. *Ms.*

barkeri was 10-fold lower at 0.87 nmol/min/mg protein, and *Mc. thermolithotrophicus* was barely detectable at 0.06 nmol/min/mg protein. Again, *Mc. voltae* and *Ml. tindarius* showed no detectable activity for $NADP^+$ reduction.

The 0-65% $(NH_4)_2SO_4$ pellet revealed a different trend. *Mb. thermoautotrophicum* had the highest total activity of 6.03 nmol/min/ml CFE, followed by *Mc. voltae* and *Msph. stadtmanae* without 2-propanol having 2.6 and 2.9 nmol/min/ml CFE, respectively. *Mst. concilli* was lower at 1.69 nmol/min/ml CFE. *Ml. tindarius* and *Msph. caniculi* were both below 1.0 nmol/min/ml CFE and *Mc. thermolithotrophicus*, *Msph. stadtmanae* with 2-propanol, *Msp. hungatei*, *Ms. barkeri*, and *Mb.* G2R all had total activity below 0.2 nmol/min/ml CFE.

Again the specific activity findings were comparable to the total activity findings. *Mb. thermoautotrophicum* was highest at 12.45 nmol/min/mg protein, with *Mc. voltae*, *Msph. stadtmanae* without 2-propanol, and *Mst. concilli* ranging between 3.0-4.4 nmol/min/mg protein. *Msph. caniculi*, and *Ml. tindarius* were 3-4-fold lower at 1.04 and 0.71 nmol/min/mg protein, respectively. *Mc. thermolithotrophicus*, *Msph. stadtmanae* without 2-propanol, *Msp. hungatei*, *Ms. barkeri*, and *Mb.* G2R were approximately 5-fold lower, ranging between 0.05-0.21 nmol/min/mg protein.

For the 90% $(NH_4)_2SO_4$ supernatant, *Mst. concilli* showed a huge difference from all others with 43.13 nmol/min/ml CFE. This was over 3-fold higher than *Msph. stadtmanae* without 2-propanol at 13 nmol/min/ml CFE. *Msp. hungatei* and *Mb.* G2R ranged from 1.3-1.5 nmol/min/ml CFE. *Mb. thermoautotrophicum*, *Msph. stadtmanae* with 2-propanol, *Msph. caniculi*, *Ml. tindarius*, and *Ms. barkeri* were all below 1.0 nmol/min/ml CFE. Interestingly though, both members of Methanococcales showed no detectable total

activity for $NADP^+$ reduction in this $(NH_4)_2SO_4$ fraction whereas in the opposite direction, there was activity in both *Mc. thermolithotrophicus* and *Mc. voltae* (Table III.1.).

With respect to specific activity, *Mst. concilli* had the highest activity at 249.0 nmol/min/mg protein; 5.3-fold higher than *Msph. caniculi* at 47.05 nmol/min/mg protein. The interesting development here is that *Msph. caniculi* had such a high specific activity when its total activity was one of the lowest at 0.85 nmol/min/ml CFE. This would indicate that although the total activity is low, the enzyme was highly enriched in this fraction. *Msph. stadtmanae* without 2-propanol, *Mb.* G2R, and *Msp. hungatei* showed significant activities of 36.95, 11.45, and 9.48 nmol/min/mg protein. *Mb. thermoautotrophicum*, *Msph. stadtmanae* with 2-propanol, *Ml. tindarius*, and *Ms. barkeri* all had approximately 10-fold less activities ranging from 2.1-3.88 nmol/min/mg protein. Again, there was no detectable specific activity in either member of Methanococcales for $NADP^+$ reduction in this $(NH_4)_2SO_4$ fraction.

In the final fraction, the 65-90% $(NH_4)_2SO_4$ pellet, *Msph. caniculi and Msph. stadtmanae* without 2-propanol had the highest total activities of 5.0 and 4.5 nmol/min/ml CFE, respectively. This was almost 5-fold higher than *Mb. thermoautotrophicum* with 1.16 nmol/min/ml CFE. *Msph. stadtmanae* with 2-propanol, *Ms. barkeri*, and *Mst. concilli* were all about 2-fold lower ranging between 0.45-0.65 nmol/min/ml CFE. *Mc. voltae* had a total activity of 0.16 nmol/min/ml CFE while *Mc. thermolithotrophicus*, *Msp. hungatei*, *Ml. tindarius*, and *Mb.* G2R were all below 0.1 nmol/min/ml CFE.

As was seen for the specific activity of *Msph. caniculi* in the 90% $(NH_4)_2SO_4$ supernatant, *Msph. stadtmanae* without 2-propanol showed very high specific activity. It

yielded a specific activity of 546.0 nmol/min/mg protein which was 5-fold higher than the significant activity in *Msph. caniculi* of 107.5 nmol/min/mg protein. *Mb. thermoautotrophicum* and *Mst. concilli* were approximately 10-fold lower at 14.6 and 13.05 nmol/min/mg protein, respectively. *Mc. voltae*, *Msph. stadtmanae* with 2-propanol, *Msp. hungatei*, and *Ms. barkeri* all ranged between about 1.0-2.0 nmol/min/mg protein, while *Mb.* G2R and *Ml. tindarius* were 2-4-fold lower at 0.88 and 0.41 nmol/min/mg protein, respectively. For this fraction, *Mc. thermolithotrophicus* was the only organism below 0.1 at 0.07 nmol/min/mg protein. A point of note is that in this direction, the sum of the total activity in the 65% $(NH_4)_2SO_4$ supernatant and the 0-65% $(NH_4)_2SO_4$ pellet roughly equals the total activity seen in the CFE.

III.3.2. Dominant direction of enzymatic activity in each microorganism under *in vitro* conditions

The dominant direction of the enzyme(s) found in each organism was determined by a direct comparison of total and specific activities for $NADP^+$ reduction and NADPH oxidation in CFE (Table III.3.). In all organisms tested, except *Msph. stadtmanae* with 2-propanol, the dominant direction under the conditions tested was the reduction of $NADP^+$. In some cases the difference was quite small as seen in *Mb.* G2R and *Mc. thermolithotrophicus* where the difference was consistently 2-3 fold. In the other cases of *Msph. stadtmanae* without 2-propanol, *Mb. thermoautotrophicum*, *Mst. concilli*, *Msp. hungatei*, *Msph. caniculi*, *Ml. tindarius*, *Ms. barkeri*, and *Mc. voltae* there was a consistently large difference between $NADP^+$ reduction and NADPH oxidation. When the ratio of activities in many of the organisms were compared in the various $(NH_4)_2SO_4$

Table III.3.: Specific Activities* of Various Methanogens for Determination of Dominant Direction under *in vivo* Conditions

Organism	Reaction	CFE	65%sup	0-65%pel	90%sup	65-90%pel
Mb. thermoautotrophicum	NADPH ox.	0.11	1.57	0.08	4.14	0.25
	NADP red.	8.24	8.50	12.45	2.10	14.60
Mb. G2R	NADPH ox.	0.39	2.01	0.06	4.72	0.20
	NADP red.	0.53	6.26	0.11	11.45	0.88
Msph. stadtmanae[a]	NADPH ox.	0.94	29.10	0.20	11.70	11.70
	NADP red.	15.60	222.50	3.48	36.95	546.00
Msph. stadtmanae[b]	NADPH ox.	0.94	8.10	0.15	3.28	4.25
	NADP red.	0.64	1.99	0.05	3.80	1.55
Msph. caniculi	NADPH ox.	1.20	20.55	0.11	4.44	13.55
	NADP red.	18.15	87.30	1.04	47.05	107.50
Msp. hungatei	NADPH ox.	0.19	3.24	0.03	3.47	0.29
	NADP red.	1.33	8.78	0.15	9.48	1.30
Mst. concilli	NADPH ox.	0.08	1.52	0.07	1.59	0.17
	NADP red.	6.92	355.50	3.00	249.00	13.05
Ml. tindarius	NADPH ox.	0.07	0.56	0.01	1.73	0.16
	NADP red.	0.16	ND	0.71	2.96	0.41
Ms. barkeri	NADPH ox.	0.06	0.15	0.01	1.72	0.06
	NADP red.	1.47	0.87	0.17	3.29	2.13
Mc. thermolithotrophicus	NADPH ox.	0.11	0.23	0.09	0.80	0.04
	NADP red.	0.22	0.06	0.21	<0.01	0.07
Mc. voltae	NADPH ox.	0.03	0.65	0.01	0.49	0.02
	NADP red.	1.69	0.90	4.39	<0.01	1.06

a=2-propanol not added to growth medium
b=2-propanol added to growth medium
ND = not determined
* values are in nmol/min/mg protein

fractions, these varied leading to the thought that different activities were being enriched in different $(NH_4)_2SO_4$ fractions.

III.3.3. Determination of the Presence of 1 or 2 Enzymes for $NADP^+$ Reduction and NADPH Oxidation

Determining the presence of 1 or 2 distinct enzymes for $NADP^+$ reduction/NADPH oxidation was done in a number of ways. First the $(NH_4)_2SO_4$ fraction displaying the greatest specific activity was compared in each direction (Table III.4.). If the fraction was the same in both directions, this indicated that there could be 1 enzyme present since it would obviously precipitate in the same $(NH_4)_2SO_4$ fraction whereas with more than 1 enzyme present, this would not necessarily be the case.

Msp. hungatei, *Mst. concilli*, *Ml. tindarius*, and *Ms. barkeri* all had the greatest total and specific activity in the 90% $(NH_4)_2SO_4$ supernatant for both $NADP^+$ reduction and NADPH oxidation. This indicated that there could be 1 enzyme present in all members of family Methanomicrobiales. A difficulty in determining this for *Ml. tindarius* arose in that the 65% $(NH_4)_2SO_4$ supernatant showed no activity for $NADP^+$ reduction, but there was activity in the fractions generated from this supernatant, i.e. the 90% $(NH_4)_2SO_4$ supernatant and 65-90% $(NH_4)_2SO_4$ pellet fractions for $NADP^+$ reduction and in the 65% $(NH_4)_2SO_4$ supernatant for NADPH oxidation. At first glance this would indicate that perhaps there are 2 enzymes present, however, excluding the 65% $(NH_4)_2SO_4$ supernatant data, the ratios of NADPH oxidation to $NADP^+$ reduction are similar to the CFE.

In family Methanococcales, both *Mc. thermolithotrophicus* and *Mc. voltae* had the greatest total and specific activity for $NADP^+$ reduction in the 0-65% $(NH_4)_2SO_4$ pellet,

Table III.4.: $(NH_4)_2SO_4$ Fractions with Highest Specific Activity for $NADP^+$ Reduction/NADPH Oxidation in Various Methanogens Indicating Presence of 1 or 2 Enzyme(s)

Organism	NADPH Oxidation	$NADP^+$ Reduction	# Enz
Family Methanobacteriales			
Mb. thermoautotrophicum	90% Supernatant	0-65% Pellet	2
Mb. G2R	90% Supernatant	90% Supernatant	1
Msph. stadtmanae[a]	65-90% Pellet	65-90% Pellet	1
Msph. stadtmanae[b]	65-90% Pellet	90% Supernatant	2
Msph. caniculi	65-90% Pellet	65-90% Pellet	1
Family Methanomicrobiales			
Msp. hungatei	90% Supernatant	90% Supernatant	1
Mst. concilli	90% Supernatant	90% Supernatant	1
Ml. tindarius	90% Supernatant	90% Supernatant	1
Ms. barkeri	90% Supernatant	90% Supernatant	1
Family Methanococcales			
Mc. thermolithotrophicus	90% Supernatant	0-65% Pellet	2
Mc. voltae	90% Supernatant	0-65% Pellet	2

a=2-propanol not added to growth medium
b=2-propanol added to growth medium

while the greatest amount of NADPH oxidation activity was found in the 90% $(NH_4)_2SO_4$ supernatant. This indicated that while the Methanomicrobiales tested appeared to possess 1 enzyme for both $NADP^+$ reduction and NADPH oxidation, the Methanococcales tested appeared to have 2 separate enzymes.

Family Methanobacteriales was variable. Of those tested *Methanobacterium* G2R, *Msph. stadtmanae* without 2-propanol, and *Msph. caniculi* all appeared to have 1 enzyme for $NADP^+$ reduction and NADPH oxidation. The greatest amount of activity for both directions was found in the 65-90% $(NH_4)_2SO_4$ pellet for both *Msph. stadtmanae* without 2-propanol and *Msph. caniculi*. *Mb.* G2R had the greatest activity for both directions in the 90% supernatant. *Mb. thermoautotrophicum* however, clearly appeared to have 2 separate enzymes. The greatest NADPH oxidation activity was found in the 90% $(NH_4)_2SO_4$ supernatant, while the greatest $NADP^+$ reduction activity was found in the 0-65% $(NH_4)_2SO_4$ pellet. Although there is a clear difference between *Mb. thermoautotrophicum* and *Mb.* G2R, it is interesting to note that for NADPH oxidation, both had the greatest activity in the 90% $(NH_4)_2SO_4$ supernatant. In fact, with the exception of genus *Methanosphaera*, all NADPH oxidation activity was found in the 90% $(NH_4)_2SO_4$ supernatant, while again with the exception of genus *Methanosphaera*, $NADP^+$ reduction activity was found in either the 90% $(NH_4)_2SO_4$ supernatant or the 0-65% $(NH_4)_2SO_4$ pellet.

The addition of 2-propanol to *Msph. stadtmanae* again indicates a change in the enzyme activity from that in the absence of 2-propanol. Although NADPH oxidation activity was still found in the 65-90% $(NH_4)_2SO_4$ pellet, the greatest amount of $NADP^+$ reduction activity was found in the 90% $(NH_4)_2SO_4$ supernatant. This indicates that

although there appears to be 1 enzyme in the absence of 2-propanol, there appears to be 2 separate enzymes when 2-propanol is added to the growth medium. There is also the possibility that in the absence of 2-propanol, both the $NADP^+$:F_{420} oxidoreductase and another similar but unidirectional enzyme are present with a lower activity in the similar enzyme. When 2-propanol is present, this other enzyme may remain while the $NADP^+$:F_{420} oxidoreductase is replaced with a unidirectional enzyme for the opposite direction.

This prompted a second method of determination. If only one enzyme is present in the CFE, then the ratio of total activity between $NADP^+$ reduction and NADPH oxidation should be somewhat consistent between the CFE and each $(NH_4)_2SO_4$ fraction. The results were consistent with the results from Table III.4. As well, if there is only one enzyme present, then all fractions should either be greater than 1 or less than 1 for a given organism. *Mb.* G2R, *Msph. stadtmanae* without 2-propanol, *Msph. caniculi, Msp. hungatei, Ms. barkeri, Mst. concilli,* and all had $NADP^+$ reduction/NADPH oxidation values above 1 (Table III.5.). *Mb. thermoautotrophicum* and *Msph. stadtmanae* with 2-propanol all had at least one fraction with a ratio value of less than one. This meant that while some $(NH_4)_2SO_4$ fractions had higher activity for NADPH oxidation, some $(NH_4)_2SO_4$ fractions had higher activity for $NADP^+$ reduction. *Mb. thermoautotrophicum* had a ratio value less than 1 in the 90% $(NH_4)_2SO_4$ supernatant, *Mc. thermolithotrophicus* in the 65% $(NH_4)_2SO_4$ supernatant, and *Msph. stadtmanae* with 2-propanol in all but the 90% $(NH_4)_2SO_4$ supernatant. In the case of *Msph. stadtmanae* with 2-propanol, 4 of the 5 fractions were below 1 as was expected since the dominant *in vitro* direction was determined to be NADPH oxidation (Table III.3).

Table III.5.: Ratio of Total Activity * for $NADP^+$ Reduction to NADPH Oxidation in Various Methanogens

Organism	Reaction	CFE	65%sup	0-65%pel	90%sup	65-90%pel
Mb. thermoautotrophicum	red / ox.	76.00	5.45	150.75	0.50	58.00
Mb. G2R	red / ox.	1.38	2.75	6.00	2.03	8.00
Msph. stadtmanae[a]	red / ox.	16.44	7.68	17.24	43.33	3.96
Msph. stadtmanae[b]	red / ox.	0.69	0.25	0.27	1.21	0.37
Msph. caniculi	red / ox.	14.98	4.23	8.80	1.05	7.94
Msp. hungatei	red / ox.	7.05	2.76	5.00	2.73	3.50
Ml. tindarius	red / ox.	2.08	N.A.	86.00	1.71	4.00
Ms. barkeri	red / ox.	25.79	6.20	8.50	1.98	30.00
Mst. concilli	red / ox.	91.79	235.17	40.50	154.04	65.00
Mc. thermolithotrophicus	red / ox.	2.00	0.27	2.00	N.A.	1.00
Mc. voltae	red / ox.	6.09	7.50	258.00	N.A.	8.00

a=2-propanol not added to growth medium
b=2-propanol added to growth medium
N.A. = not applicable
* values are in nmol/min/ml CFE

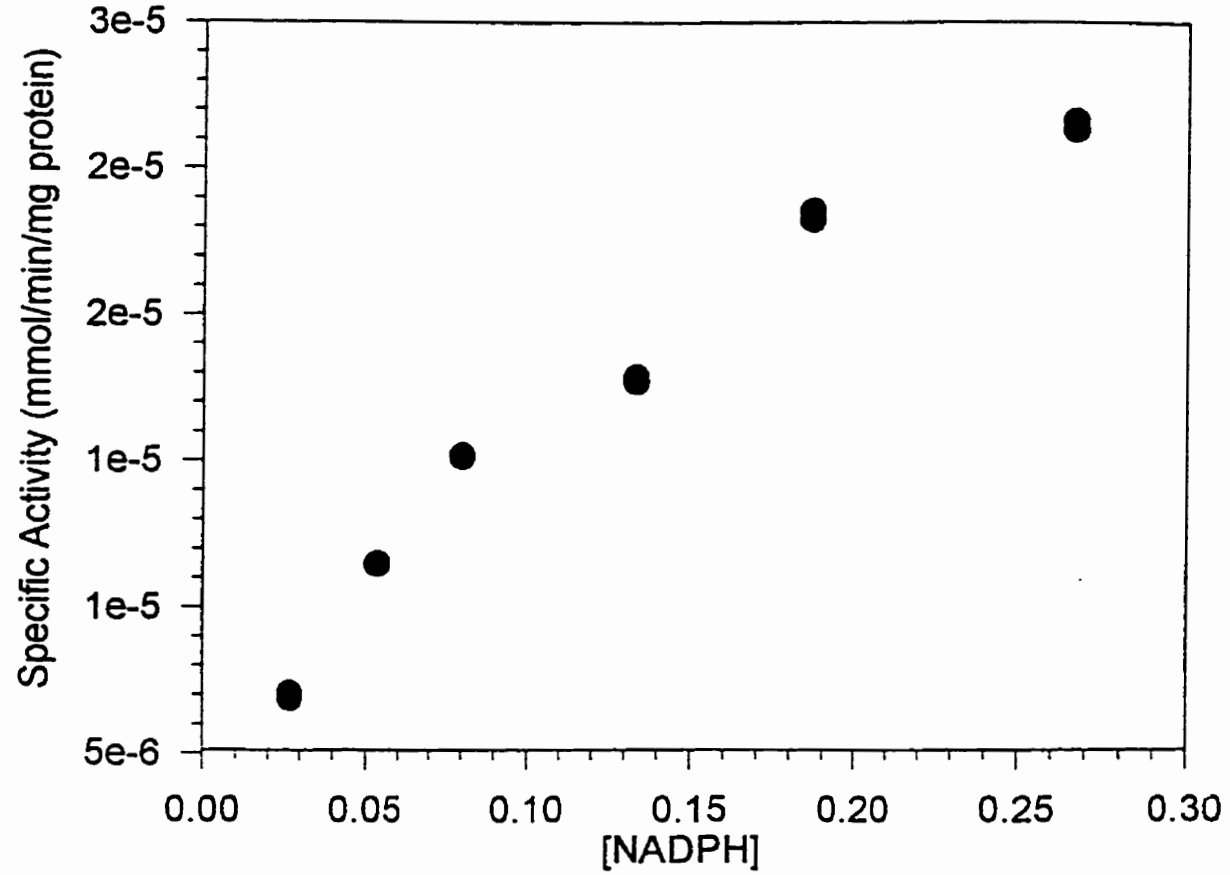
Ml tindarius, *Mc. thermolithotrophicus*, and *Mc. voltae* were another matter. For *Mc. voltae*, there was no measurable activity for NADPH oxidation in the 65-90% $(NH_4)_2SO_4$ pellet and no detectable $NADP^+$ reduction activity in the 65% $(NH_4)_2SO_4$ supernatant and 90% $(NH_4)_2SO_4$ supernatant. This did not allow for an accurate comparison of the ratios. However, the lack of both activities in different $(NH_4)_2SO_4$ fractions is itself convincing for the presence of two separate enzymes. A similar result was seen for *Ml. tindarius* and *Mc. thermolithotrophicus*.

In addition to the above comparisons, K_m's for NADPH in the NADPH oxidation assays were also performed on *Msp. hungatei* and *Mb. thermoautotrophicum* in order confirm the findings that some methanogens possess more than 1 activity. Due to the absence of activity in some $(NH_4)_2SO_4$ fractions in *Mc. voltae*, K_m's were performed for NADPH, $NADP^+$, and F_{420}.

Msp. hungatei was tested in the 90% $(NH_4)_2SO_4$ supernatant and the 65-90% $(NH_4)_2SO_4$ pellet for NADPH. For the 90% $(NH_4)_2SO_4$ supernatant, the Lineweaver-Burke plot gave a K_m of 0.37 mM NADPH (Figure III.1.), while the Eadie-Hofstee plot gave a K_m of 0.41 mM NADPH (Figure III.2.). For the 65-90% $(NH_4)_2SO_4$ pellet the Lineweaver-Burke plot gave a K_m of 0.278 mM NADPH (Figure III.3.), while the Eadie-Hofstee plot showed a K_m of only 0.095 mM NADPH (Figure III.4.). This suggested that the regression was poor for the Eadie-Hofstee plot for the 65-90% $(NH_4)_2SO_4$ pellet.

For *Mb. thermoautotrophicum*, the 65-90% $(NH_4)_2SO_4$ pellet and the 90% $(NH_4)_2SO_4$ supernatant were tested. The 65-90% $(NH_4)_2SO_4$ pellet gave a non-first order K_m for the Lineweaver-Burke plot, but a second order regression for the Lineweaver-Burke plot gave a very good fit (Figure III.5.). The Michealis-Menton plot showed a parabolic curve while

Figure III.1. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for NADPH in the 90% $(NH_4)_2SO_4$ supernatant of *Msp. hungatei*. The Lineweaver-Burke plot showed K_m of 370 μM. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
3e-5
2e-5
2e-5
1e-5
1e-5
5e-6
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADPH]

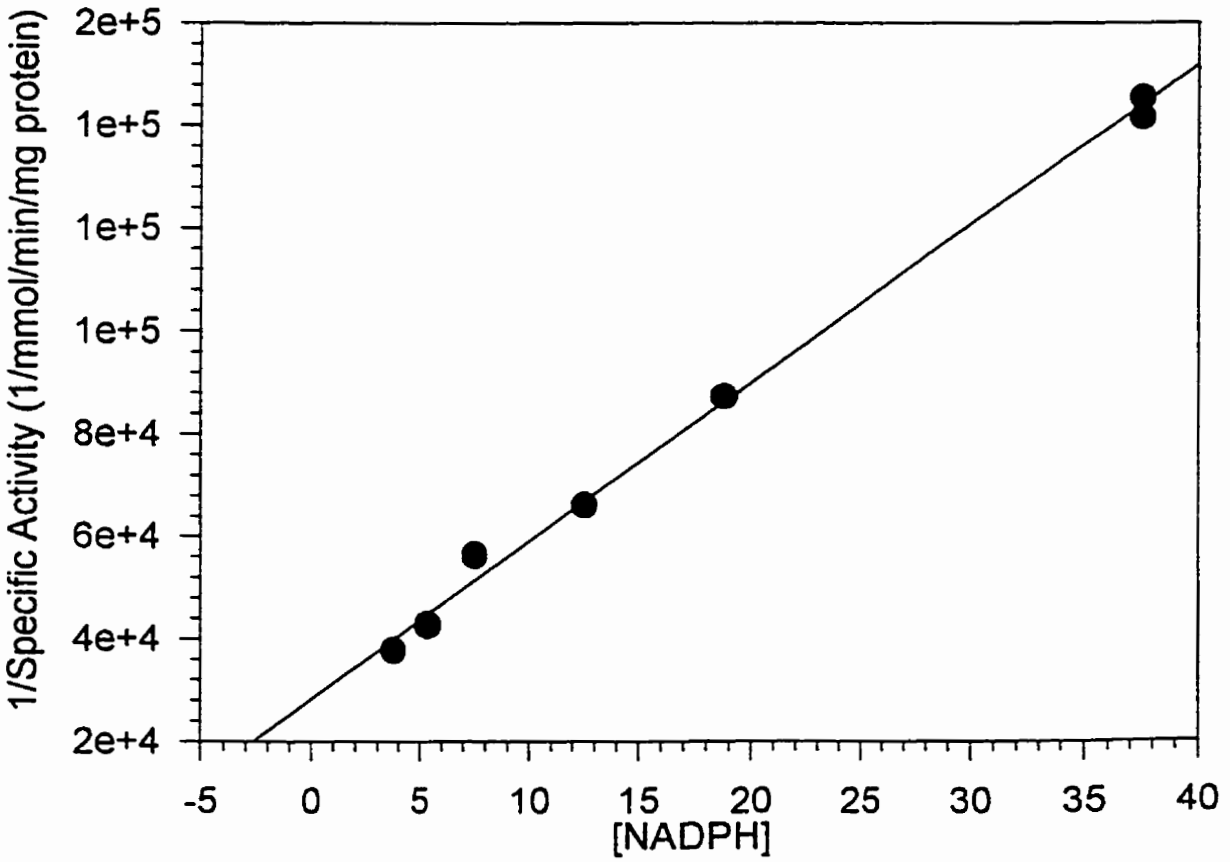

1/Specific Activity (1/mmol/min/mg protein)
2e+5
1e+5
1e+5
1e+5
8e+4
6e+4
4e+4
2e+4
-5
0
5
10
15
20
25
30
35
40
[NADPH]

Figure III.2. Eadie-Hofstee Plot for NADPH in the 90% $(NH_4)_2SO_4$ supernatant of *Msp. hungatei.* The plot showed a K_m of 410 μM. The experiment was performed twice in duplicate.

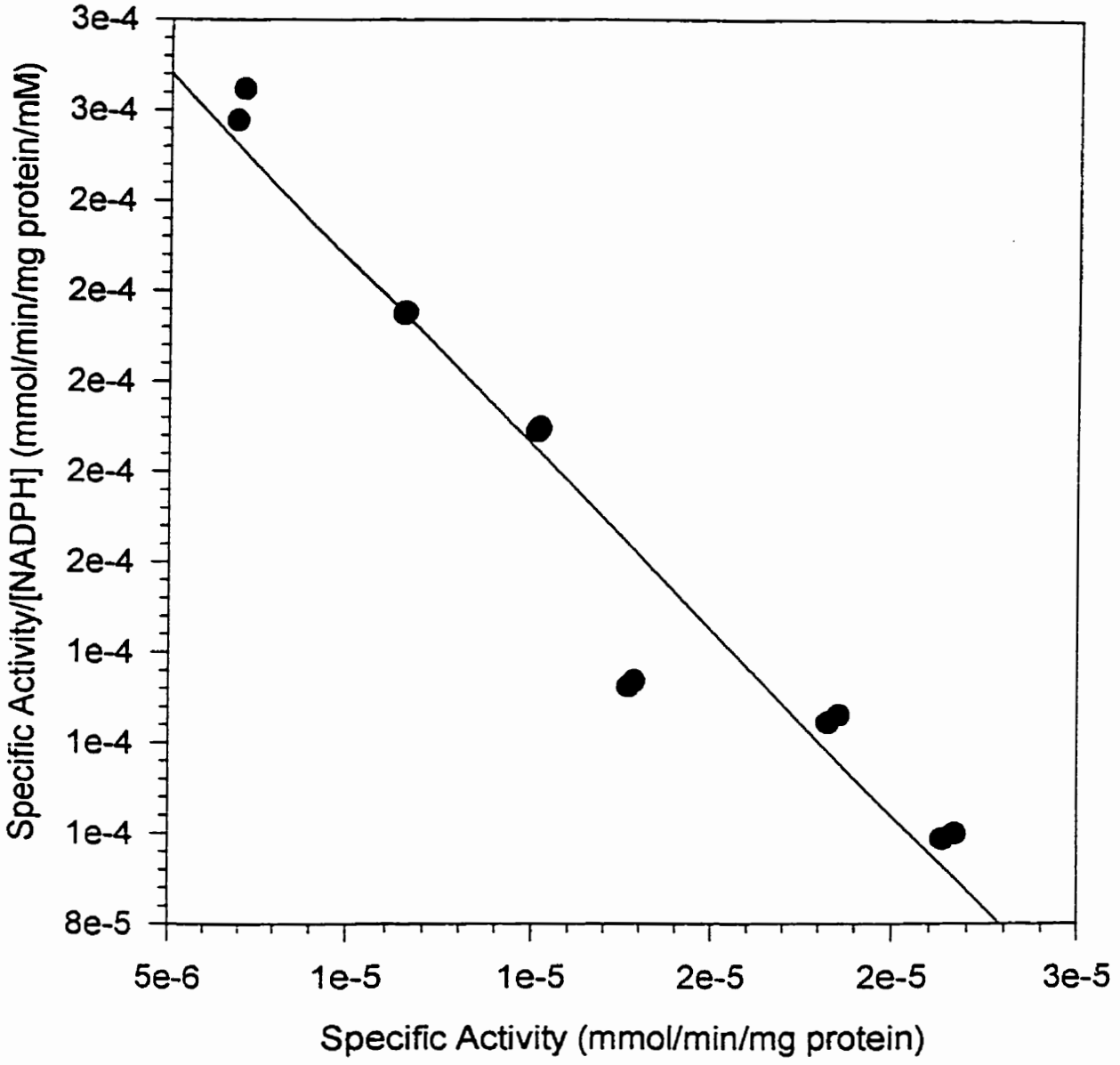
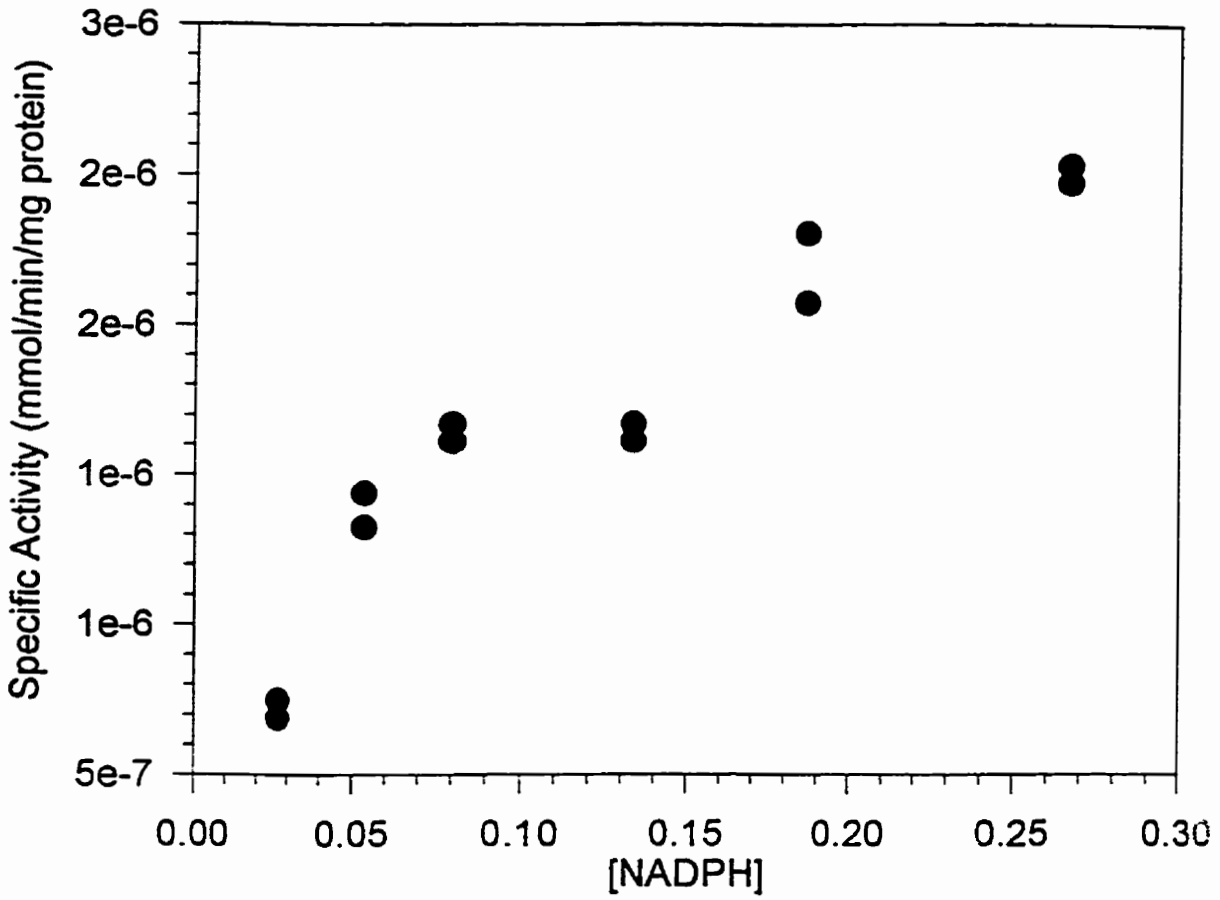

3e-4
3e-4
2e-4
2e-4
2e-4
2e-4
2e-4
1e-4
1e-4
1e-4
8e-5
Specific Activity/[NADPH] (mmol/min/mg protein/mM)
5e-6
1e-5
1e-5
2e-5
2e-5
3e-5
Specific Activity (mmol/min/mg protein)

Figure III.3. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for NADPH in the 65-90% $(NH_4)_2SO_4$ pellet of *Msp. hungatei*. The Lineweaver-Burke plot showed a K_m of 278 μM. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
3e-6
2e-6
2e-6
1e-6
1e-6
5e-7
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADPH]

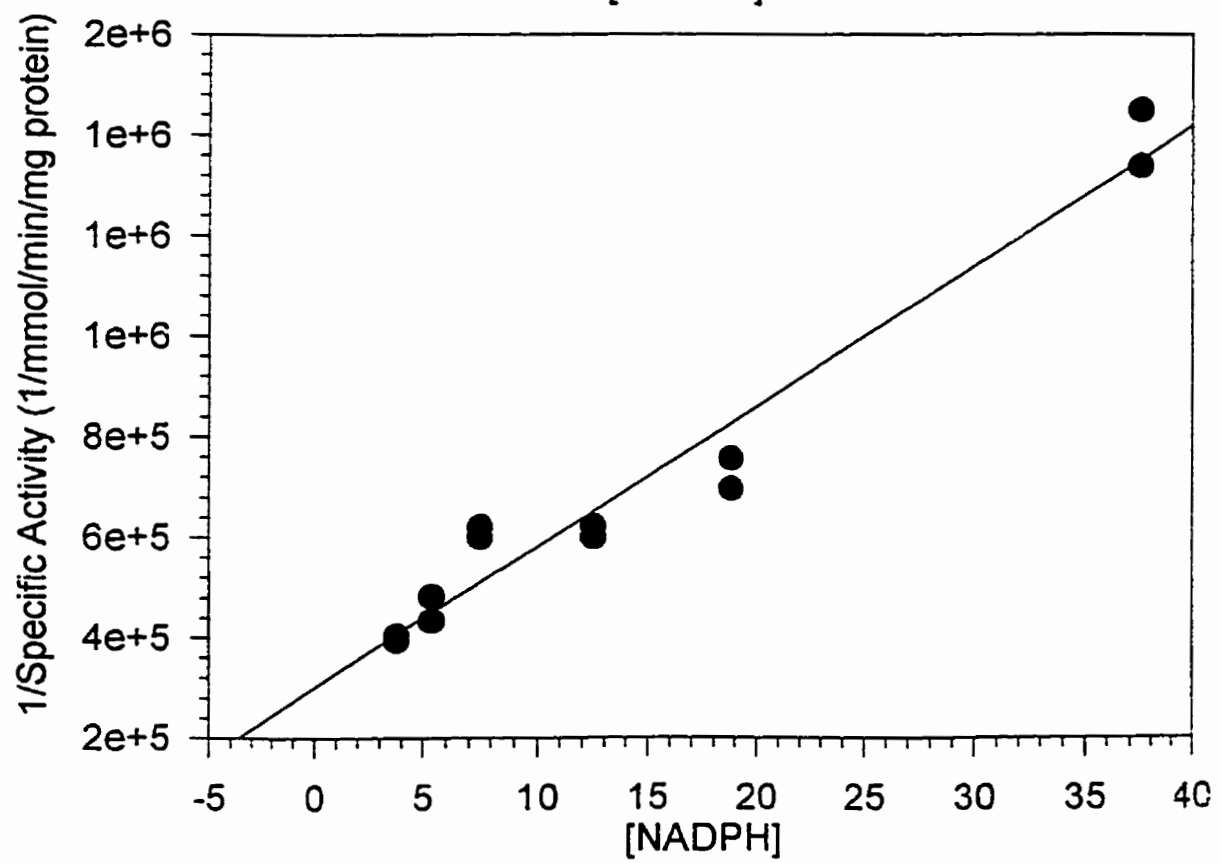

1/Specific Activity (1/mmol/min/mg protein)
2e+6
1e+6
1e+6
1e+6
8e+5
6e+5
4e+5
2e+5
-5
0
5
10
15
20
25
30
35
40
[NADPH]

Figure III.4. Eadie-Hofstee Plot for NADPH in the 65-90% $(NH_4)_2SO_4$ pellet of *Msp. hungatei*. The plot showed a K_m of 95 μM. The experiment was performed twice in duplicate.

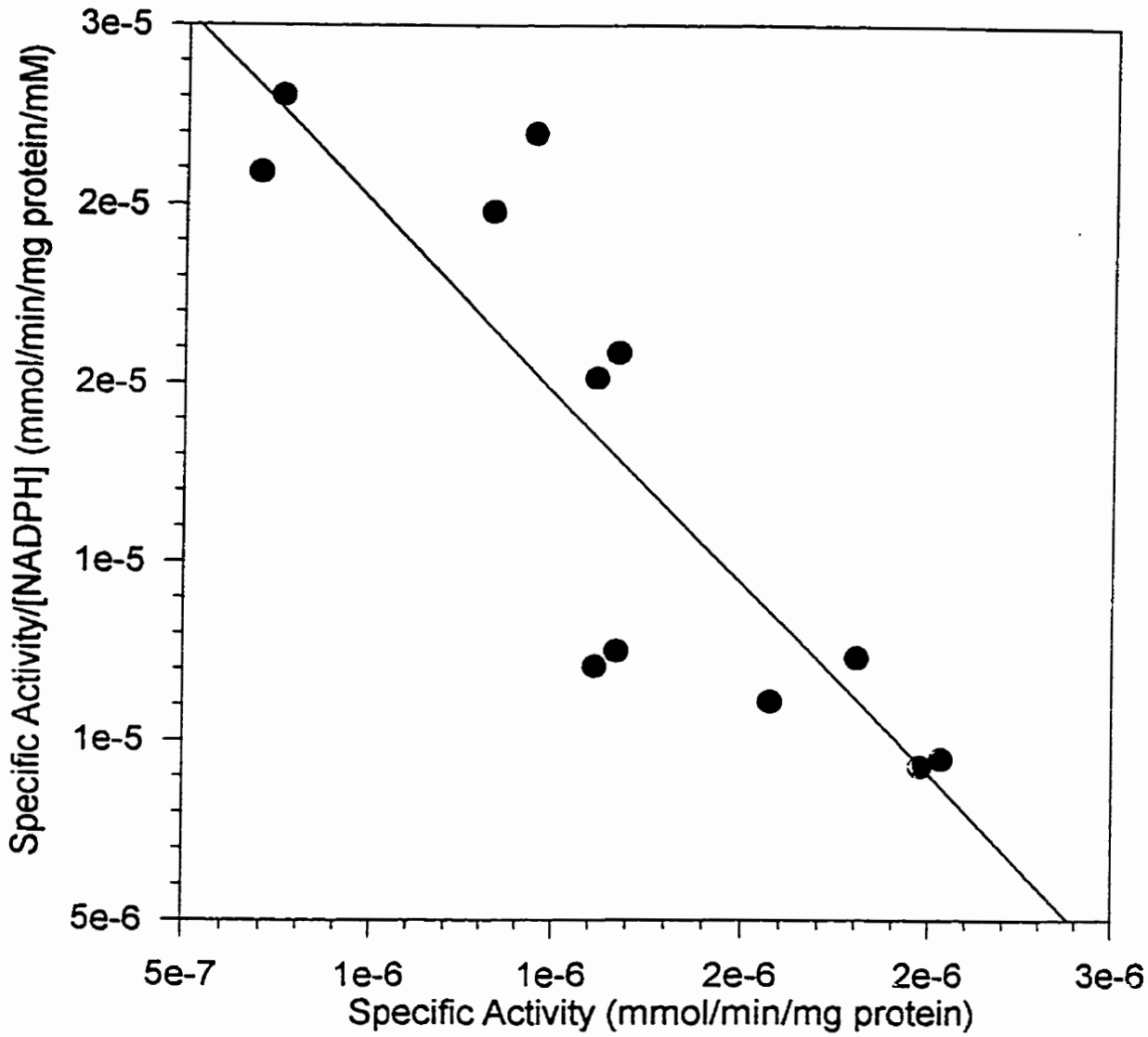
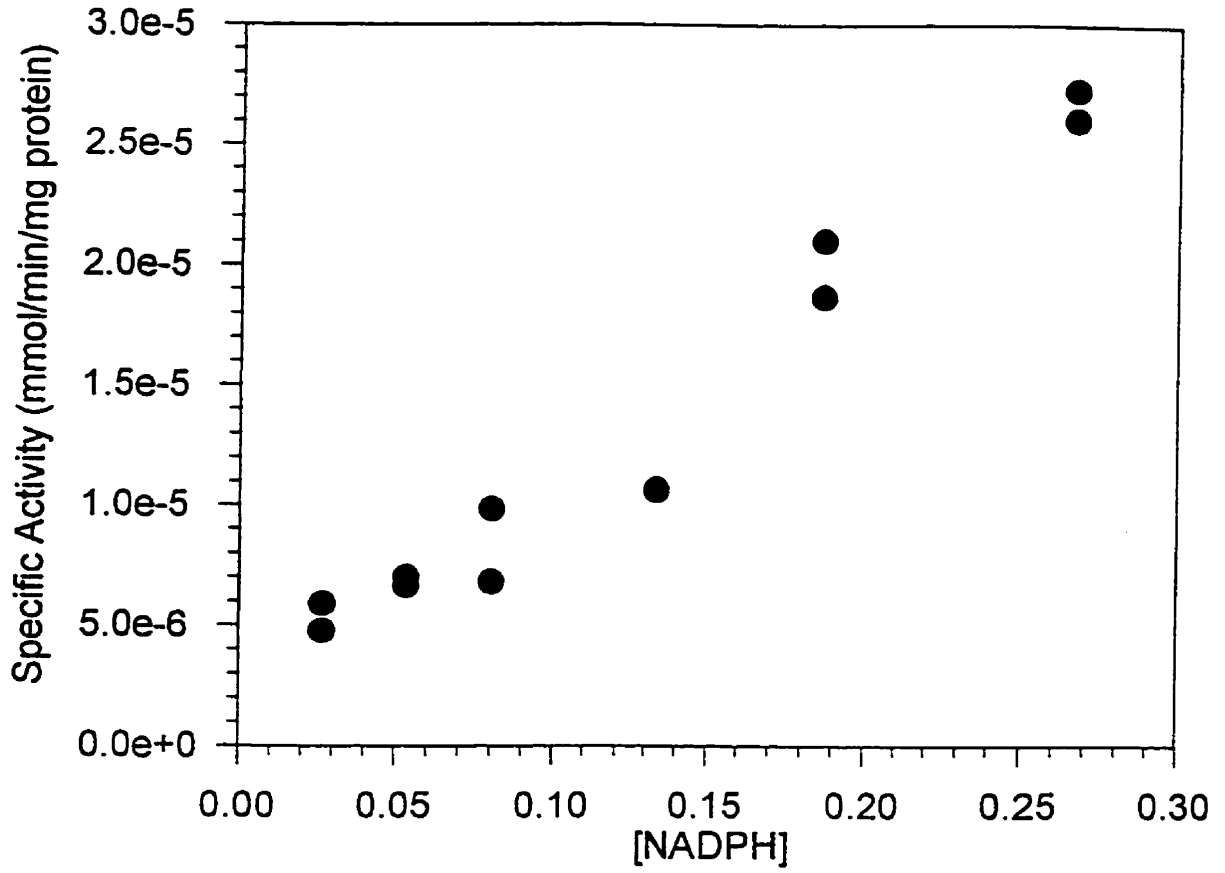
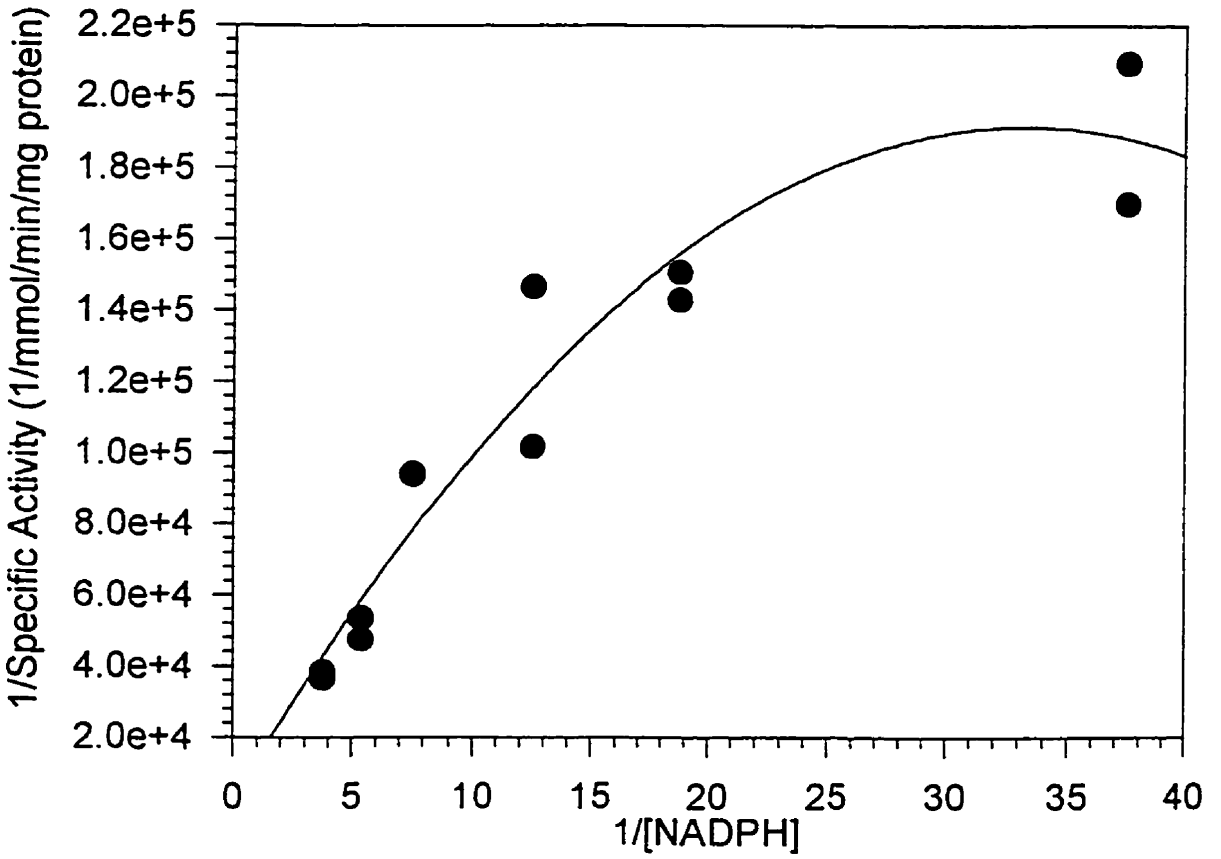

3e-5
2e-5
2e-5
1e-5
1e-5
5e-6
Specific Activity/[NADPH] (mmol/min/mg protein/mM)
5e-7
1e-6
1e-6
2e-6
2e-6
3e-6
Specific Activity (mmol/min/mg protein)

Figure III.5. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for the 65-90% $(NH_4)_2SO_4$ pellet of *Mb. thermoautotrophicum*. The Lineweaver-Burke plot showed a non-first order reaction. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
3.0e-5
2.5e-5
2.0e-5
1.5e-5
1.0e-5
5.0e-6
0.0e+0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADPH]


1/Specific Activity (1/mmol/min/mg protein)
2.2e+5
2.0e+5
1.8e+5
1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0
5
10
15
20
25
30
35
40
1/[NADPH]

the Lineweaver-Burke plot showed a hyperbolic curve (Figure III.5.). The Eadie-Hofstee plot confirmed that this was a non-first order reaction (Figure III.6.). The 90% $(NH_4)_2SO_4$ supernatant showed a fairly straight line for the Michaelis-Menton plot (Figure III.7.). The Lineweaver-Burke plot showed a straight line with a K_m of 1 mM NADPH (Figure III.7.). The Eadie-Hofstee plot concurred with this high K_m with a value of 1.09 mM NADPH (Figure III.8.). Therefore, while the 65-90% $(NH_4)_2SO_4$ pellet gave non-first order characteristics for both plots, the 90% $(NH_4)_2SO_4$ supernatant gave typical first order kinetics for both plots. This strengthened the observation that *Mb. thermoautotrophicum* possessed two separate enzymes for $NADP^+$ reduction and NADPH oxidation.

Because of the lack of activity in the 65% $(NH_4)_2SO_4$ supernatant and the 90% $(NH_4)_2SO_4$ supernatant for $NADP^+$ reduction, *Mc. voltae* was tested for both NADPH and F_{420}. NADPH was tested in the 65% $(NH_4)_2SO_4$ supernatant and the 65-90% $(NH_4)_2SO_4$ pellet, while F_{420} was tested in the 90% $(NH_4)_2SO_4$ supernatant and the 0-65% $(NH_4)_2SO_4$ pellet so that all four $(NH_4)_2SO_4$ fractions could be tested. Also, $NADP^+$ reduction activity was tested in the 0-65% and 65-90% $(NH_4)_2SO_4$ pellets.

NADPH K_m's appeared similar in both $(NH_4)_2SO_4$ fractions. The 65% $(NH_4)_2SO_4$ supernatant showed a parabolic curve for the Michealis-Menton plot and a hyperbolic curve for the Lineweaver-Burke plot (Figure III.9.), and the Eadie-Hofstee plot confirmed a non-first order reaction by showing a hyperbolic curve (Figure III.10.). The 65-90% $(NH_4)_2SO_4$ pellet showed similar characteristics in the Michealis-Menton and Lineweaver-Burke plots (Figure III.11.), while the Eadie-Hofstee plot was also non-first order (Figure III.12.). This indicated that there were non-first order kinetics in both

Figure III.6. Eadie-Hofstee Plot for NADPH in the 65-90% $(NH_4)_2SO_4$ pellet of *Mb. thermoautotrophicum*. The plot showed a non-first order reaction. The experiment was performed twice in duplicate.

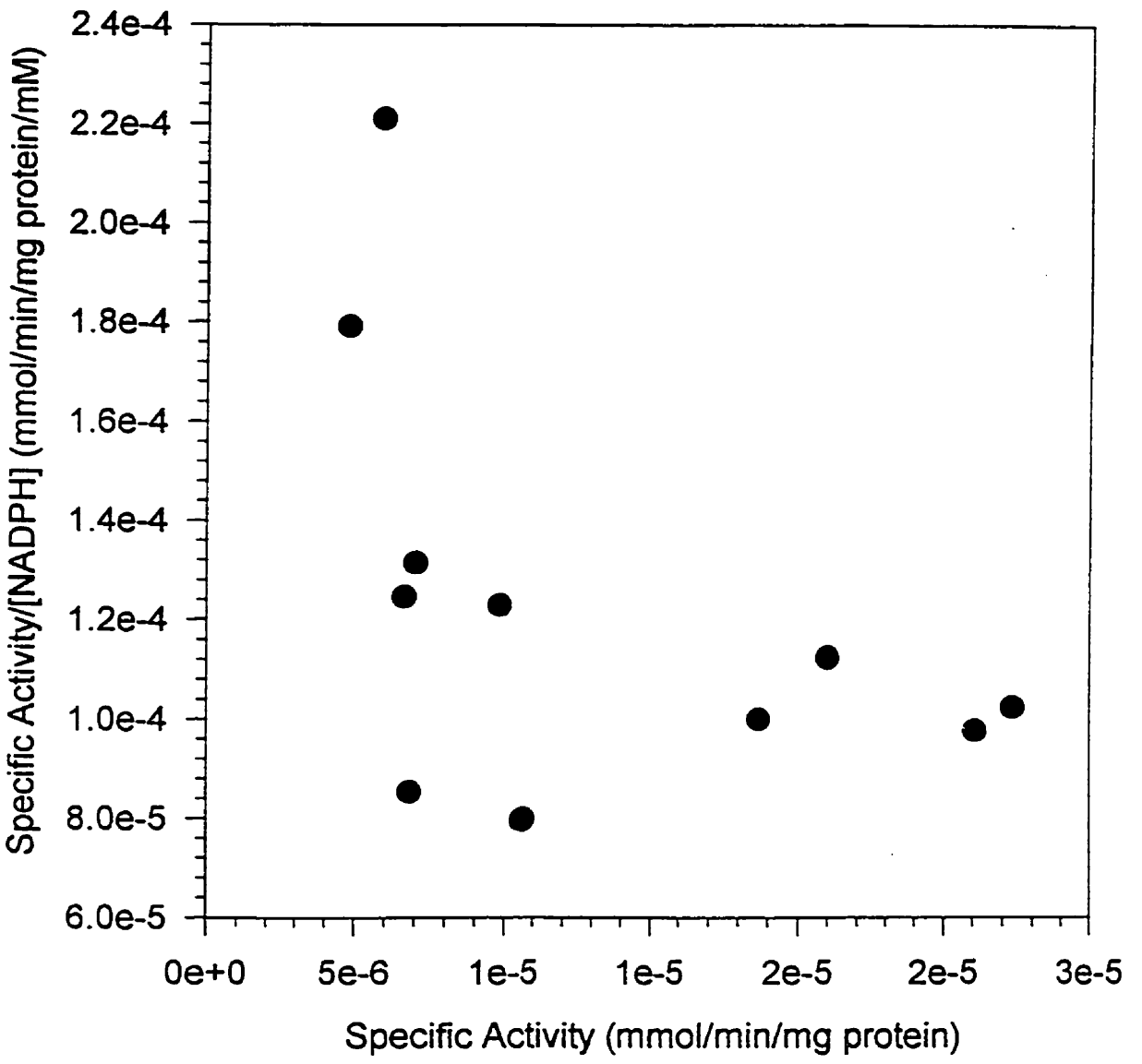
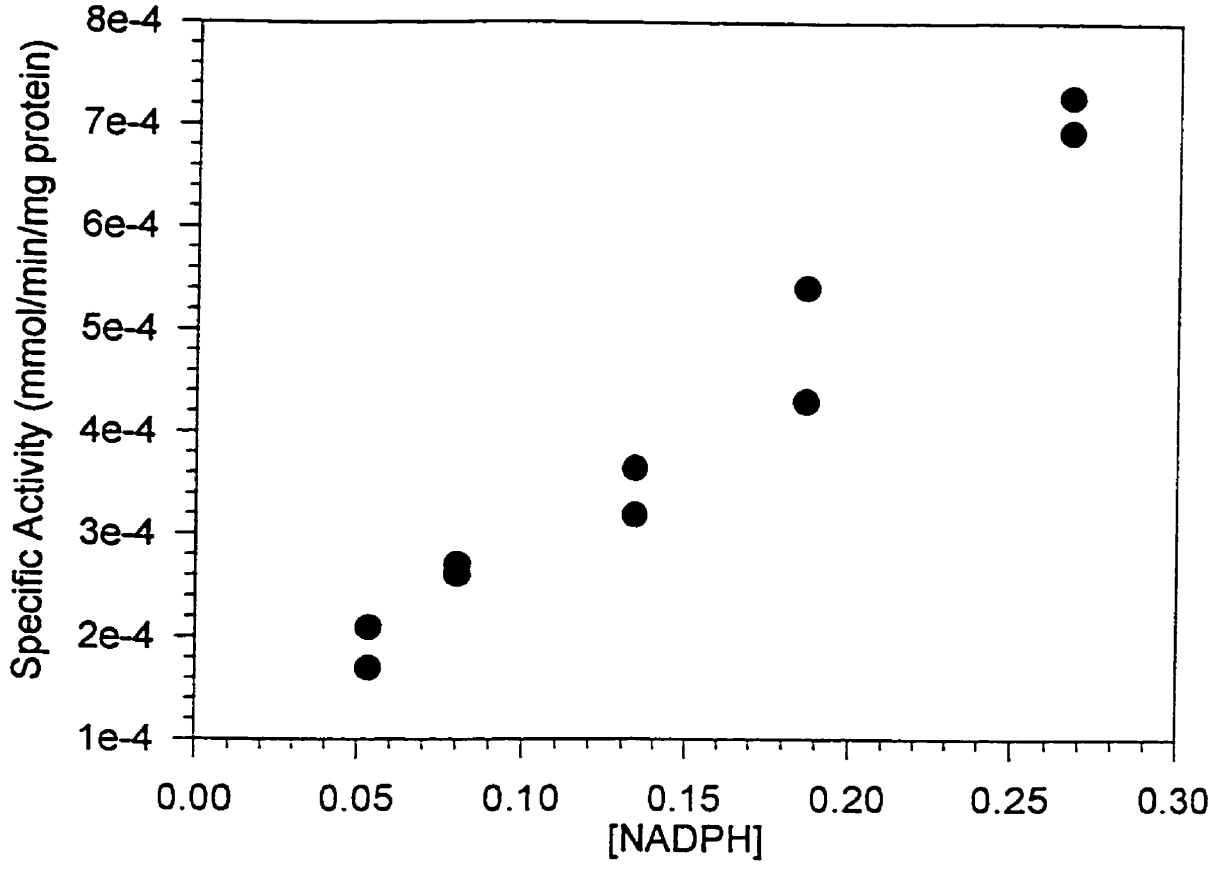

2.4e-4
2.2e-4
2.0e-4
1.8e-4
1.6e-4
1.4e-4
1.2e-4
1.0e-4
8.0e-5
6.0e-5
Specific Activity/[NADPH] (mmol/min/mg protein/mM)
0e+0
5e-6
1e-5
1e-5
2e-5
2e-5
3e-5
Specific Activity (mmol/min/mg protein)

Figure III.7. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for the 90% $(NH_4)_2SO_4$ supernatant of *Mb. thermoautotrophicum*. The Lineweaver-Burke plot showed a K_m of 1 mM. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
8e-4
7e-4
6e-4
5e-4
4e-4
3e-4
2e-4
1e-4
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADPH]

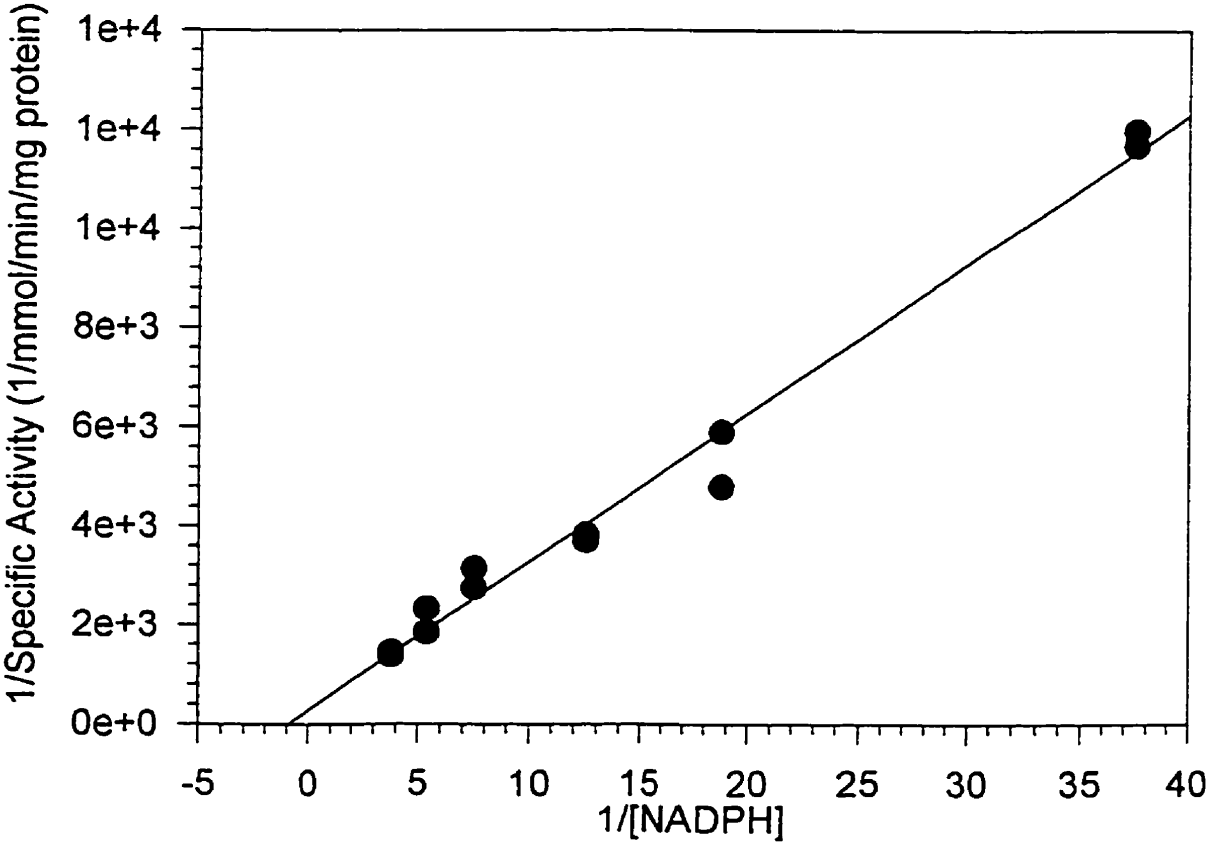

1/Specific Activity (1/mmol/min/mg protein)
1e+4
1e+4
1e+4
8e+3
6e+3
4e+3
2e+3
0e+0
-5
0
5
10
15
20
25
30
35
40
1/[NADPH]

Figure III.8. Eadie-Hofstee Plot for NADPH in the 90% $(NH_4)_2SO_4$ supernatant of *Mb. thermoautotrophicum*. The plot showed a K_m of 1.09 mM. The experiment was performed twice in duplicate.

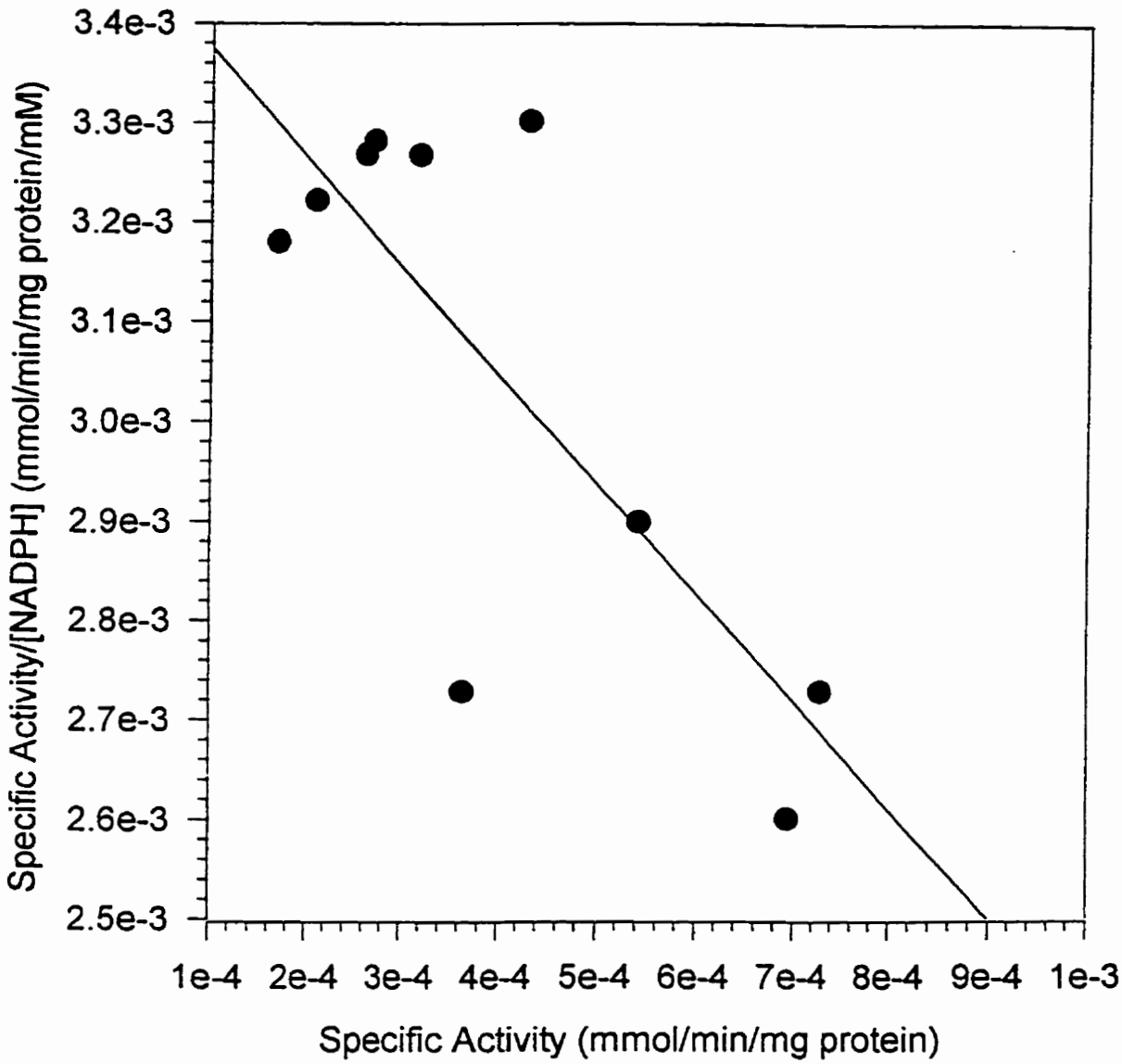
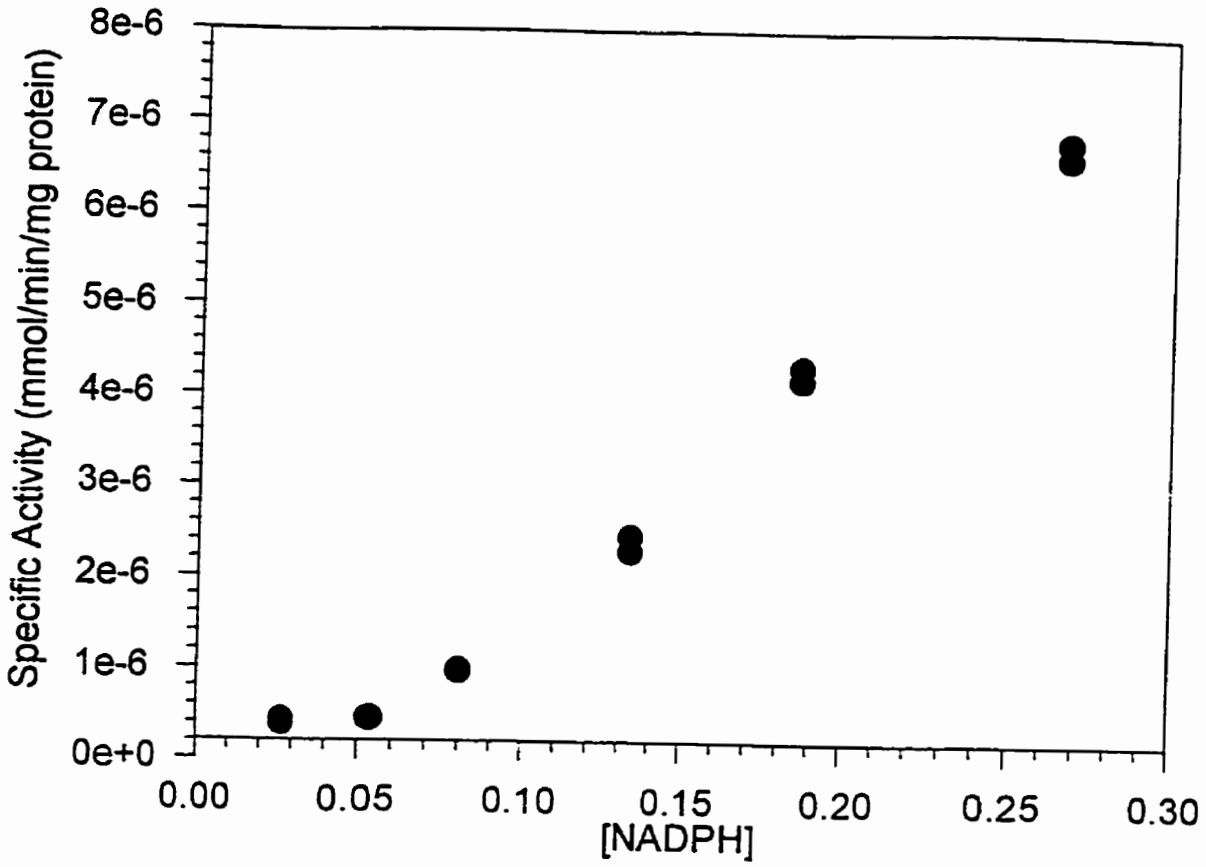

3.4e-3
3.3e-3
3.2e-3
3.1e-3
3.0e-3
2.9e-3
2.8e-3
2.7e-3
2.6e-3
2.5e-3
Specific Activity/[NADPH] (mmol/min/mg protein/mM)
1e-4
2e-4
3e-4
4e-4
5e-4
6e-4
7e-4
8e-4
9e-4
1e-3
Specific Activity (mmol/min/mg protein)

Figure III.9. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for the 65% $(NH_4)_2SO_4$ supernatant of *Mc. voltae*. The Lineweaver-Burke plot showed a non-first order reaction. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
8e-6
7e-6
6e-6
5e-6
4e-6
3e-6
2e-6
1e-6
0e+0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADPH]

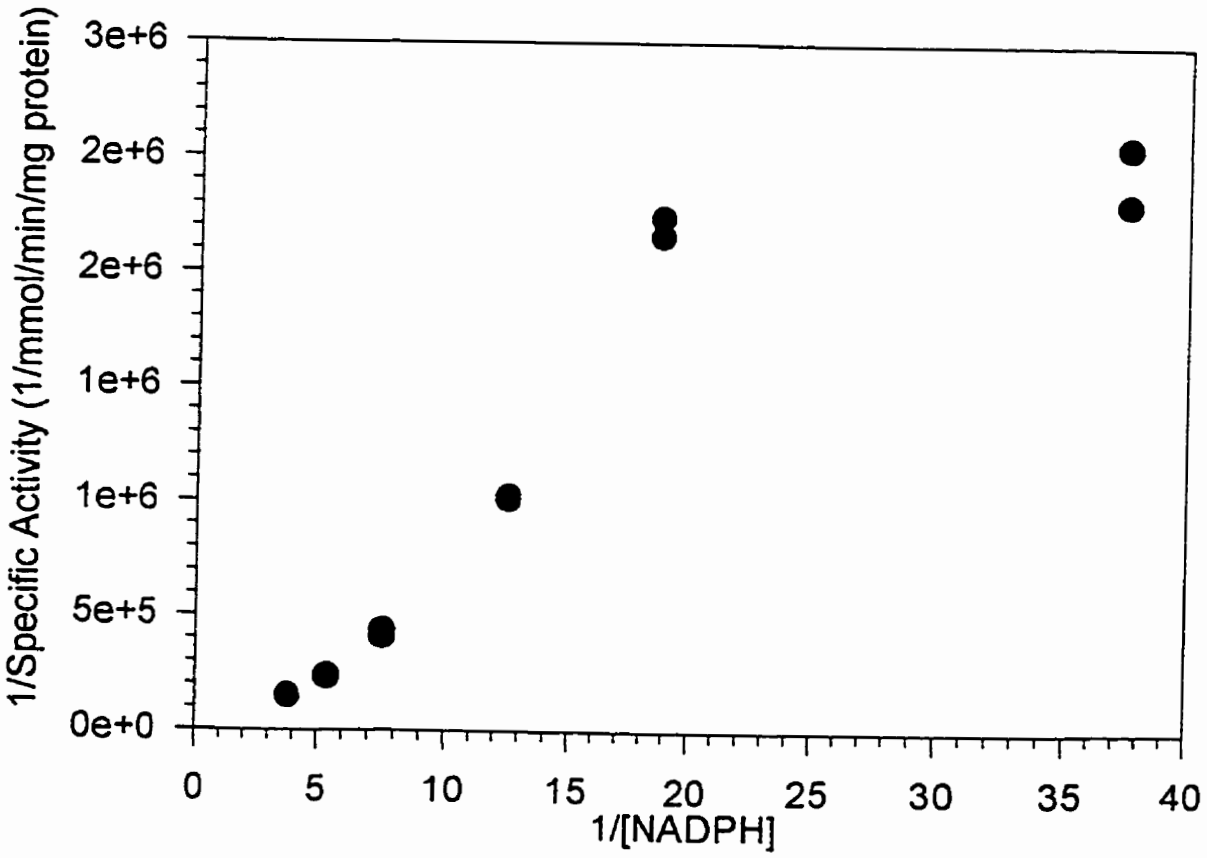

1/Specific Activity (1/mmol/min/mg protein)
3e+6
2e+6
2e+6
1e+6
1e+6
5e+5
0e+0
0
5
10
15
20
25
30
35
40
1/[NADPH]

Figure III.10. Eadie-Hofstee Plot for NADPH in the 65% $(NH_4)_2SO_4$ supernatant of *Mc. voltae*. The plot showed a non-first order reaction. The experiment was performed twice in duplicate.

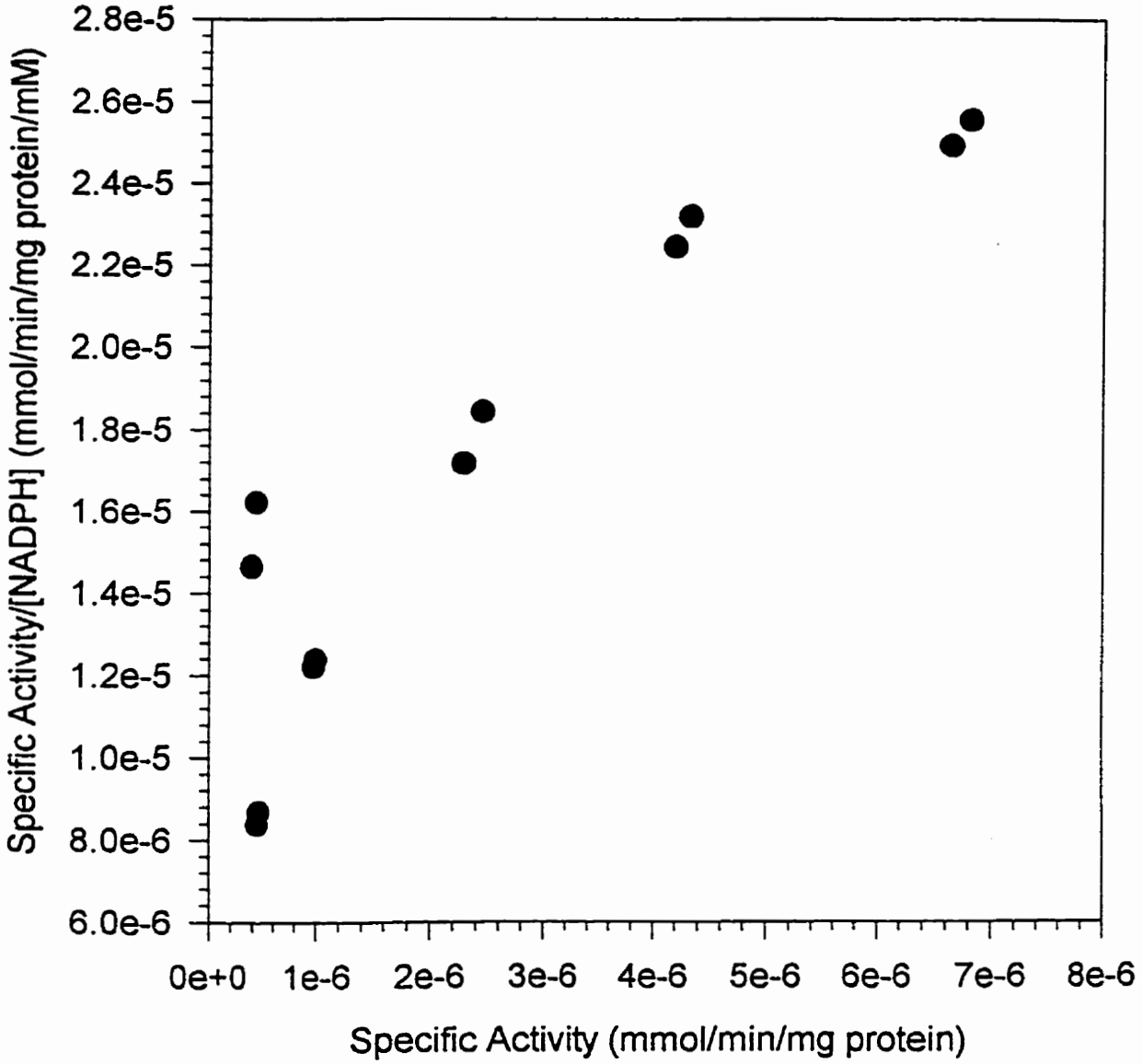
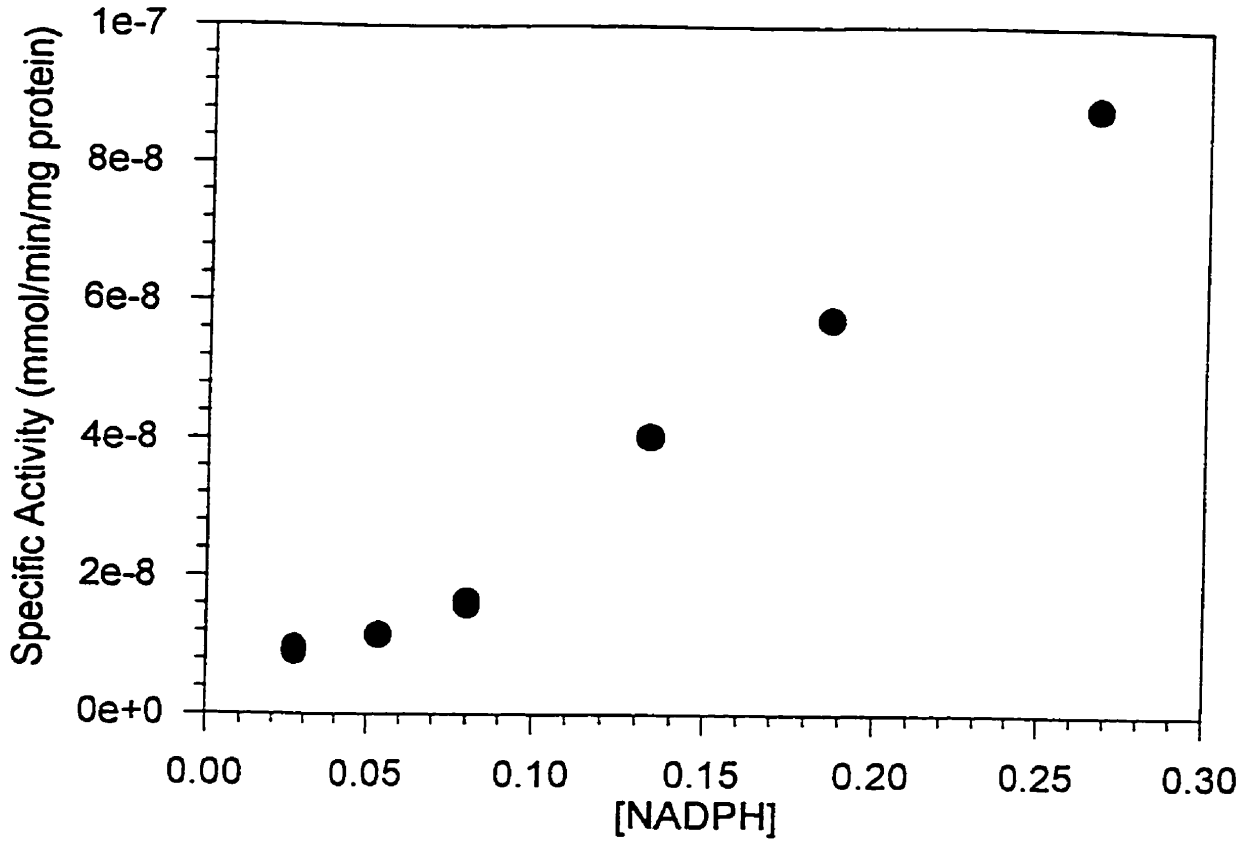

Specific Activity/[NADPH] (mmol/min/mg protein/mM)
2.8e-5
2.6e-5
2.4e-5
2.2e-5
2.0e-5
1.8e-5
1.6e-5
1.4e-5
1.2e-5
1.0e-5
8.0e-6
6.0e-6
0e+0
1e-6
2e-6
3e-6
4e-6
5e-6
6e-6
7e-6
8e-6
Specific Activity (mmol/min/mg protein)

Figure III.11. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for the 65-90% $(NH_4)_2SO_4$ pellet of *Mc. voltae*. The Lineweaver-Burke plot showed a sigmoidal curve indicating a non-first order reaction. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
1e-7
8e-8
6e-8
4e-8
2e-8
0e+0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADPH]

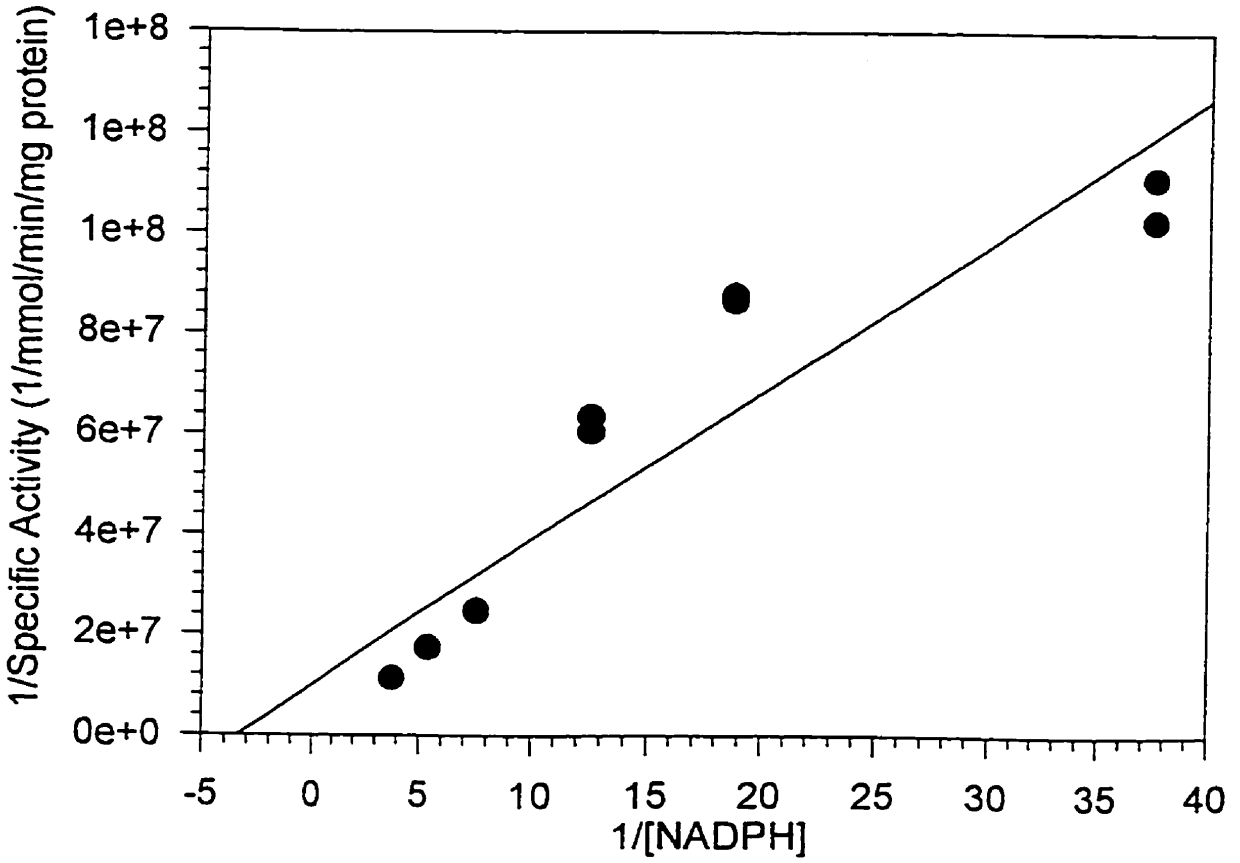
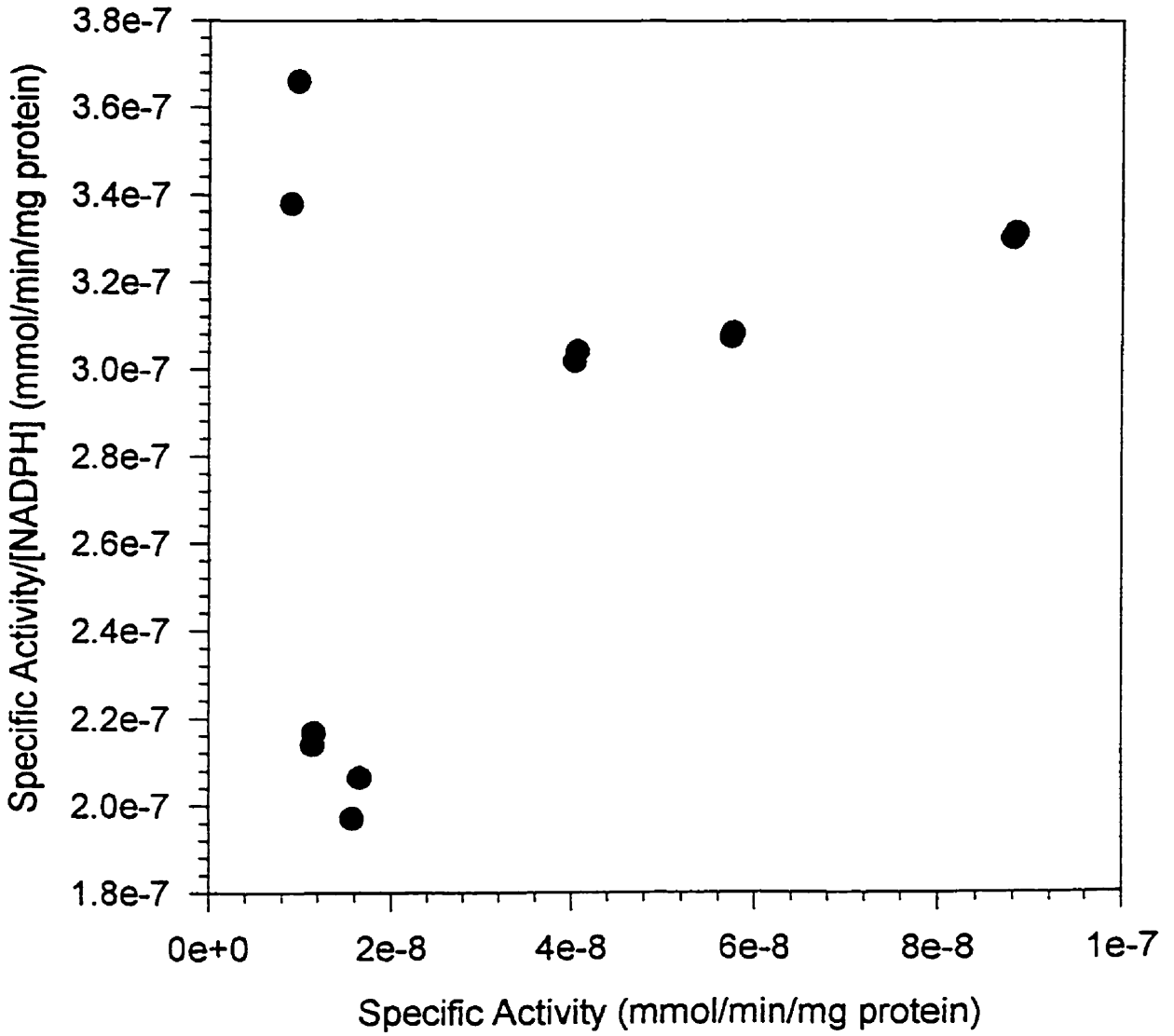

1/Specific Activity (1/mmol/min/mg protein)
1e+8
1e+8
1e+8
8e+7
6e+7
4e+7
2e+7
0e+0
-5
0
5
10
15
20
25
30
35
40
1/[NADPH]

Figure III.12. Eadie-Hofstee Plot for NADPH in the 65-90% $(NH_4)_2SO_4$ pellet of *Mc. voltae*. The plot showed a non-first order reaction. The experiment was performed twice in duplicate.

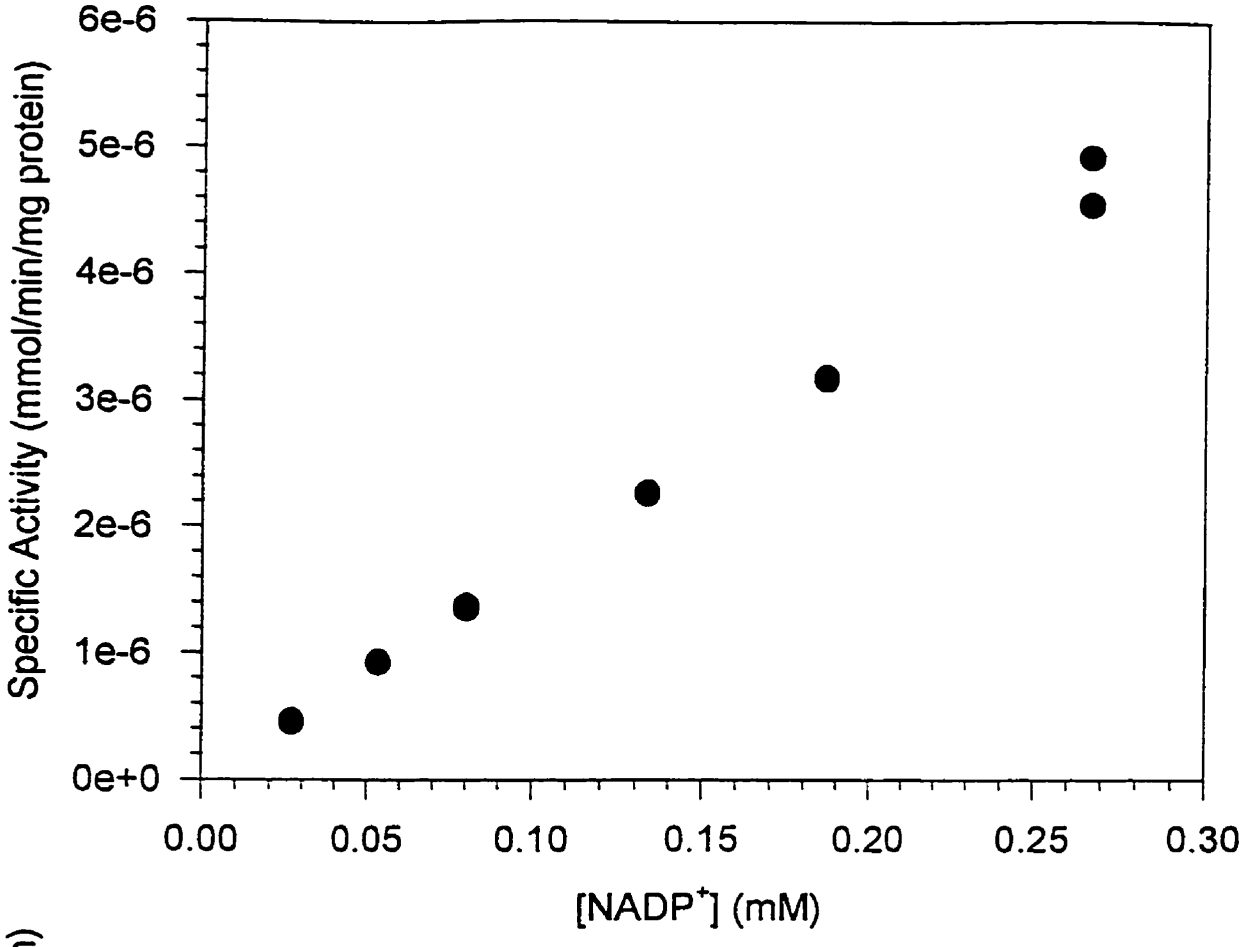

3.8e-7
3.6e-7
3.4e-7
3.2e-7
3.0e-7
2.8e-7
2.6e-7
2.4e-7
2.2e-7
2.0e-7
1.8e-7
Specific Activity/[NADPH] (mmol/min/mg protein)
0e+0
2e-8
4e-8
6e-8
8e-8
1e-7
Specific Activity (mmol/min/mg protein)

fractions and that they were similar. As well there was a similar trend in the points along the curve, further indicating it is the same enzyme being tested. However, the lack of activity for $NADP^+$ reduction in the 90% $(NH_4)_2SO_4$ supernatant and the large difference in ratios between $(NH_4)_2SO_4$ fractions indicates there are two separate enzymes.

The $NADP^+$ K_m's were also non-first order. For the 0-65% $(NH_4)_2SO_4$ pellet, both the Michealis-Menton and Lineweaver-Burke plots gave straight lines (Figure III.13.). Also, although the Lineweaver-Burke plot showed a straight line it intercepted at just above 0. This indicated a non-first order reaction. The Eadie-Hofstee plot confirmed this by giving an L-shaped curve (Figure III.14.). The 65% $(NH_4)_2SO_4$ supernatant K_m's for $NADP^+$ gave almost identical plots as the 65-90% $(NH_4)_2SO_4$ pellet for NADPH for Michealis-Menton and Lineweaver-Burke (Figure III.15.), and Eadie-Hofstee (Figure III.16.).

The F_{420} K_m's showed similar curves to those seen for NADPH. The 90% $(NH_4)_2SO_4$ supernatant showed parabolic curves for the Michealis-Menton plot while the Lineweaver-Burke plot showed a straight line (Figure III.17). The Eadie-Hofstee again confirmed that this was a non-first order reaction (Figure III.18.), and also appeared similar to the Eadie-Hofstee plots seen for NADPH. The 0-65% $(NH_4)_2SO_4$ pellet showed the same characteristics for both the Michealis-Menton and Lineweaver-Burke plots (Figure III.19.). The Eadie-Hofstee again appeared irregular (Figure III.20). Again, the similarity of the plots between $(NH_4)_2SO_4$ fractions strengthened the argument for two separate enzymes in *Mc. voltae*. However, the lack of activity in one direction while having activity in the other direction is the strongest evidence indicating that *Mc. voltae* possessed 2 separate enzymes.

Figure III.13. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for $NADP^+$ in the 0-65% $(NH_4)_2SO_4$ pellet of *Mc. voltae*. The Lineweaver-Burke plot showed a non-first order reaction as the x-intercept was above zero. The experiment was performed twice in duplicate.


6e-6
5e-6
4e-6
3e-6
2e-6
1e-6
0e+0
Specific Activity (mmol/min/mg protein)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADP+] (mM)

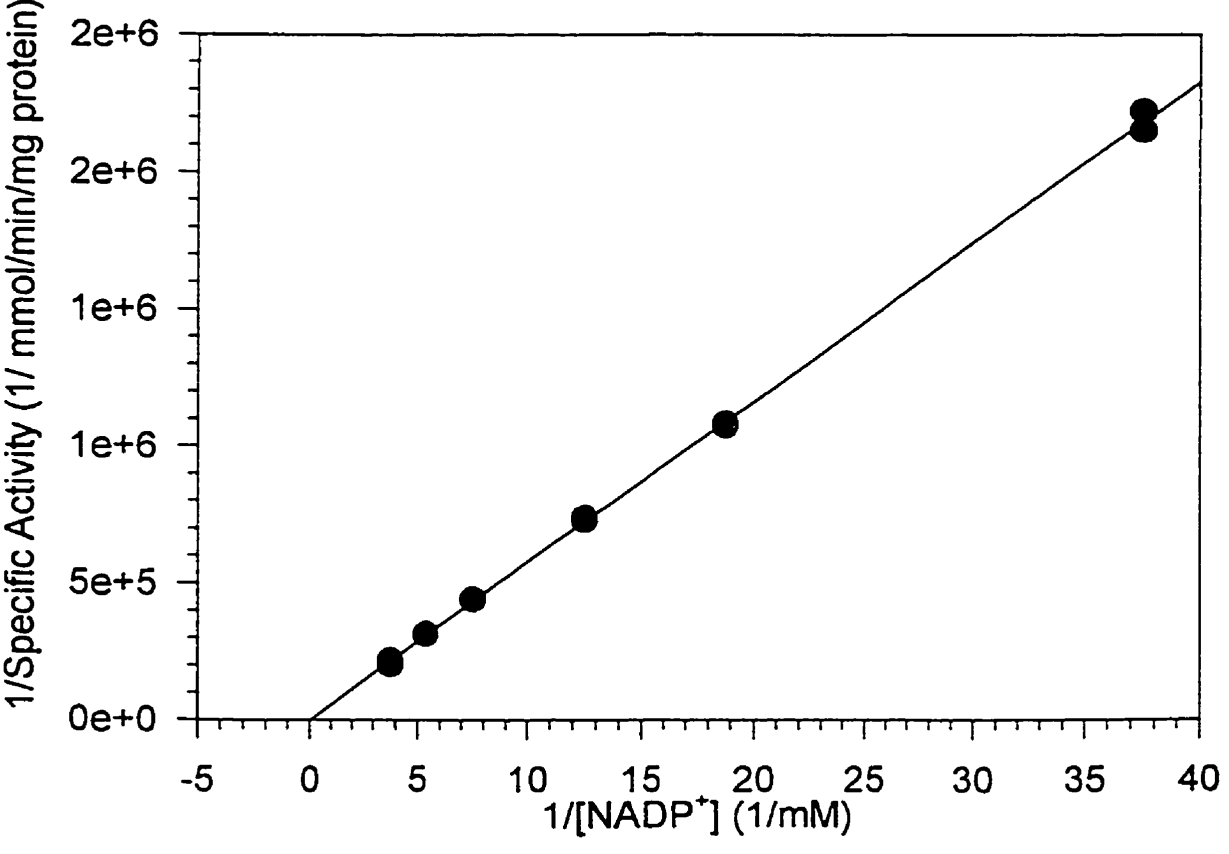

2e+6
2e+6
1e+6
1e+6
5e+5
0e+0
1/Specific Activity (1/ mmol/min/mg protein)
-5
0
5
10
15
20
25
30
35
40
1/[NADP+] (1/mM)

Figure III.14. Eadie-Hofstee Plot for $NADP^+$ in the 0-65% $(NH_4)_2SO_4$ pellet of *Mc. voltae*. The plot showed a non-first order reaction. The experiment was performed twice in duplicate.

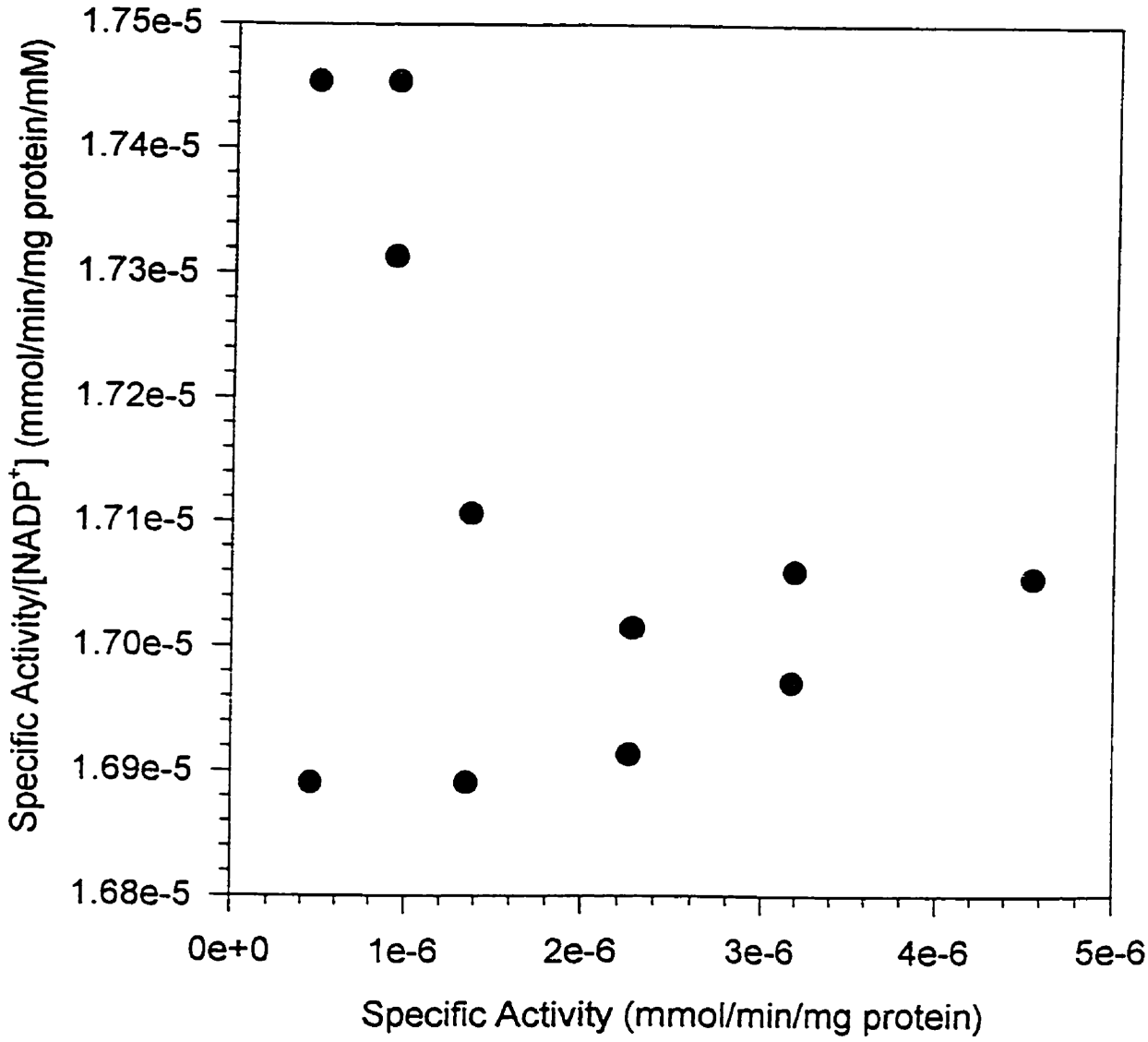
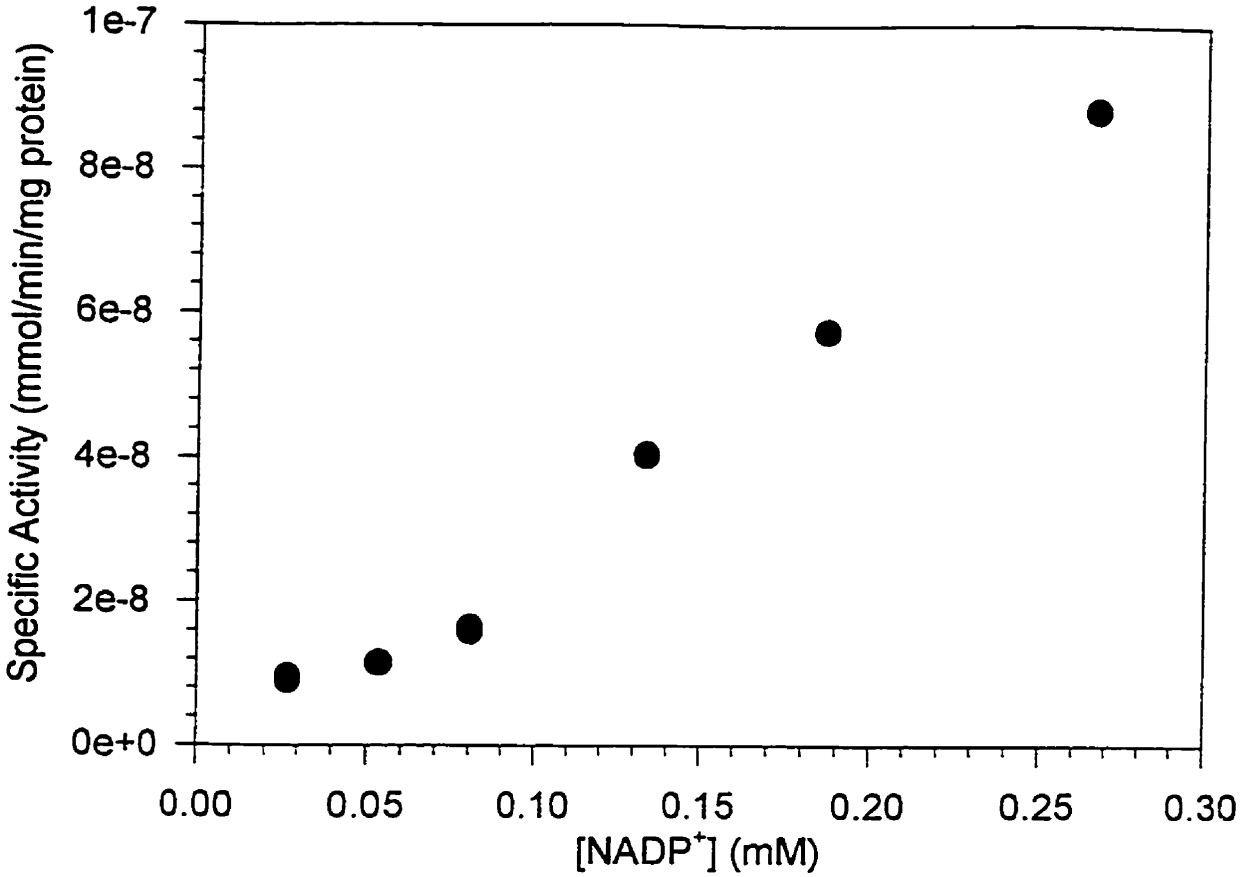

1.75e-5
1.74e-5
1.73e-5
1.72e-5
1.71e-5
1.70e-5
1.69e-5
1.68e-5
Specific Activity/[NADP+] (mmol/min/mg protein/mM)
0e+0
1e-6
2e-6
3e-6
4e-6
5e-6
Specific Activity (mmol/min/mg protein)

Figure III.15. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for $NADP^+$ in the 65% $(NH_4)_2SO_4$ supernatant of *Mc. voltae*. The Lineweaver-Burke plot showed a sigmoidal curve indicating a non-first order reaction. The experiment was performed twice in duplicate.


1e-7
8e-8
6e-8
4e-8
2e-8
0e+0
Specific Activity (mmol/min/mg protein)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADP+] (mM)

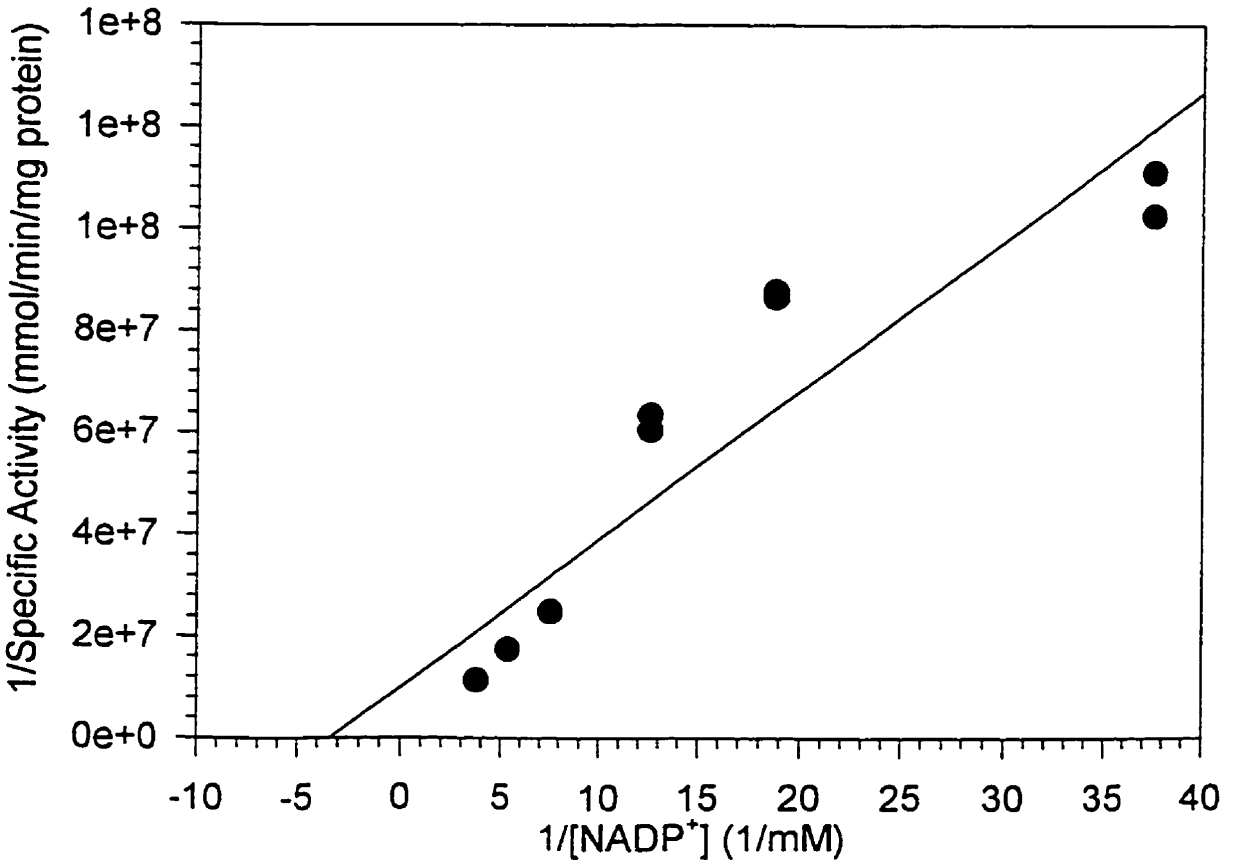

1e+8
1e+8
1e+8
8e+7
6e+7
4e+7
2e+7
0e+0
1/Specific Activity (mmol/min/mg protein)
-10
-5
0
5
10
15
20
25
30
35
40
1/[NADP+] (1/mM)

Figure III.16 Eadie-Hofstee Plot for $NADP^+$ in the 65% $(NH_4)_2SO_4$ supernatant of *Mc. voltae*. The plot showed a non-first order reaction. The experiment was performed twice in duplicate.

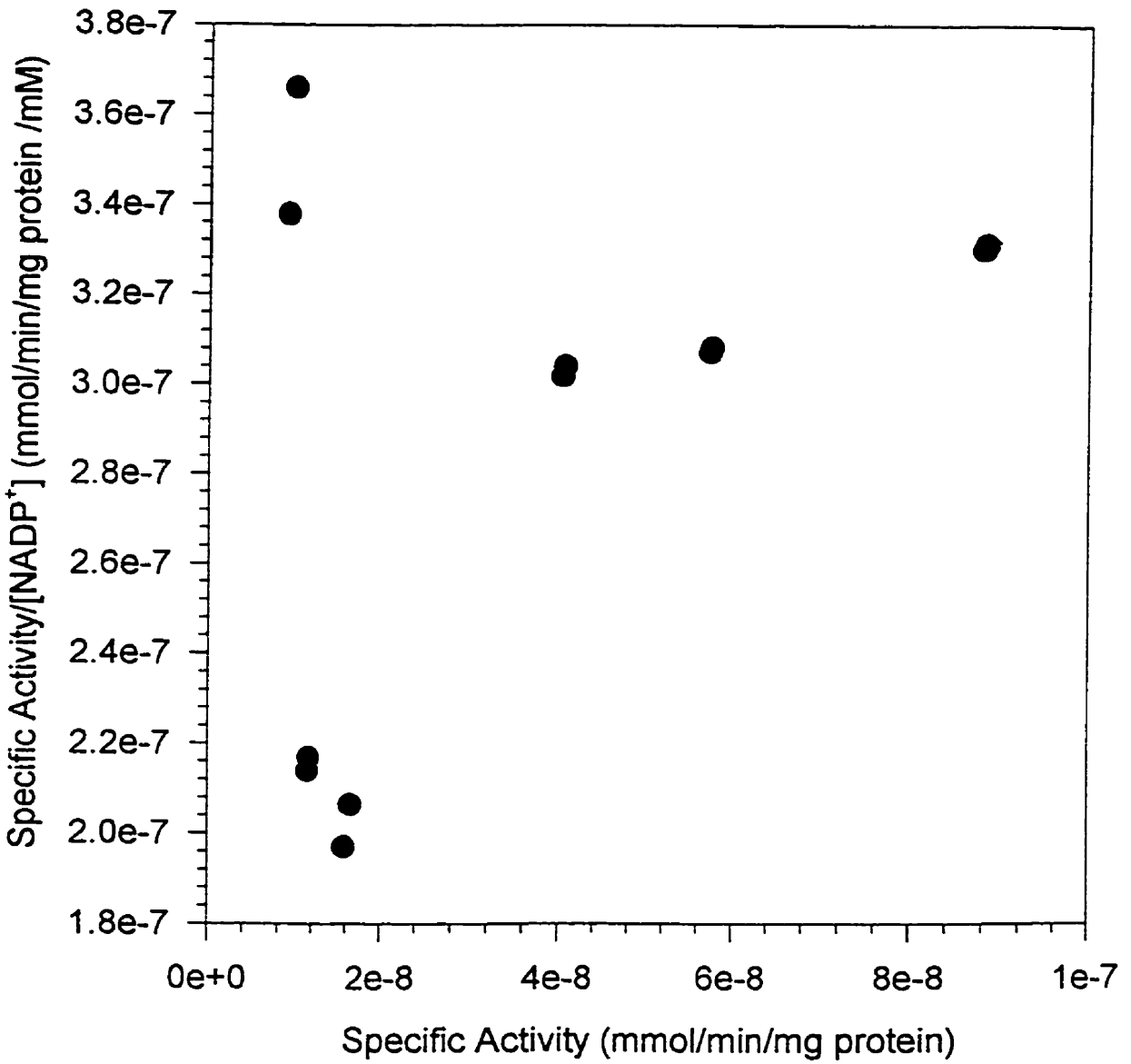
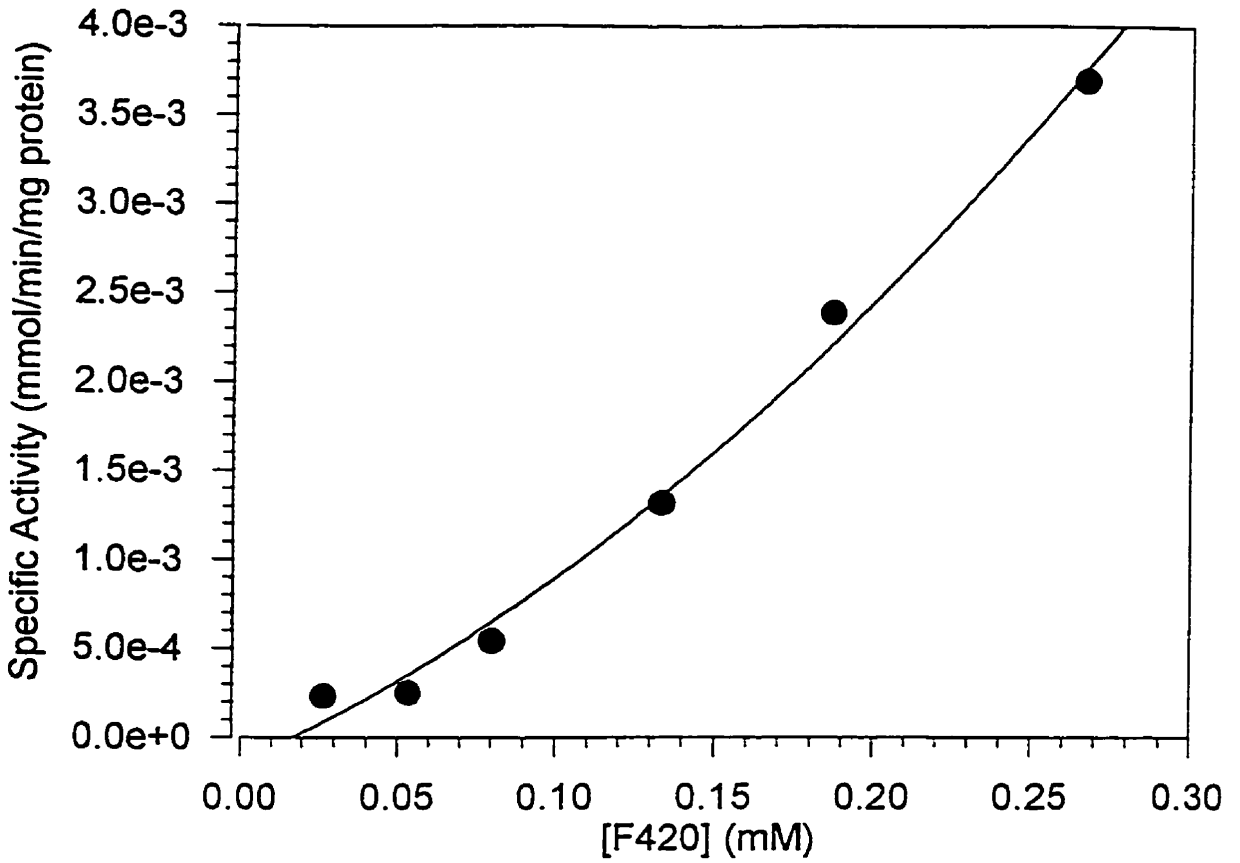

3.8e-7
3.6e-7
3.4e-7
3.2e-7
3.0e-7
2.8e-7
2.6e-7
2.4e-7
2.2e-7
2.0e-7
1.8e-7
Specific Activity/[NADP+] (mmol/min/mg protein /mM)
0e+0
2e-8
4e-8
6e-8
8e-8
1e-7
Specific Activity (mmol/min/mg protein)

Figure III.17. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for F_{420} in the 90% $(NH_4)_2SO_4$ supernatant of *Mc. voltae*. The Lineweaver-Burke plot showed a sigmoidal curve indicating a non-first order reaction. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
4.0e-3
3.5e-3
3.0e-3
2.5e-3
2.0e-3
1.5e-3
1.0e-3
5.0e-4
0.0e+0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[F420] (mM)

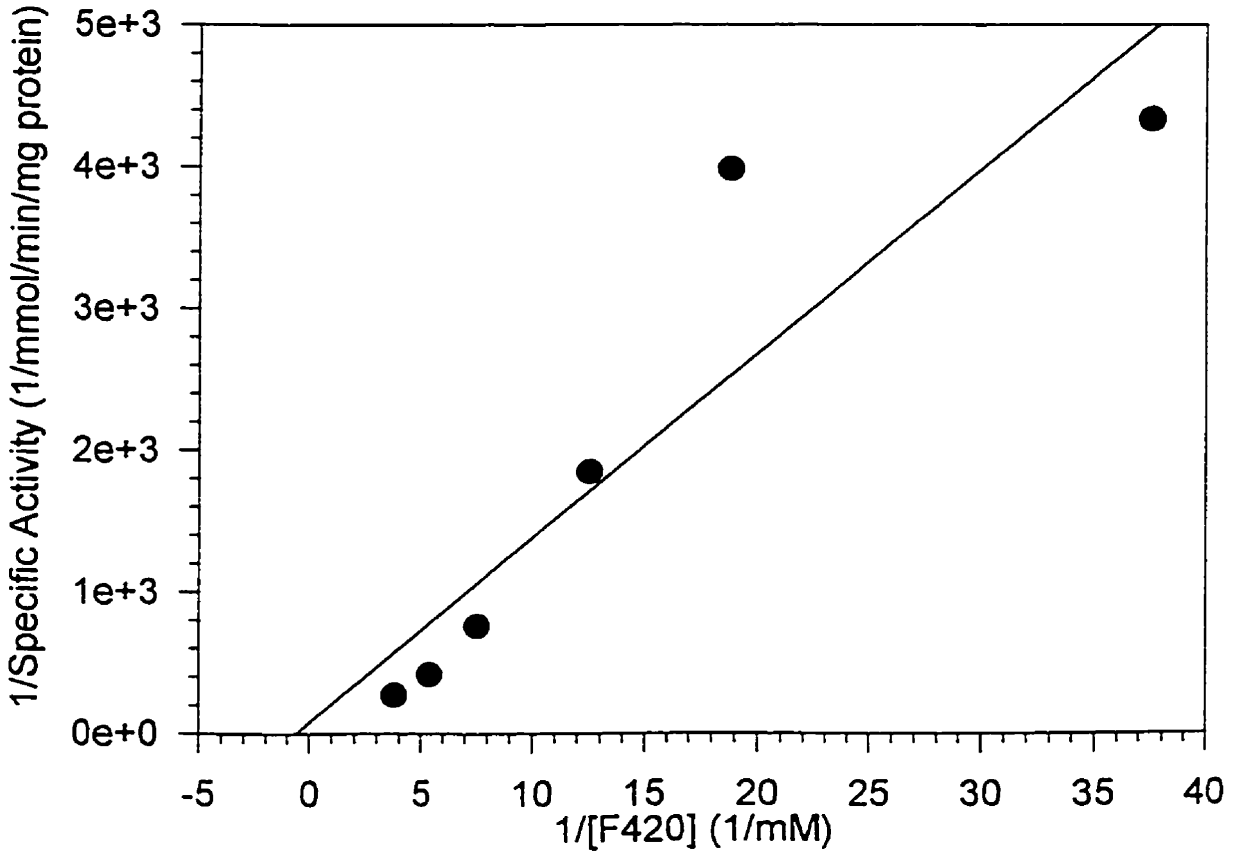

1/Specific Activity (1/mmol/min/mg protein)
5e+3
4e+3
3e+3
2e+3
1e+3
0e+0
-5
0
5
10
15
20
25
30
35
40
1/[F420] (1/mM)

Figure III.18 Eadie-Hofstee Plot for F_{420} in the 90% $(NH_4)_2SO_4$ supernatant of *Mc. voltae*. The plot showed a non-first order reaction. The experiment was performed twice in duplicate.

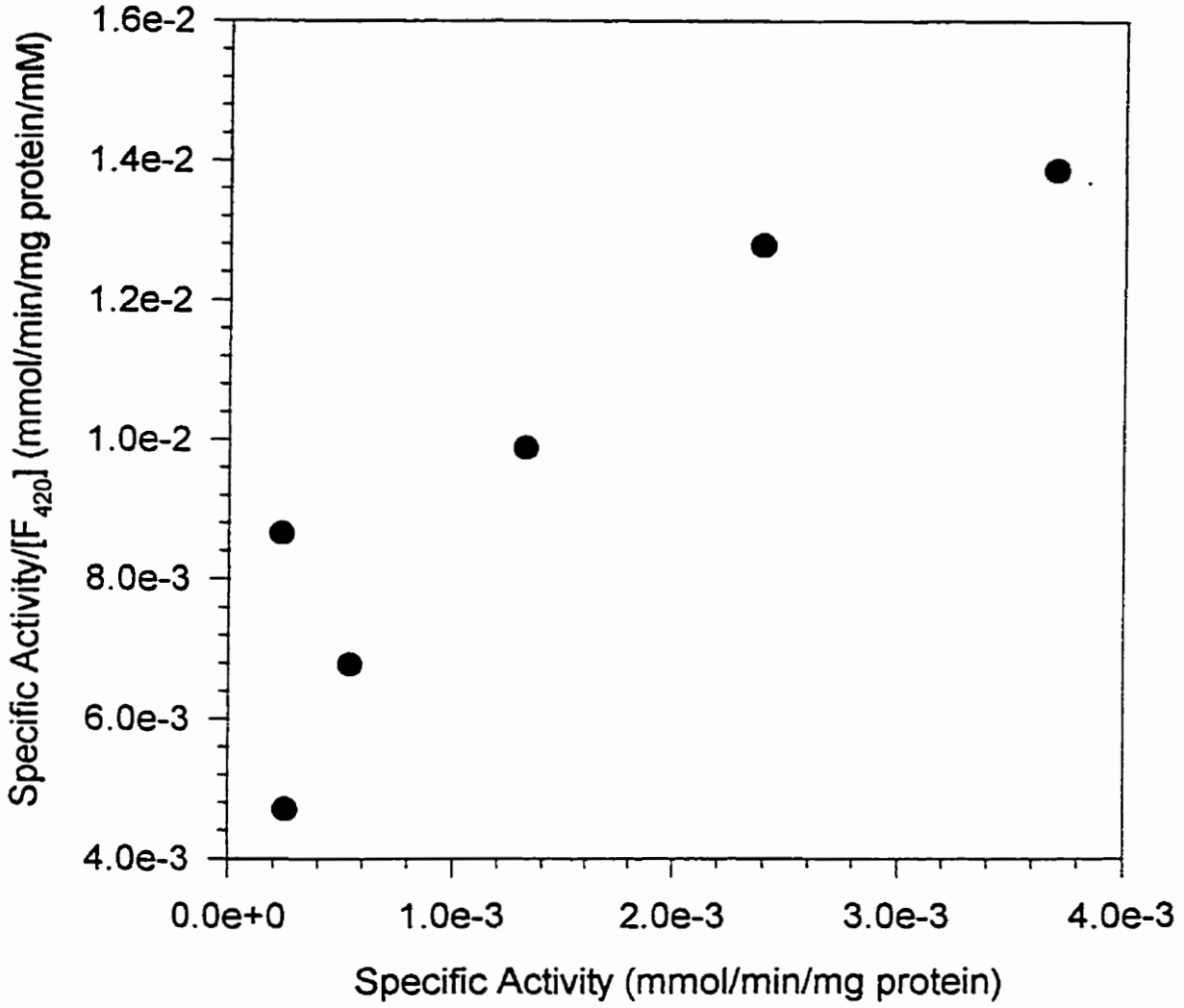
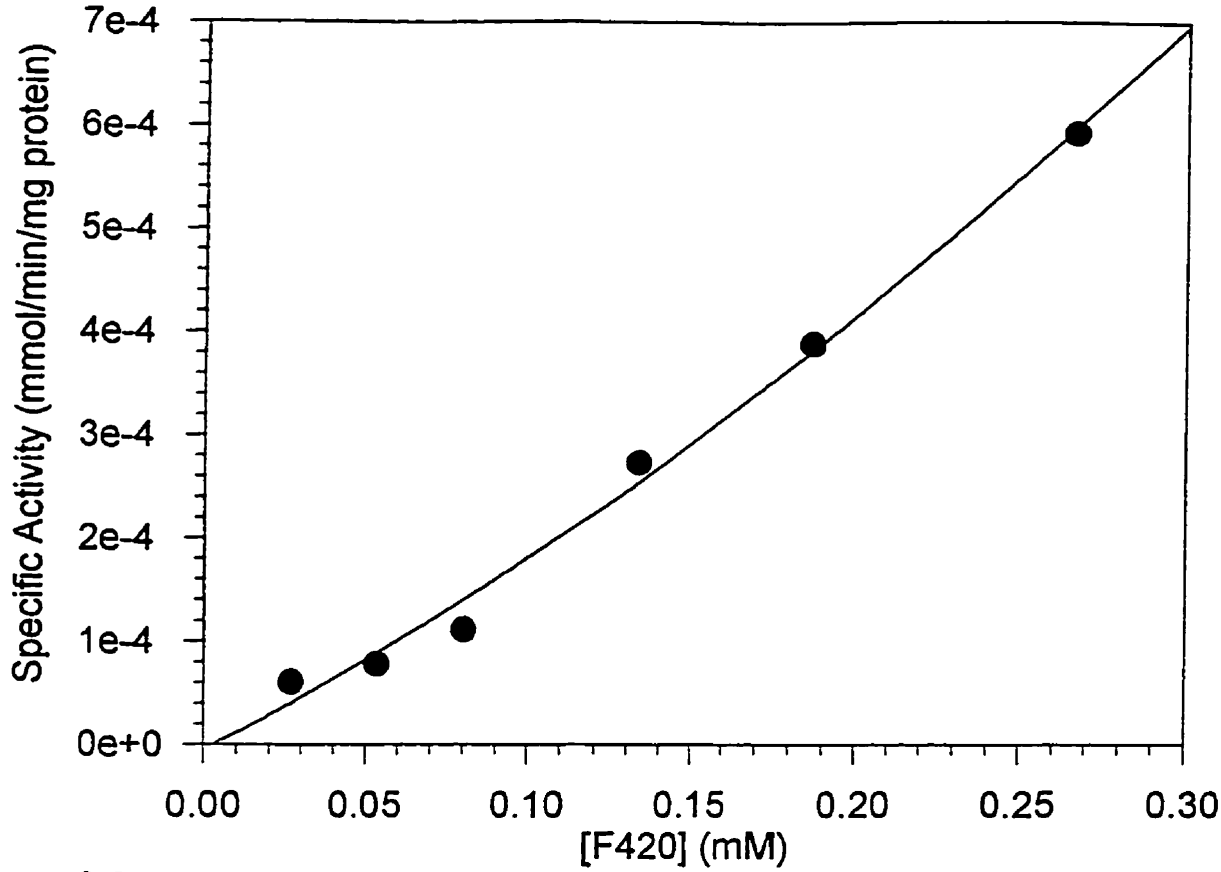

1.6e-2
1.4e-2
1.2e-2
1.0e-2
8.0e-3
6.0e-3
4.0e-3
Specific Activity/[F420] (mmol/min/mg protein/mM)
0.0e+0
1.0e-3
2.0e-3
3.0e-3
4.0e-3
Specific Activity (mmol/min/mg protein)

Figure III.19. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for F_{420} in the 0-65% $(NH_4)_2SO_4$ pellet of *Mc. voltae*. The Lineweaver-Burke plot showed a sigmoidal curve indicating a non-first order reaction. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
7e-4
6e-4
5e-4
4e-4
3e-4
2e-4
1e-4
0e+0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[F420] (mM)

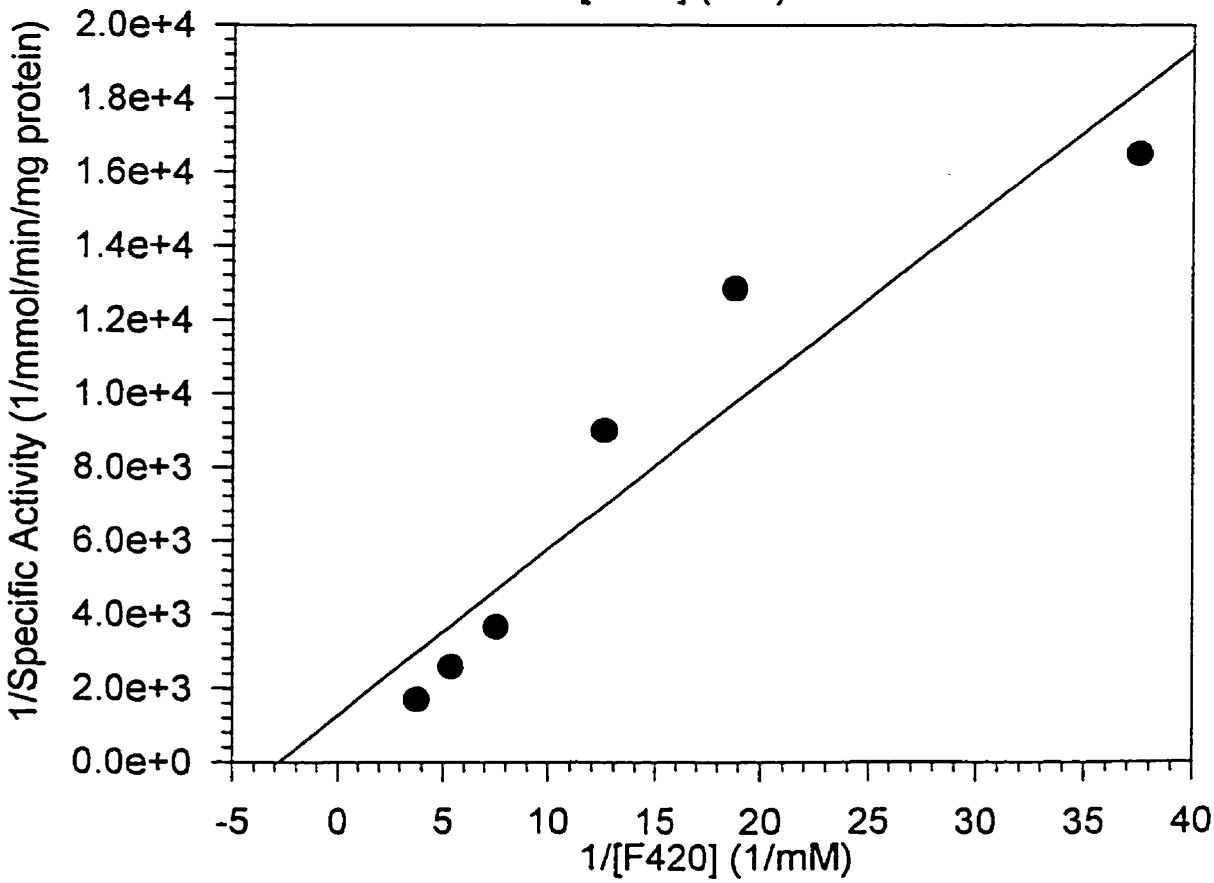
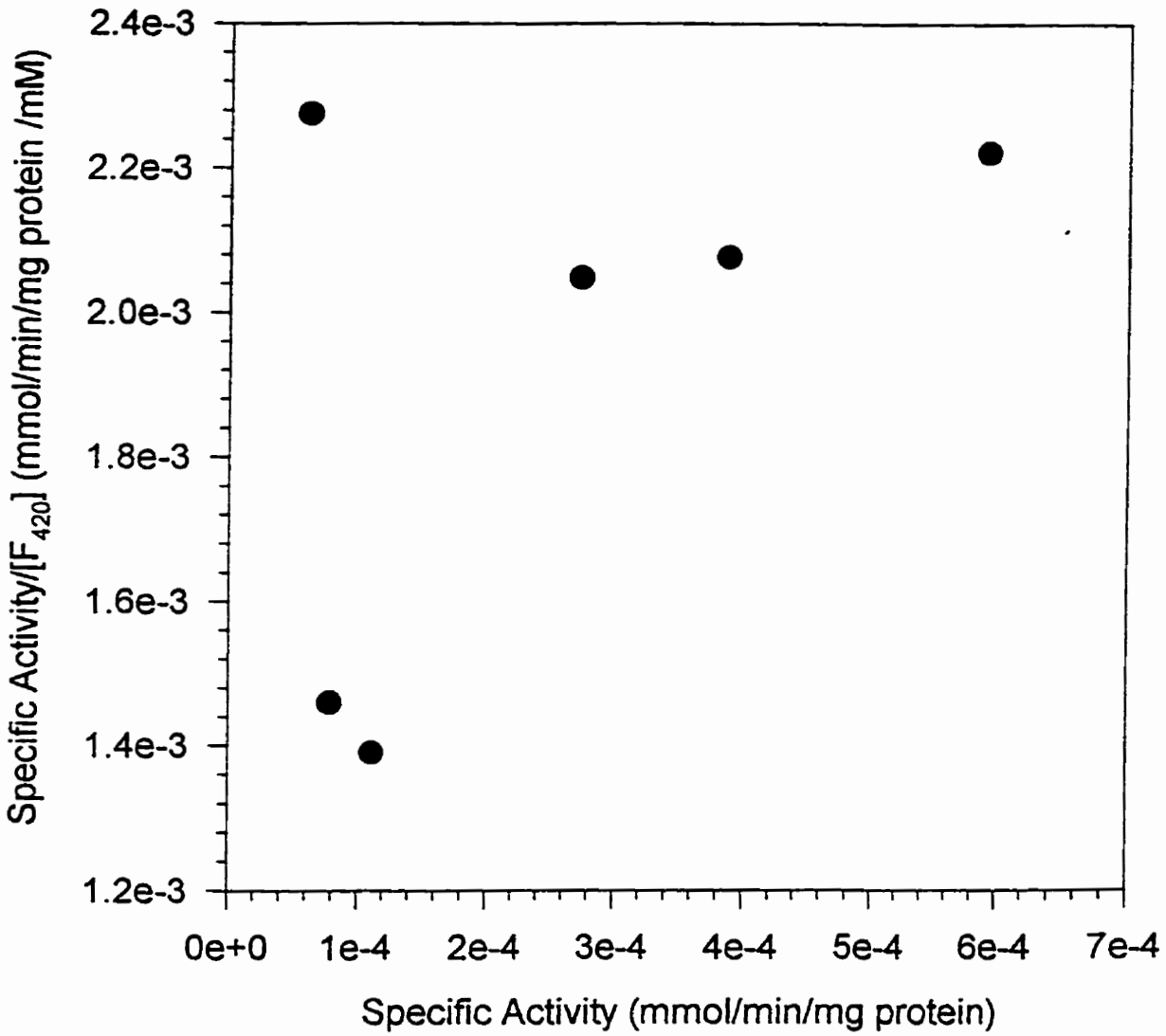

1/Specific Activity (1/mmol/min/mg protein)
2.0e+4
1.8e+4
1.6e+4
1.4e+4
1.2e+4
1.0e+4
8.0e+3
6.0e+3
4.0e+3
2.0e+3
0.0e+0
-5
0
5
10
15
20
25
30
35
40
1/[F420] (1/mM)

Figure III.20 Eadie-Hofstee Plot for F_{420} in the 0-65% $(NH_4)_2SO_4$ pellet of *Mc. voltae*. The plot showed a non-first order reaction. The experiment was performed twice in duplicate.


2.4e-3
2.2e-3
2.0e-3
1.8e-3
1.6e-3
1.4e-3
1.2e-3
Specific Activity/[F420] (mmol/min/mg protein /mM)
0e+0
1e-4
2e-4
3e-4
4e-4
5e-4
6e-4
7e-4
Specific Activity (mmol/min/mg protein)

III.4. Discussion

The survey of *Methanobacterium thermoautotrophicum* strain Marburg, *Methanobacterium* strain G2R, *Methanococcus thermolithotrophicus*, *Methanococcus voltae*, *Methanosphaera stadtmanae* (grown both on H_2:CO_2/methanol and H_2:CO_2/methanol/2-propanol), *Methanosphaera caniculi*, *Methanospirillum hungatei*, *Methanolobus tindarius*, *Methanosarcina barkeri* strain Fusaro, and *Methanosaeta concilli* yielded some interesting results.

These can be summarized in the following statements First, *Msph. stadtmanae* had highest activity overall, and secondly that genus *Methanosphaera* was only one to precipitate in 65-90% pellet, while most others were found in the 90% supernatant. Third, all organisms tested showed higher activity for $NADP^+$ reduction than NADPH oxidation with the exception *Msph. stadtmanae* with 2-propanol which indicates a change in *Msph. stadtmanae* in the presence of 2-propanol. Fourth, *Mc. voltae* and *Mc. thermolithotrophicus* showed no $NADP^+$ reduction activity in the 90% $(NH_4)_2SO_4$ supernatant. Finally, it appears that in the organisms tested Methanomicrobiales possessed 1 enzyme while Methanococcales possessed 2 enzymes, and Methanobacteriales may have 1 or 2 enzymes depending on the organism and growth conditions.

It is apparent that there is some degree of diversity in the way various methanogens control their respective intracellular $NADP^+$/NADPH pool with respect to $F_{420}/F_{420}H_2$ pools. This assumption can be made considering the differences in the pattern of activity in the various $(NH_4)_2SO_4$ fractions precipitation, and a comparison of $NADP^+$ reduction/NADPH oxidation activity, and K_m's support this supposition.

For the tested Methanococcales, it appears that they require separate enzymes to control their intracellular $NADP^+$/NADPH pool, while the Methanomicrobiales tested apparently use only one enzyme. Further, the comparisons within Methanobacteriales yield mixed observations. *Mb.* G2R appears to use only one enzyme while *Mb. thermoautotrophicum* uses two. Reasons for this are not obvious.

Genus *Methanosphaera* is a separate matter unto itself. In all other organisms tested, the major amount of activity was found in either the 0-65% $(NH_4)_2SO_4$ pellet or the 90% $(NH_4)_2SO_4$ supernatant. In both *Msph. stadtmanae* without 2-propanol and *Msph. caniculi* the major activity for both $NADP^+$ reduction and NADPH oxidation was found in the 65-90% $(NH_4)_2SO_4$ pellet. As well, in *Msph. stadtmanae* without 2-propanol and *Msph. caniculi* there was a large increase in specific activity from the CFE to the 65-90% $(NH_4)_2SO_4$ pellet. These two findings increase the possibility that the particular enzyme for genus *Msph.* may possess some unique structural features different from others tested. Also the fact that the ADH's purified to date are homodimers (Zellner and Winter (1987), Widdel et al., (1988), Widdel and Wolfe (1989), Winter (1989)) and the secondary ADH of *Msph. stadtmanae* is a heterodimer further increases this possibility (F. Lee, personal communication). This is consistent with the unique life style of *Msph. stadtmanae* as an obligate H_2/CH_3OH methanogen with low F_{420}.

In *Msph. stadtmanae* with 2-propanol, NADPH oxidation was also found in the 65-90% $(NH_4)_2SO_4$ pellet, but $NADP^+$ reduction was found in the 65% $(NH_4)_2SO_4$ supernatant. Therefore, the addition of 2-propanol to the growth medium, and thus the oxidation/reduction of NADP(H) by the secondary ADH appears to alter the activity of the $NADP^+$:F_{420} oxidoreductase in three ways. First, there appears to be an absence of the

increase in activity seen in the absence of 2-propanol, and secondly it appears that there are two separate enzymes in the presence of 2-propanol as opposed to one enzyme in the absence of 2-propanol. Third, in all other organisms tested, including *Msph. stadtmanae* without 2-propanol the dominant direction under the conditions tested was $NADP^+$ reduction whereas the addition of 2-propanol reverses this. NADPH oxidation is then the dominant direction under the conditions tested. This agrees well with recent findings that show the dominant direction for the secondary ADH is NADPH oxidation in the conversion of acetone to 2-propanol (F. Lee, personal communication).

Finally, the work performed here gives insight into the differences within the methanogens for controlling NADP(H) and should be continued. Continuing work could include a similar treatment of more methanogens. Further, it would be useful to attempt purification of the two enzymes of *Msph. stadtmanae* in the presence of 2-propanol and compare the N-terminal amino acid sequences to that of *Msph. stadtmanae* in the absence of 2-propanol. If the above assumption is true, this could validate the present work, may give further insight into the metabolism of *Msph. stadtmanae*, and possibly *Mc. voltae* or *Mc. thermolithotrophicus*. It would be very helpful to include *Mc. vannielii* in this survey in order to give a comparison between the three organisms from Methanococcales. Using *Mc. vannielii* would indicate the validity of the protocols used since the enzyme has already been purified in this organism, (Yamazaki et. al., 1980).

Chapter IV

Purification and Characterization of the $NADP^+$:F_{420} Oxidoreductase in *Methanosphaera stadtmanae*

IV.1. Introduction

Once it became apparent that *Methanosphaera stadtmanae* possessed an $NADP^+$:F_{420} oxidoreductase, the purification of the enzyme began. The enzyme of *Msph. stadtmanae* showed a 10-fold increase in total and a concurrent 30-fold increase in specific activity, as well as appearing allosteric based on work performed in chapter II. The purification protocol that was to be employed was based on the literature dealing with *Methanococcus vannielii*, *Methanobacterium thermoautotrophicum*, and the work of previous undergraduates employed by Dr. R. Sparling.

The $NADP^+$:F_{420} oxidoreductases *Methanococcus vannielii* and *Methanobacterium thermoautotrophicum* were purified using quite different strategies. *Methanococcus vannielii* employed $(NH_4)_2SO_4$ precipitation, substrate (NADPH) affinity and ion-exchange chromatography, (Yamazaki et. al., 1980). *Methanobacterium thermoautotrophicum* did not use $(NH_4)_2SO_4$ precipitation (Eirich and Dugger, 1984). Instead, a combination of dye-affinity, gel filtration, and ion-exchange chromatography was used.

These strategies were considered along with the work done on the $NADP^+$:F_{420} oxidoreductase of *Methanosphaera stadtmanae* by the previous undergraduates. The $(NH_4)_2SO_4$ treatment was considered as a first step considering the previous results of the

undergraduates yielded such a large enrichment of the enzyme. However, this was as far as the undergraduate work had successfully proceeded. Further purification was attempted using various dye-affinity and $NADP^+$ affinity. However, none of these were successful. This left several possibilities including gel filtration and ion-exchange with DEAE Cellulose. Therefore, the initial strategy after cell lysis was to use $(NH_4)_2SO_4$ precipitation followed by gel filtration and ion-exchange chromatography.

Several procedures were attempted but proved unsuccessful. These included dialysis, gel filtration chromatography (Sephacryl S-200), cation chromatography (Cellulose Phosphate), various forms of dye affinity chromatography, $NADP^+$-affinity chromatography, 2',5'-ADP affinity chromatography, hydrophobic chromatography, and Biorad Biologic cation chromatography. All were unsuccessful overall in that either there was a low enrichment of the enzyme, or there was an unacceptably low percent recovery of the enzyme as discussed further in the following results sections.

IV.2. Materials and Methods

IV.2.1. Chemicals

All chemicals and DEAE Cellufine column resin were purchased from Sigma Chemical Co. The Q Biorad Biologic column resin and Biorad Biologic equipment were purchased from BIORAD.

IV.2.2. Growth and Harvesting of cells

Methanosphaera stadtmanae (DSM 3091) was grown at 37°C, pH 7.0 under H_2:CO_2 (80:20, v/v) as described in chapter II.2.2. For a typical purification run, 10 L. of cells were used. Cells were centrifuged and resuspended in ~65 ml of harvesting buffer.

IV.2.3. $NADP^+$:F_{420} Oxidoreductase assay and calculation of activity

Unless otherwise specified, the assay conditions and calculation of activity is the same as described in chapter II.2.6.

IV.2.4. Purification of the $NADP^+$:F_{420} Oxidoreductase in *Methanosphaera stadtmanae*

The following purification procedures were all performed aerobically at 4°C, except for the Q Biorad Biologic column which was conducted at room temperature.

IV.2.4.1. $(NH_4)_2SO_4$ Precipitation

The first step of the purification protocol was to perform an $(NH_4)_2SO_4$ treatment on freshly pressed cells. This was done as previously described in chapter II.2.5. with one exception. The $(NH_4)_2SO_4$ treatment was extended to include a 75% saturation step. The 65% $(NH_4)_2SO_4$ supernatant was saturated to 75% and centrifuged at 47 000 x g for 20 minutes. The 65-75% supernatant was then subjected to a 90% saturation and the centrifugation repeated. The 75-90% $(NH_4)_2SO_4$ pellet was kept for further analysis.

IV.2.4.2. DEAE Cellufine

Once the activity of the 75-90% $(NH_4)_2SO_4$ pellet was confirmed, the enzyme was loaded onto a DEAE Cellufine column and 2.0ml fractions were collected in 4 inch test tubes using a Biorad 2110 Fraction collector.

The 20 mM Tris-HCl was the loading buffer for the DEAE Cellufine column. The column (1.5 cm. x 11.5 cm.) flowrate was 25 ml/hr for loading. A wash through of 50 ml of the loading buffer was used.

Binding of the enzyme to the DEAE Cellufine column was determined by concentrating the eluted volume after loading, resuspending in 2.0 ml of loading buffer, and testing for activity and protein content. This was repeated for the eluted volume of the wash through. The range of the appropriate positive KCl gradient for elution was determined by performing 50ml discontinuous bumps of 0.4 M, 0.7 M, 1.0 M, and 2.0 M KCl.

Each of these was collected, concentrated, resuspended in 2.0 ml of loading buffer, and checked for activity and protein content. The salt gradient used was a 0 - 0.3 M KCl gradient over 60 ml. Fractions were collected in 2.0 ml volumes and tested for activity. Those with activity were pooled, concentrated, and resuspended in 2.0 ml of loading buffer for protein content determination, and further analysis.

IV.2.4.3. Q Biorad Biologic

The Biorad biologic used was purchased from Biorad, and used a Q anion resin for the column. All procedures were controlled using the computer controlled interface which came with the Biorad Biologic. The loading buffer used was Tris-Cl, pH 8.1 as

previously. The salt gradient was determined in the same manner as for the DEAE Cellufine step of the protocol. For the Biorad Biologic step, the final salt gradient consisted of 0-0.3M KCl and 0.3-0.5M KCl. The run began with 10 ml isocratic flow of 20mM Tris-Cl over 10ml to equilibrate the column, a 10 ml injection of the sample using a 5 ml injection loop and 6 ml isocratic flow through the column. The 0-0.3M KCl gradient was 40 ml with an 8 ml hold at 0.3M KCl. Next was a 20ml gradient of 0.3-0.5M KCl and a hold of 6 ml of 0.5M KCl.

IV.2.5. Electrophoresis and purity criteria of the $NADP^+$:F_{420} Oxidoreductase

SDS-PAGE was performed during the purification of the $NADP^+$:F_{420} oxidoreductase in order to determine the molecular weight of subunits of the enzyme, and the relative purity of the enzyme as compared to previous purification steps. This was done using a Tris/glycine buffer system (Laemmli, 1970). The protein standards used were from a MW-SDS-200 kit purchased from Sigma Chemical Co. These included myosin of rabbit muscle (205kDa), β-galactosidase of *E. coli* (116 KDa), phosphorylase B of rabbit muscle (97 KDa), Bovine Serum Albumin (66 KDa), egg albumin (45 KDa), and carbonic anhydrase (29 KDa).

A 10% slab gel was used which contained 10.0 ml separating gel buffer (30:0.8 (v:v) acrylamide:N'-N'-methylenebisacrylamide), 3.75 ml resolving gel buffer (3.0 M Tris-HCl pH(8.8)), 0.3 ml 10% SDS (10 g SDS/ 100 ml ddH_2O), 15.85 ml ddH_2O, 50 µl TEMED, and 50 µl of 1.5% ammonium persulfate (1.5g/100 ml ddH_20). The solution was injected between the glass plates to form the gel using a 50 cc syringe. The solution was allowed to harden for 20 minutes. The ammonium persulfate was added just before injection as it

acted as the catalyst for the hardening reaction. Ethanol (95%) was placed on top of the gel immediately after injection in order to form a smooth edge on the gel.

When the gel had hardened, the ethanol was poured off and the 3% stacking gel was injected in the same manner. A well comb for formation of the sample loading wells was placed in the stacking gel at this time. The 3% stacking gel consisted of 1.5 ml acrylamide:N'-N'-methylenebisacrylamide, (30:0.8, v:v), 2.5 ml stacking gel buffer stock (6% Tris, 50 µl TEMED per 100 ml ddH2O, pH = 6.7), 50 µl of 10% SDS, 6.25 ml ddH_2O, 60 µl TEMED, and 50 µl ammonium persulfate. Again, the ammonium persulfate was added immediately before injection as it was the catalyst for the hardening reaction. The solution was allowed to harden for 15 minutes.

The samples for the SDS-PAGE gel were diluted in 2X sample buffer. The 2X sample buffer consisted of; Tris, 1.51 g; glycerol, 20.0 ml; dissolved to 35.0 ml total volume, and the pH adjusted to 6.75 with HCl. The SDS (Lauryl Sulfate) was added, 4.0 g; 2-mercaptoethanol, 0.010 g; and bromophenol blue, 0.002 g; and diluted to a total volume of 100 ml.

The samples were diluted in the following ratios, and mixed in 1.5 ml Eppendorf tubes; CFE 100 µl: 400 µl buffer, 75-90% $(NH_4)_2SO_4$ pellet 100 µl: 400 µl buffer, DEAE Cellufine fractions 100 µl: 200 µl buffer, Q Biorad Biologic fractions 100 µl: 100 µl buffer. Once mixed, the samples were vortexed briefly, and boiled at 80°C for 1.5-2.0 minutes, and allowed to cool for 5 minutes. The samples, 80 µl each, were injected into the wells which were submerged in electrode buffer. The electrode buffer consisted of; Tris, 6.05 g; glycine, 28.8 g; SDS (Lauryl Sulfate) 2.0 g, diluted to a final volume of 2.0 Litres, and the pH adjusted to 8.3. The entire apparatus was submerged into the

electrophoresis tank filled with electrode buffer. Electrical leads were attached to a Fisher Biotech FB135 power unit. The unit was set to run at a constant amperage of 22 milliamps. The procedure lasted approximately 5.5 hours.

Once the dye front had reached approximately one inch from the bottom of the gel, the power was disconnected. For the purpose of determining the molecular weight of the enzyme, the gel submerged in fixative solution (methanol, glacial acetic acid, ddH_2O; 40:7:53, v:v:v) overnight. The purpose of the fixative solution was to prevent any loss of protein during the destaining procedure, or any diffusion of the bands into the gel. The fixative solution was poured off and the gel submerged in Coomassie Brilliant Blue Stain (Coomassie Blue R250, 0.2 g; methanol, 80.0 ml; glacial acetic acid, 20.0 ml; ddH_2O, 100 ml) for one hour. The gel was then destained (methanol, glacial acetic acid, ddH_2O; 35:10:55, v:v) until all background coloring was removed, (Fairbanks et. al., 1971).

When all background coloring was removed, the gel was submerged in ddH_2O for 1 hour 3 times. The gel was dried at 80°C for 1.5 hours on a Savant SGD4050 gel dryer using a Savant GP100 vacuum pump.

Along with SDS-PAGE, a native-PAGE gel with a 2.8-19.6% acrylamide gradient was also used. This was also done using a Tris-glycine buffer system. The 2.8% acrylamide solution consisted of 15.9 ml ddH2O, 2 ml of 30:0.8 acrylamide:bisacrylamide solution, 5 µl TEMED, and 2 ml resolving buffer (48 ml 1N HCl and 36.3 g Tris/100 ml ddH2O), pH= 8.9. The 19.6% acrylamide solution consisted of 2 ml resolving buffer, 14 ml of 30:0.8 acrylamide:bisacrylamide solution, 4 ml ddH2O, 5 µl TEMED. The 2.8% acrylamide solution and 19.6% acrylamide solution were mixed in a gradient mixer and poured in between the glass plates. Just prior to putting the

solutions into the gradient mixer, 60 µl ammonium persulfate was added to each solution. Once the gel was poured, 95% ethanol was added to the top to ensure an even edge to the top of the gel, and the gel was allowed to harden for approximately 30 minutes.

Once the gel was hardened, the stacking gel and well comb were added and allowed to harden as in the SDS-PAGE protocol. From each sample 40 µl was added to 60 µl of glycerol to ensure that the sample remained in the well and there was minimal diffusion of the protein during the running of the gel. From this, 100 ml of sample/glycerol was added to each well. The protein standards were injected (5 µl) into the well at each end of the gel. Standards were purchased from Pharmacia. they consisted of; Thyroglobulin, 669 kDa (hog thyroid); Ferritin, 440 kDa (horse spleen); Catalase, 232 kDa (beef liver); Lactate dehydrogenase, 140 kDa (beef heart); and Albumin, 67 kDa (bovine serum).

The rest of the running of the gel, staining and destaining was as for the SDS-PAGE gel, except that the native-PAGE gel was run for 8 hours.

IV.2.6. Electroelution

For continuation of the purification procedure, the gel was cut on either side and the standard ladder and 1 lane containing enzyme were stained and destained as above. The rest of the gel was placed in ddH_2O while the staining was proceeding.

Once the cut away sides of the gel were stained and destained, the three pieces were placed together and a line cut across the unstained portion of the gel in the area containing the stained protein band on either side. The area containing the unstained protein was kept and the rest discarded. This was cut up into fine pieces and divided into five equal amounts. These were loaded into the five vessels and placed in the

electroelution apparatus containing a buffer of Tris-glycine (25 mM Tris, 192 mM Glycine, 0.1% SDS). The whole apparatus was placed into the fridge at 4°C, and run at 45 mA for 5 hours. Once the run was completed, the volume of buffer that had passed through the membrane in each vessel was collected, and concentrated to 1 ml. This was tested for activity and protein content. To assure the enzyme was purified, it was re-run on a native PAGE, stained, and dried as above.

IV.2.7. Molecular mass determination

Molecular mass of the functional enzyme was determined in two ways. The first was by native-PAGE as described in Chapter IV.2.5. Once the gel was stained and destained, the distances were measured from top of the gel to each standard, the R_f values calculated, and a calibration curve constructed using Microsoft Excel 5.0. The R_f value for the band representing the enzyme was then plotted against the calibration curve, and the molecular weight determined.

The second method for molecular mass determination involved using a Sephacryl S-200 column (1.5 cm. x 60 cm.) which was previously equilibrated in 20 mM Tris-Cl, pH 8.1. A sample volume of 2.0 ml was loaded onto the column. A constant flow rate of 1.0 ml per minute was maintained with 2.0 ml fractions collected. The standard molecular weight marker kit used for the calibration was purchased from Sigma Chemical Co. The kit included blue dextran (2,000 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (15 kDa).

The void volume of the column was calculated by running 2ml of Blue Dextran in 20% glycerol on the column. The volume of elution (V_e) divided by the void volume (V_o) ratio was calculated for each standard and the enzyme, and plotted on a graph with the abscissa being the V_e/V_o ratio, and the ordinate being the molecular weight of each standard. A standard curve was extrapolated and the V_e/V_o of the enzyme plotted, thereby giving an estimation of the molecular weight of the functional enzyme, Andrews, (1965).

IV.2.8. Protein determination

The protein concentration of the samples was determined by the method of Bradford, (1976) as previously described in Chapter II.2.4.

IV.2.9. K_m Determinations

The K_m's were determined anaerobically using assay tubes prepared as in Chapter II.2.6. Volumes used were 2 ml of assay buffer, 50 µl of sample, 100 µl of the substrate not being tested for and 10, 20, 30, 50, 70, 100 µl of the substrate being tested. If F_{420} was being tested for, then the NADPH volume used was 100µl, while the volumes of F_{420} used were 10, 20, 30, 50 ,70, 100 µl. For $NADP^+$ and NADPH the concentration of the substrate was assumed to be equal in each tube. In the case of F_{420} and $F_{420}H_2$, the absorbance at 400 nm was recorded and divided by 25.9 (the molar extinction co-efficient of F_{420}) and this was used as the concentration of $F_{420}(H_2)$.

IV.2.10. Storage of enzyme

During the course of a purification run, the enzyme was stored at 4°C in 20mM Tris-HCl, pH 8.1. When the enzyme was not used for longer periods, it was stored at -60°C in the same buffer.

IV.3. Results

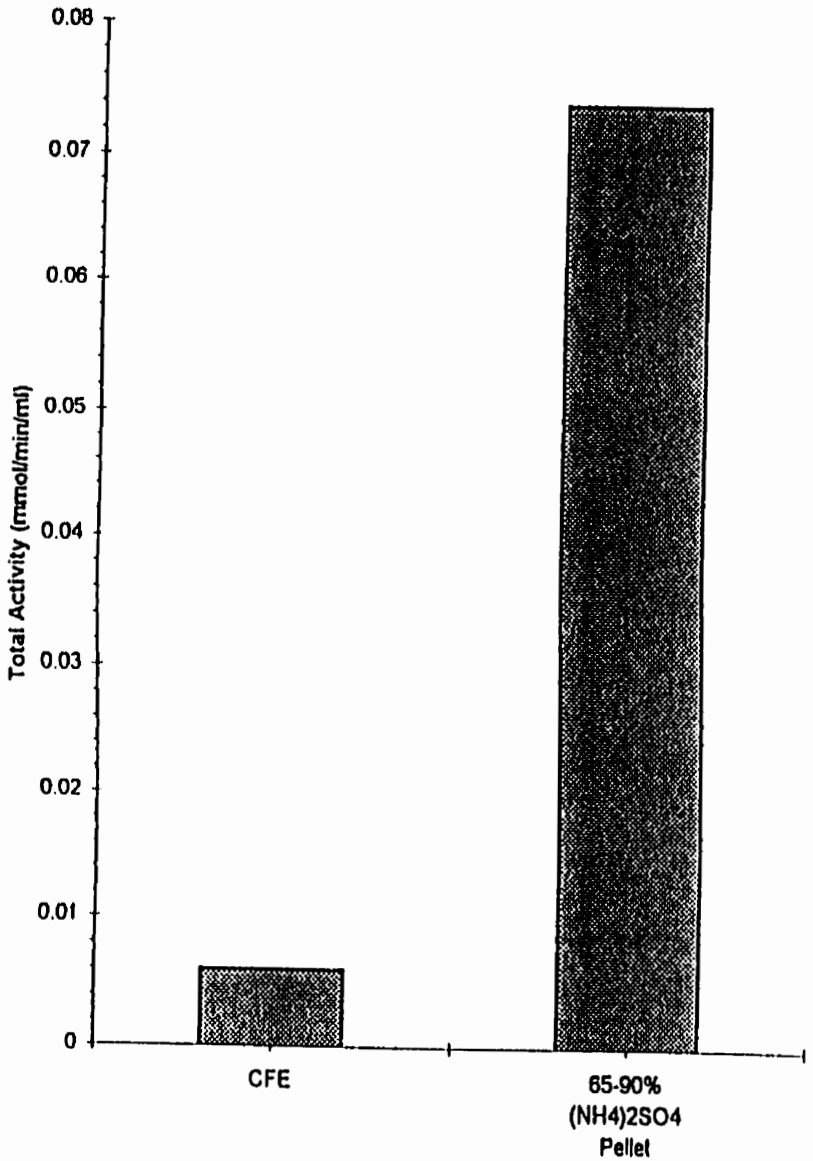
IV.3.1. Purification of the $NADP^+$:F_{420} Oxidoreductase

The $NADP^+$:F_{420} oxidoreductase was purified utilizing the following protocol; 75-90% $(NH_4)_2SO_4$ saturation, DEAE Cellufine chromatography, Biorad Biologic chromatography, native PAGE, electroelution.

The 75-90% $(NH_4)_2SO_4$ pellet resulted in an unusually large degree of enrichment. Both the total and specific activities were increased simultaneously. The total activity was increased approximately 16-fold with a concomitant increase in specific activity of approximately 43-fold (Figure IV.1.). This yielded an apparent enrichment of over 450-fold, with a 27-fold decrease in total protein (Table IV.1.). Since there was such a large increase in both total and specific activities, all percent recoveries calculated were done using the total activity found in the 75-90% $(NH_4)_2SO_4$ pellet as a reference. That is, the total activity found in the 75-90% $(NH_4)_2SO_4$ pellet was made equivalent to 1.

The DEAE Cellufine step in the purification resulted in very good resolution of the enzyme from other protein remaining in the 75-90% $(NH_4)_2SO_4$ pellet (Figure IV.2.). Although there was only an approximate 2-fold increase in enrichment of the enzyme, percent recovery was quite acceptable (Table IV.1.). A typical elution profile for this step

Figure IV.1.: Increase in total and specific activity for NADPH oxidation in the 65-90% $(NH_4)_2SO_4$ pellet vs CFE. The experiment was performed twice in triplicate with the average used.


0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Total Activity (mmol/min/ml)
CFE
65-90%
(NH4)2SO4
Pellet

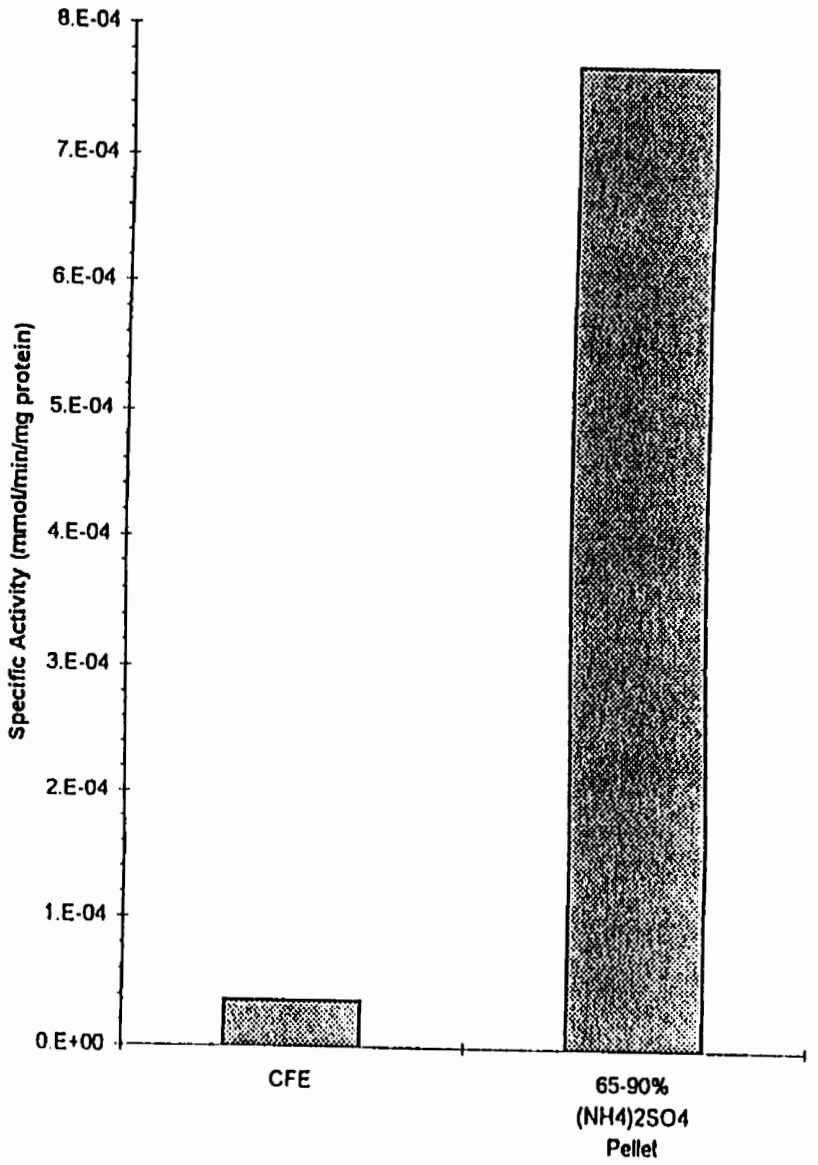
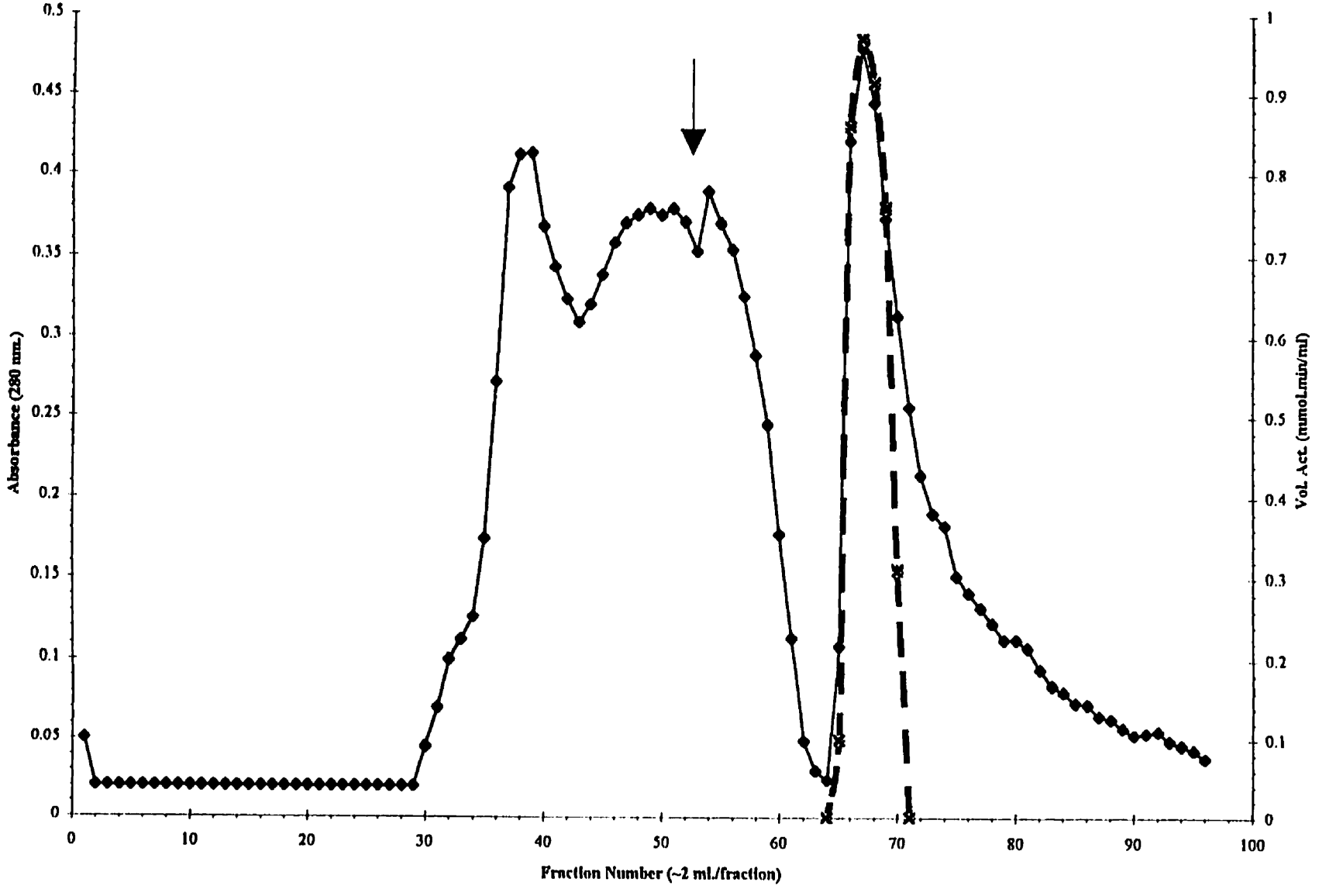

8.E-04
7.E-04
6.E-04
5.E-04
4.E-04
3.E-04
2.E-04
1.E-04
0.E+00
Specific Activity (mmol/min/mg protein)
CFE
65-90%
(NH4)2SO4
Pellet

Table IV.1.: Table Indicating Enrichment for NADPH Oxidation During the Purification of the $NADP^+$:F_{420} Oxidoreductase

Total Volume		Total Protein	Vol Act	Spec Act	Fold-Enrichment
	(ml)	(mg)	(mmol/min)	(mmol/min/mg)	
CFE	65	1561.1	0.00347	4.45E-5	
75-90% pellet	2	57.72	0.0584	0.0202	453.9
DEAE Cellufine	2	28.44	0.0277	0.0295	662.9
Q Biorad Biologic	2	0.646	0.00793	0.245	5505.6
Electroelution	1	0.297	0.00525	0.347	7711.1

Figure IV.2.: Elution profile of the $NADP^+$:F_{420} Oxidoreductase from the DEAE Cellufine column. Elution profile does not include wash-through prior to the start of gradient. (♦) indicates A_{280} and (x) indicates Total Activity. The arrow indicates the end of the 0-0.3 M KCl salt gradient and the beginning of the 0.3 M KCl hold.

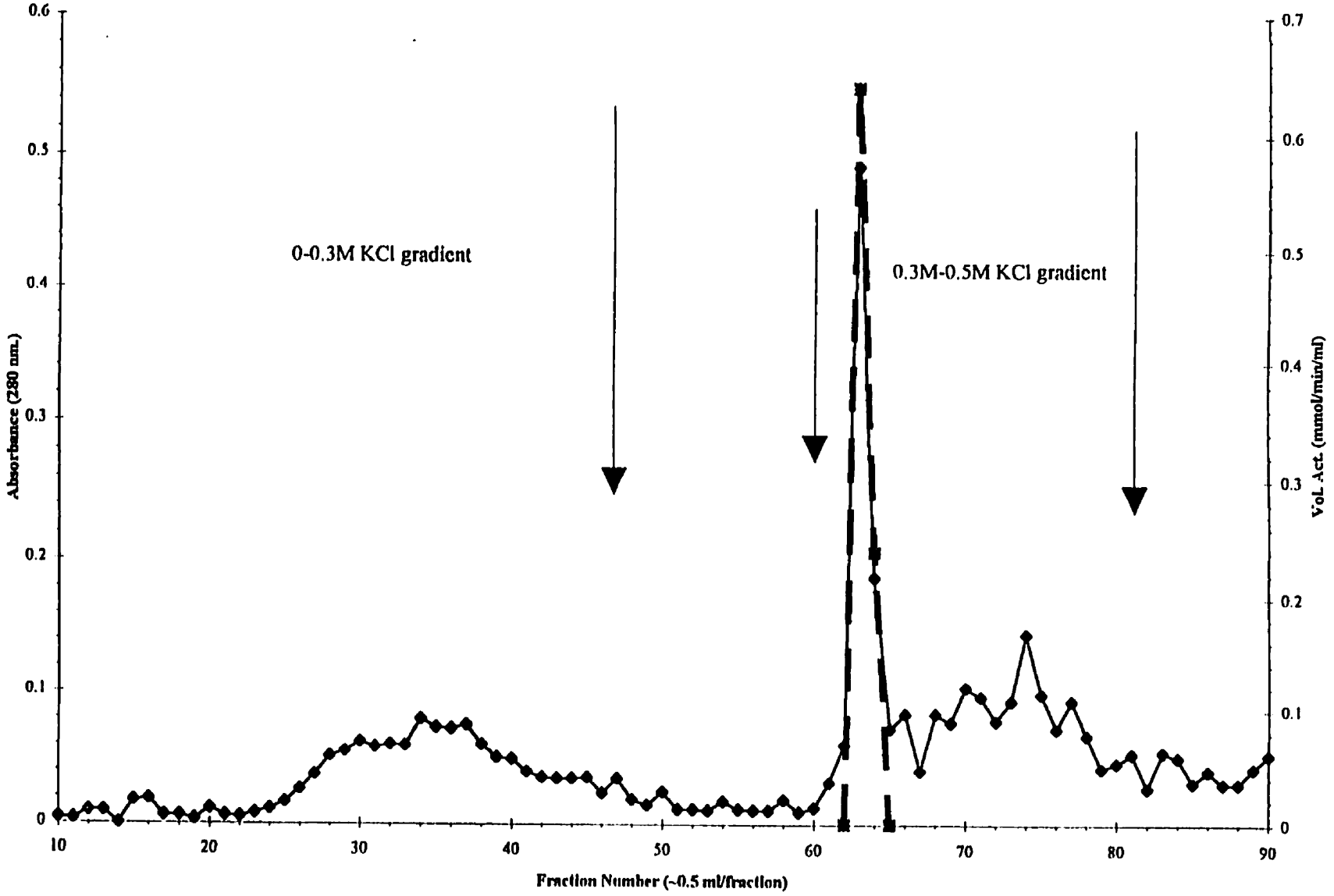

Absorbance (280 nm.)
Vol. Act. (nmoL/min/ml)
Fraction Number (~2 ml./fraction)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
80
90
100

was to see a slow building of a large protein peak eluted from the column starting at approximately 0.15M KCl and peaking just after the end of the salt gradient and into the hold of 0.3M KCl consisting of approximately 45-50ml. This was followed by a drastic drop in protein content, and followed by a small, well resolved peak of approximately 16-20ml. There were no protein peaks found after this, and no enzyme activity was found in the first large peak or anywhere else in the load eluate, wash through eluate. All activity was found in the second smaller protein peak coincided with the profile of this protein peak. There was not a significant amount of protein found in the wash through before the salt gradient.

The total recovery of the enzyme to this point was approximately 47%, with an overall enrichment from the CFE of approximately 660-fold, and a reduction in total protein of 55-fold (Table IV.1.).

The Biorad Biologic yielded good resolution of the enzyme from other proteins still remaining in the sample (Figure IV.3.). The first salt gradient of 0-0.3M KCl and hold at 0.3M KCl resulted in the elution of two distinct peaks, neither of which contained any enzyme activity. No activity was found in the load or wash through eluate. Early into the 0.3M-0.5M KCl gradient a large peak was eluted from the column. All activity found in the volume eluted from the column was found in this peak. The total and specific activity of the enzyme coincided with the protein elution profile, and the fractions containing significant amounts of activity were pooled, and concentrated to 1 ml. Total recovery from the Biorad Biologic was 29% of the activity loaded onto the column, with a total enrichment of over 5500-fold, and a total reduction in total protein of over 2400-fold from the CFE (Table IV.1.). The fractions were loaded into the wells of the native-PAGE.

Figure IV.3.: Elution profile of the $NADP^+$:F_{420} Oxidoreductase from the Biorad Biologic Q anion column. Elution profile does not include washthrough prior to the start of gradient. (♦) indicates A_{280} and (x) indicates Total Activity. The first arrow indicates the end of the 0-0.3 M KCl salt gradient and the beginning of the 0.3 M KCl hold. The second arrow indicates the beginning of the 0.3 M - 0.5 M KCl gradient, and the third arrow indicates the end of the 0.3 M - 0.5 M KCl gradient and the beginning of the 0.5 M KCl hold.


0-0.3M KCl gradient
0.3M-0.5M KCl gradient
Absorbance (280 nm.)
Vol. Act. (mmol/min/ml)
Fraction Number (~0.5 ml/fraction)
0.6
0.5
0.4
0.3
0.2
0.1
0
0.7
10
20
30
40
50
60
70
80
90

Both the $NADP^+$ and 2',5'-ADP affinity columns gave elution profiles with small amounts of activity throughout the elution gradients. The enzyme bound well to the $NADP^+$ affinity column, and elution was attempted with various salts, salt concentrations and gradients as well as $NADP^+$ gradients. In all cases $NADP^+$:F_{420} oxidoreductase activity was found throughout the elution profile in similar amounts with no peak of activity. Similarly, in the 2',5'-ADP affinity column recommended by Ostrove and Weiss (1990) for the binding of $NADP^+$-dependent enzymes, the enzyme bound well to the column, but elution with salts and 2',5'-ADP gradients was unsuccessful. The elution profile was similar to the $NADP^+$ affinity column with no peak of activity found.

The native PAGE proved to be extremely powerful as a later step in the purification of the enzyme. The enzyme proved to be the only protein band in the 130 kDa to 160 kDa area. As such, it was relatively easy to cut the bands without worrying about mistakenly cutting another band with that of the enzyme.

The electroelution proved to be the appropriate step in achieving purity of the enzyme from all other protein. After concentration of the buffer containing the enzyme, activity was found and subsequent running of a second native-PAGE showed only one protein band at the same molecular weight as the first native-PAGE. At this point the enzyme was considered pure, and was kept at -60°C for further testing. The pure enzyme had been enriched over 7700-fold, with a reduction in total protein of 5250-fold from the CFE. This also showed a percent recovery from the electroelution of approximately 66%, and a total recovery of 11.2 % from the 75-90% $(NH_4)_2SO_4$ pellet (Table IV.1.).

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

The molecular mass of the enzyme was determined by both gel filtration chromatography, and native-PAGE. The two methods yielded results that were in good agreement. The gel filtration showed a molecular weight for the functional enzyme at approximately 145 kDa (Figure IV.4.), while the native-PAGE gave a result of approximately 148 kDa (Figure IV.5., Figure IV.6.). This is also in relatively good agreement with the SDS-PAGE of the purified enzyme. The SDS-PAGE showed that the purified enzyme consisted of three non-identical subunits. Thus the subunit structure of the purified enzyme is an α,β,γ with molecular subunit weights of 60 kDa, 50 kDa, and 45 kDa, respectively (Figure IV.7.). This gives a total molecular weight of 155 kDa which is in good agreement with the 145 kDa and 148 kDa given by the gel filtration and native-PAGE.

IV.3.3. K_m Determinations

The K_ms were determined for the purified enzyme for $NADP^+$, NADPH, F_{420}, and $F_{420}H_2$. Three of the four substrates showed first-order kinetics in the purified enzyme. According to Lineweaver-Burke regressions, the K_m's were; $NADP^+$ 370 μM, NADPH 142 μM, F_{420} 62.5 μM, and $F_{420}H_2$ 7.7 μM. For $NADP^+$, the Michealis-Menton plot showed a slight hyperbolic curve indicating for first-order kinetics while the Lineweaver-Burke plot yielded a straight line with an x-intercept of -2.7 giving a K_m of 370 μM (Figure IV.8.) The Eadie-Hofstee plot for $NADP^+$ was a poor regression, but overall still indicated a first-order reaction with a K_m of 538 μM (Figure IV.9.). NADPH gave a slightly irregular Michealis-Menton plot which could have been interpreted as a straight

Figure IV.4.: Plot showing determination of the functional molecular weight of the $NADP^+$:F_{420} oxidoreductase from *Msph. stadtmanae* using the Sephacryl S-200 gel filtration column.

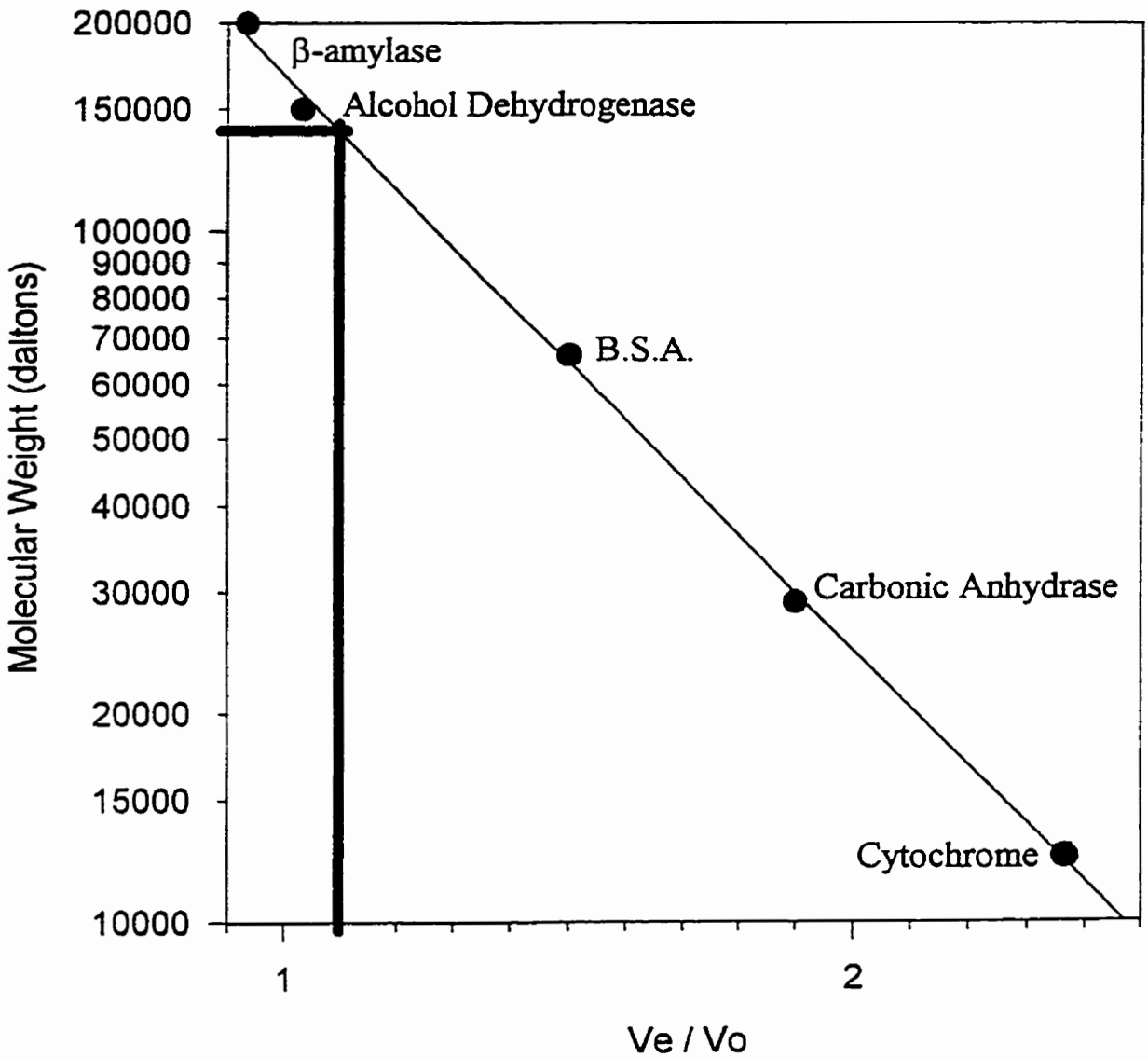

200000
150000
100000
90000
80000
70000
60000
50000
40000
30000
20000
15000
10000
Molecular Weight (daltons)
β-amylase
Alcohol Dehydrogenase
B.S.A.
Carbonic Anhydrase
Cytochrome
1
2
Ve / Vo

Figure IV.5.: native PAGE of the steps leading to the purification of the $NADP^+$:F_{420} Oxidoreductase from *Msph. stadtmanae*.

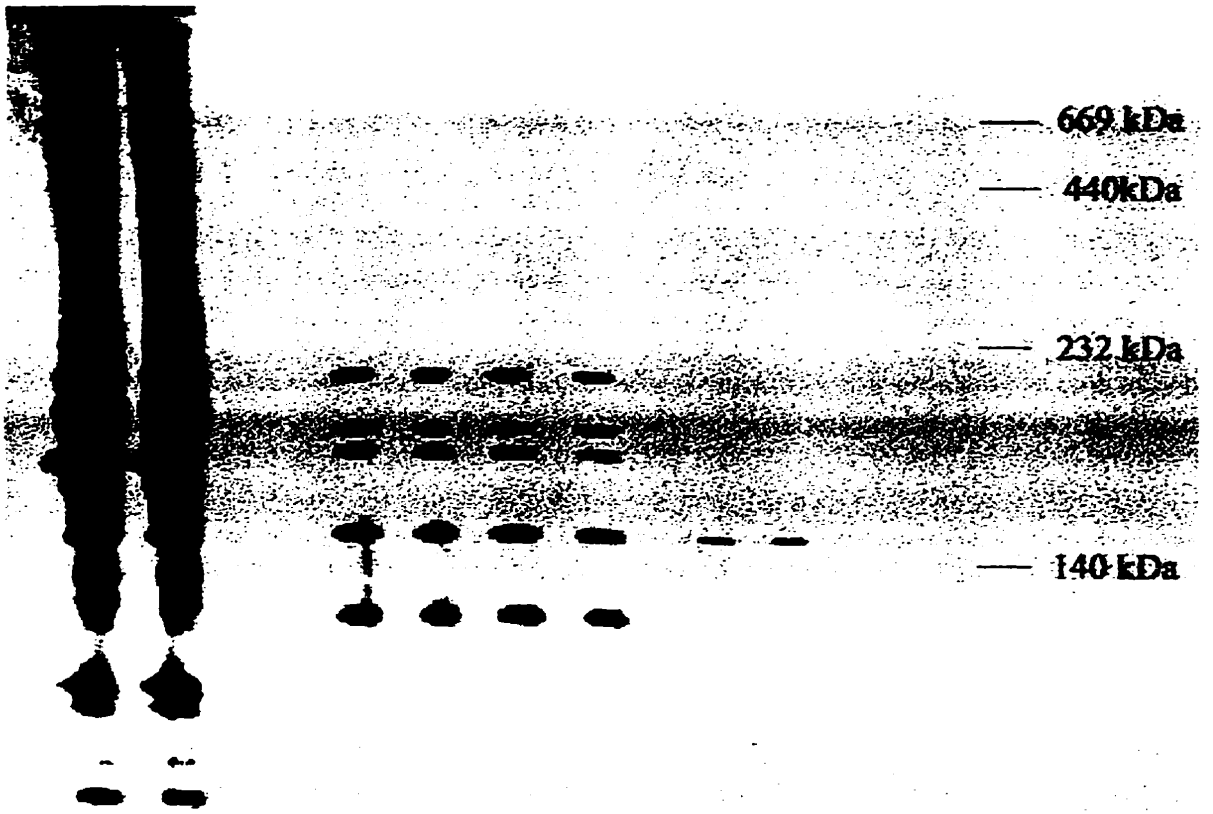

669 kDa
440kDa
232 kDa
140 kDa
DEAE Cellufine
Biorad Biologic
Electro-elution
standards
67 kDa

Figure IV.6.: Plot of the native-PAGE for Determination of the functional molecular weight of the $NADP^+$:F_{420} Oxidoreductase from *Msph. stadtmanae*

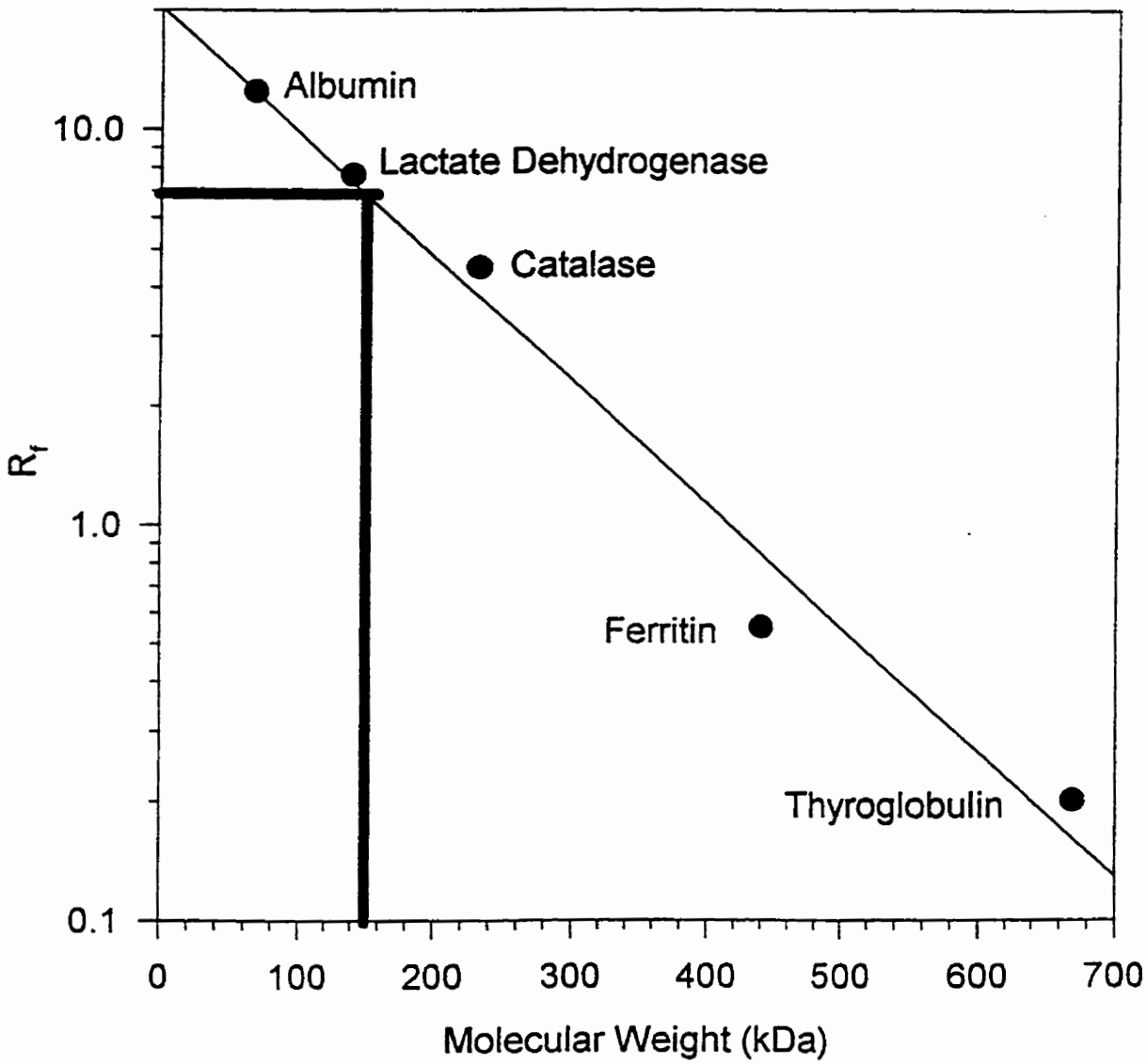

Albumin
Lactate Dehydrogenase
Catalase
Ferritin
Thyroglobulin
10.0
1.0
0.1
R_f
0
100
200
300
400
500
600
700
Molecular Weight (kDa)

Figure IV.7.: SDS-PAGE was used to determine the subunit weight of the purified $NADP^+$:F_{420} oxidoreductase. Lanes 3 and 9 contain the marker ladder, while lanes 1,2,4,5,6,7,8 contain the purified enzyme. There were three separate subunits showing the enzyme was a heterotrimer. These were designated α at 60 kDa, β at 50 kDa, and γ at 45 kDa.

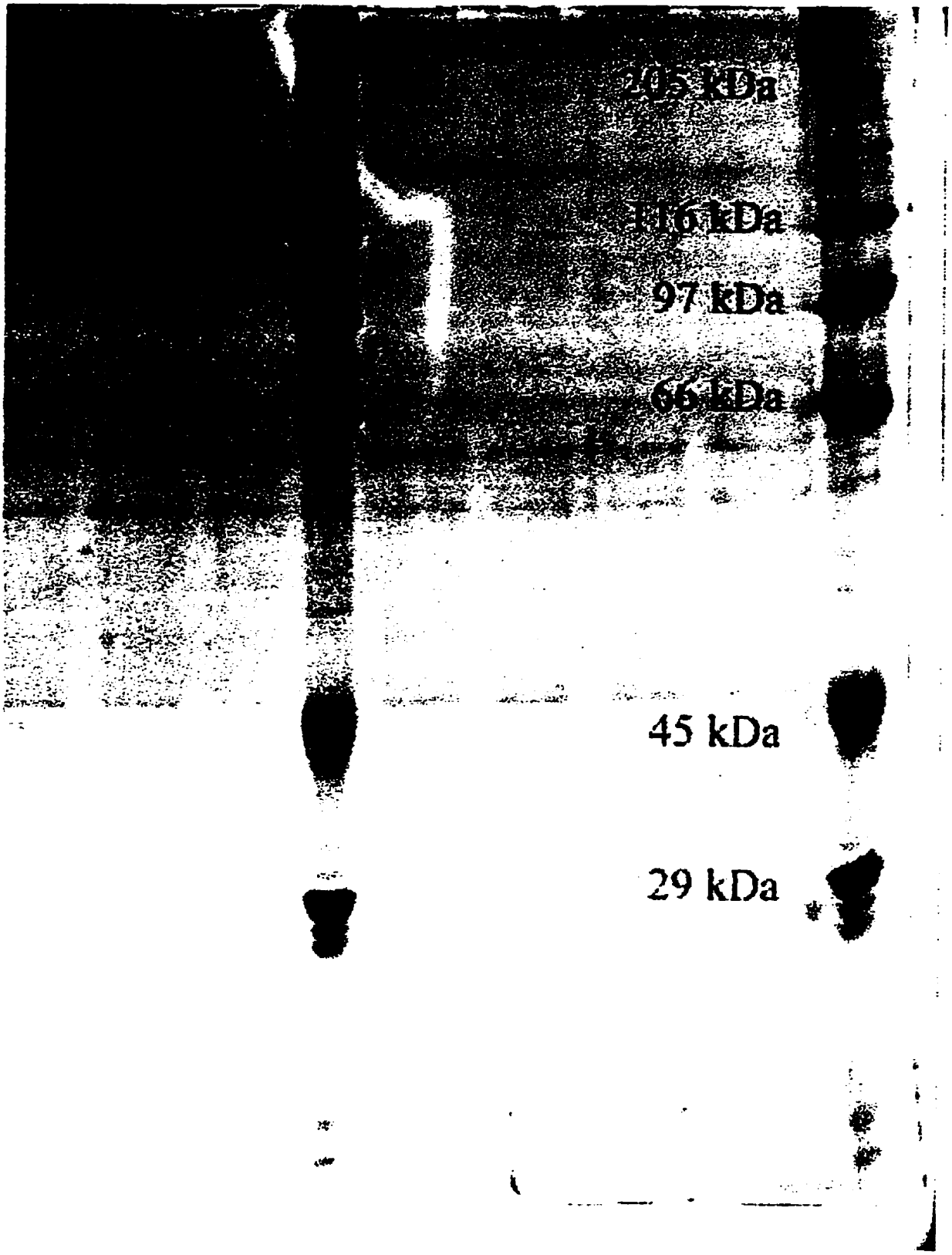
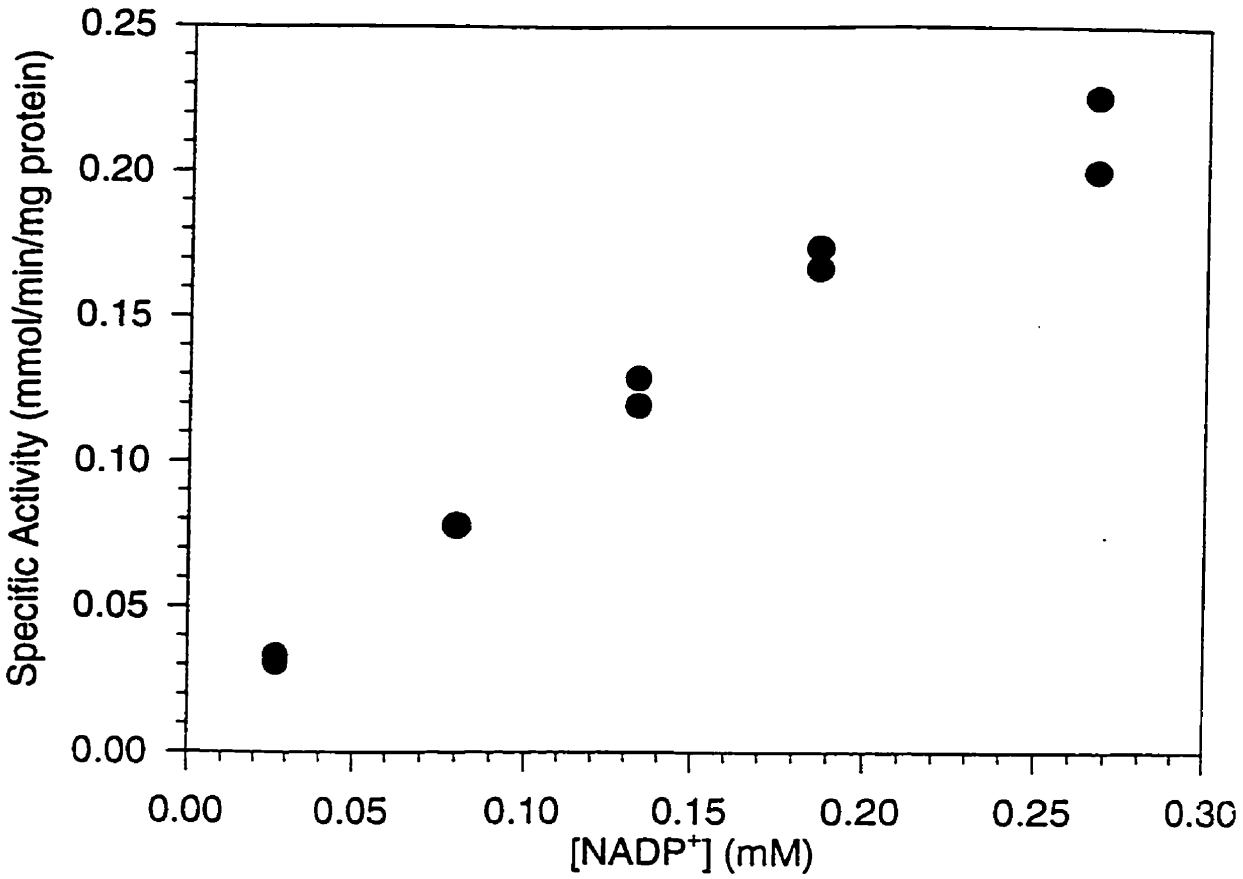

205 kDa
116 kDa
97 kDa
66 kDa
45 kDa
29 kDa

Figure IV.8. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for $NADP^+$ in the purified $NADP^+$:F_{420} Oxidoreductase of *Msph. stadtmanae*. The Lineweaver-Burke plot showed a K_m of 370 μM. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
0.25
0.20
0.15
0.10
0.05
0.00
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[NADP+] (mM)

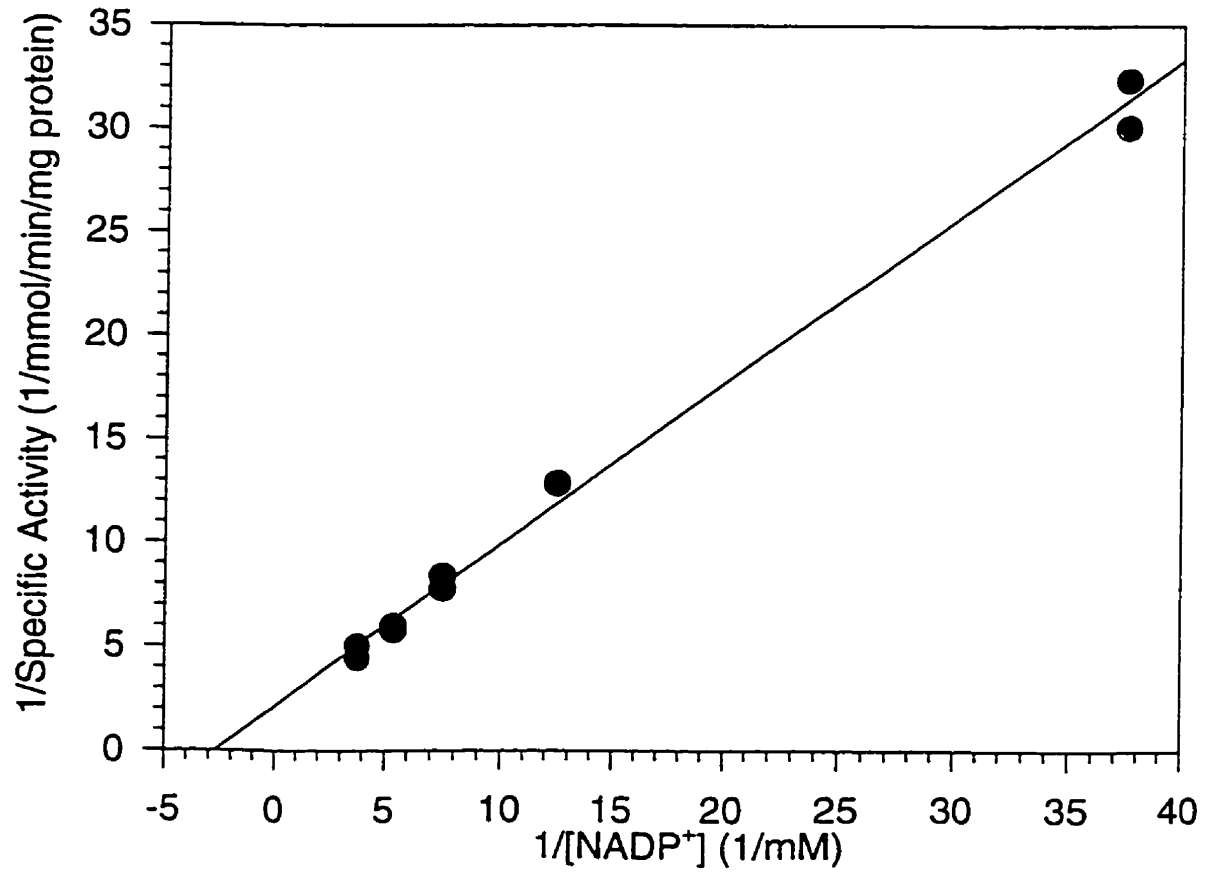
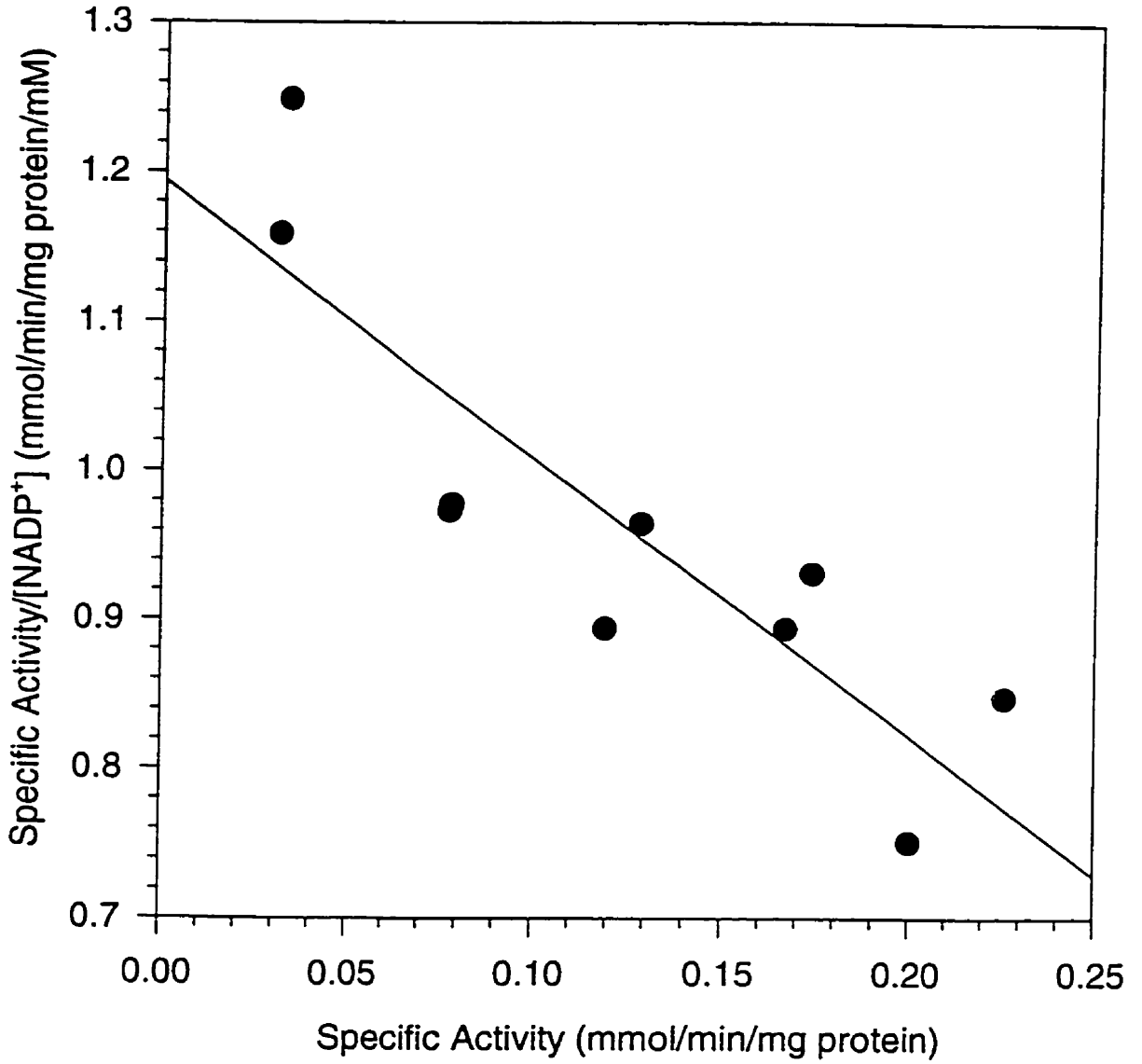

1/Specific Activity (1/mmol/min/mg protein)
35
30
25
20
15
10
5
0
-5
0
5
10
15
20
25
30
35
40
1/[NADP+] (1/mM)

Figure IV.9. Eadie-Hofstee Plot for $NADP^+$ in the purified $NADP^+$:F_{420} Oxidoreductase of *Msph. stadtmanae*. The plot showed a K_m of 538 μM. The experiment was performed twice in duplicate.


1.3
1.2
1.1
1.0
0.9
0.8
0.7
Specific Activity/[NADP+] (mmol/min/mg protein/mM)
0.00
0.05
0.10
0.15
0.20
0.25
Specific Activity (mmol/min/mg protein)

line or as a slightly hyperbolic curve (Figure IV.10.). The Lineweaver-Burke plot gave a fairly straight line with an x-intercept of -7, giving a Km of 142 μM. The Eadie-Hosftee plot gave a Km of 156 μM (Figure IV.11.).

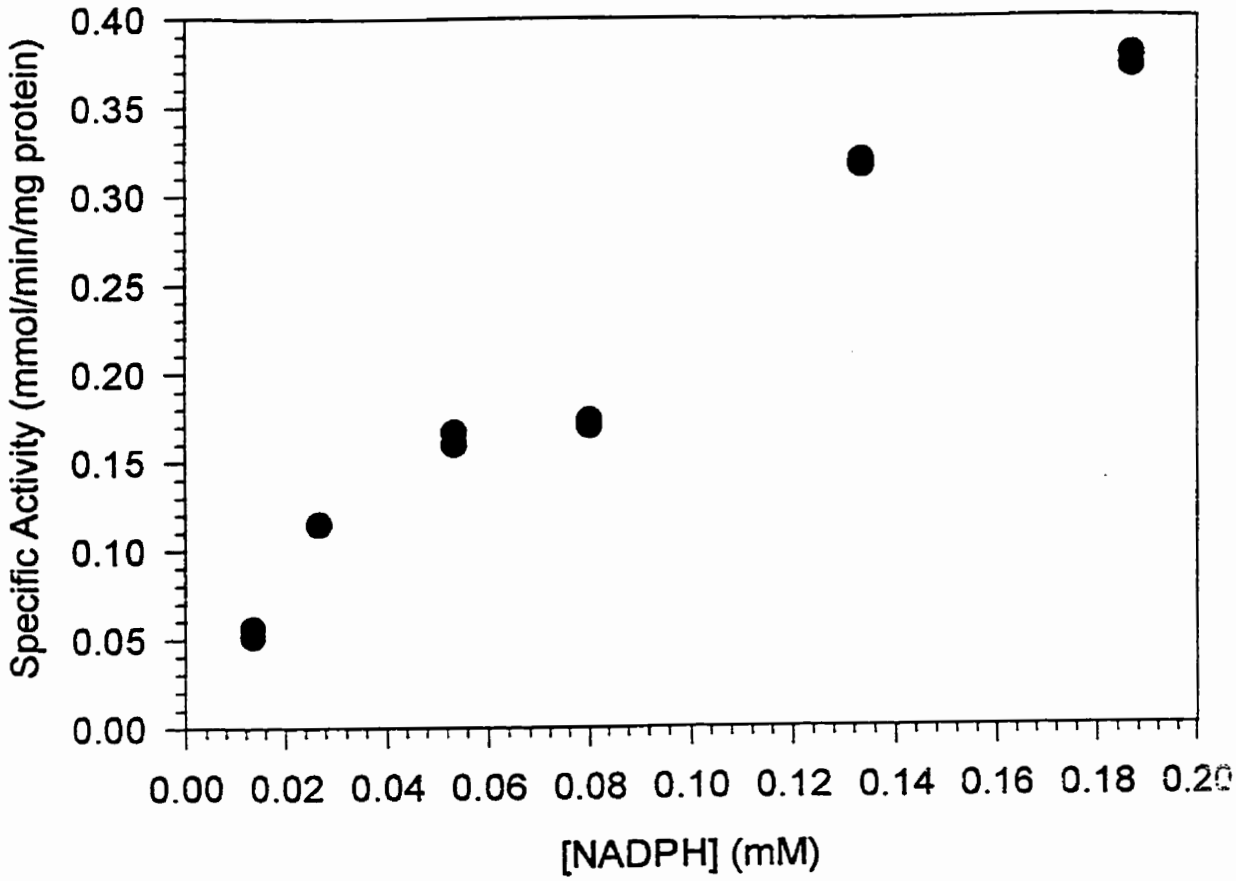
The test for F_{420} showed typical first-order kinetics for both the Michealis-Menton and Lineweaver-Burke plots (Figure IV.12.). For the Lineweaver-Burke plot was a very good regression line with r^2=0.984, and an x-intercept of -16 giving a K_m of 62.5 μM. This was also confirmed by the Eadie-Hofstee plot which gave a straight line with a K_m value of 52.3 μM (Figure IV.13.).

The plots for $F_{420}H_2$ were satisfactory. The Michealis-Menton plot was a roughly hyperbolic curve, while the Lineweaver-Burke plot showed a straight line regression of r^2=0.932 and an x-intercept of -130 which gave a K_m of 7.7 μM (Figure IV.14.). The Eadie-Hofstee plot showed a slight curvature at the higher concentrations of sample, but was overall considered a straight line and therefore a first-order reaction and gave a K_m of 7.5 μM (Figure IV.15.).

IV.4. Discussion

The purification of the $NADP^+$:F_{420} oxidoreductase was a rather difficult project to bring to a close. Various different procedures were attempted only to be unsuccessful. The final protocol for purification was less than trivial when considering the use of native-PAGE and electroelution. The final recovery of the enzyme was 9% using the total activity found in the 75-90% $(NH_4)_2SO_4$ pellet as a starting point for the reasons given previously. The total protein was reduced by 5250-fold, with an overall enrichment of over 7700-fold. It is interesting that there was such a large fold enrichment to attain purity

Figure IV.10. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for NADPH in the purified $NADP^+$:F_{420} Oxidoreductase of *Msph. stadtmanae*. The Lineweaver-Burke plot showed a K_m of 142 μM. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.00 0.02 0.04 0.06 0.08 0.10 0.12 0.14 0.16 0.18 0.20
[NADPH] (mM)

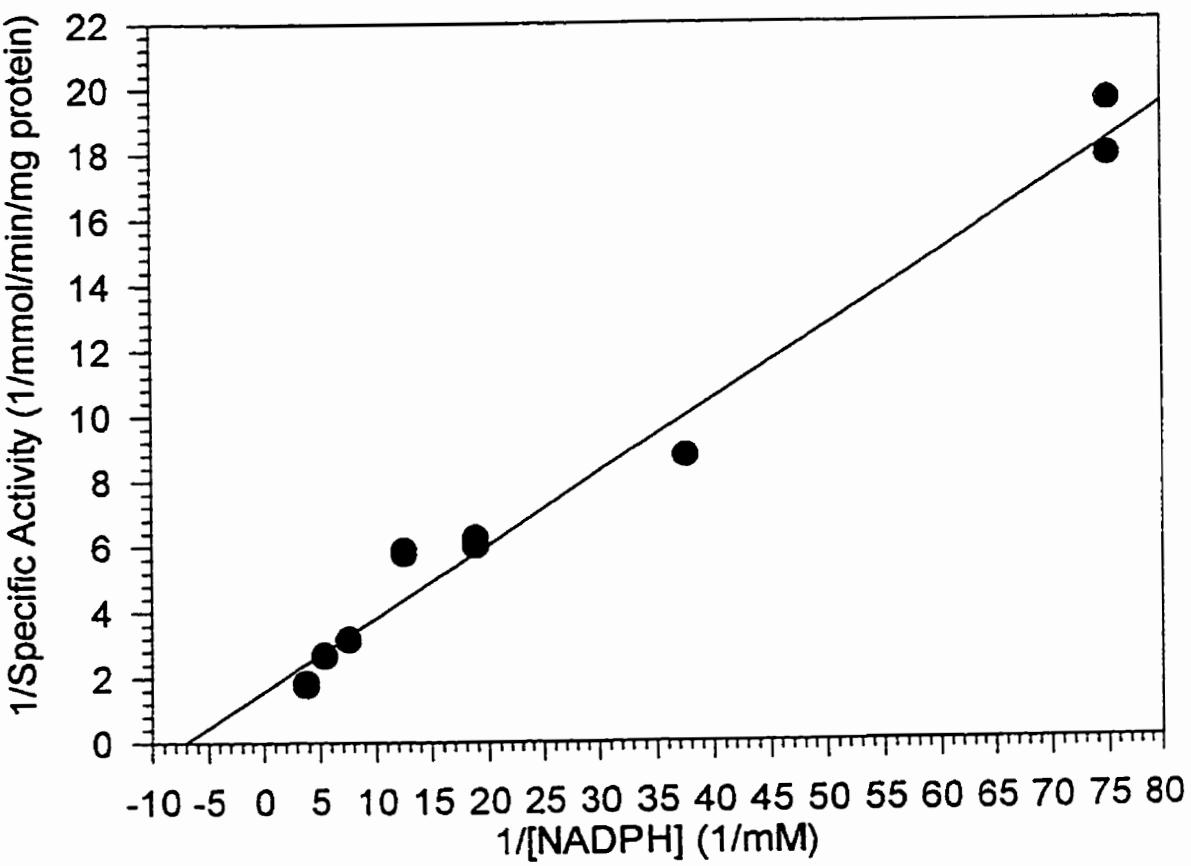

1/Specific Activity (1/mmol/min/mg protein)
22
20
18
16
14
12
10
8
6
4
2
0
-10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
1/[NADPH] (1/mM)

Figure IV.11. Eadie-Hofstee Plot for NADPH in the purified $NADP^+$:F_{420} Oxidoreductase of *Msph. stadtmanae*. The plot showed a K_m of 156 μM. The experiment was performed twice in duplicate.

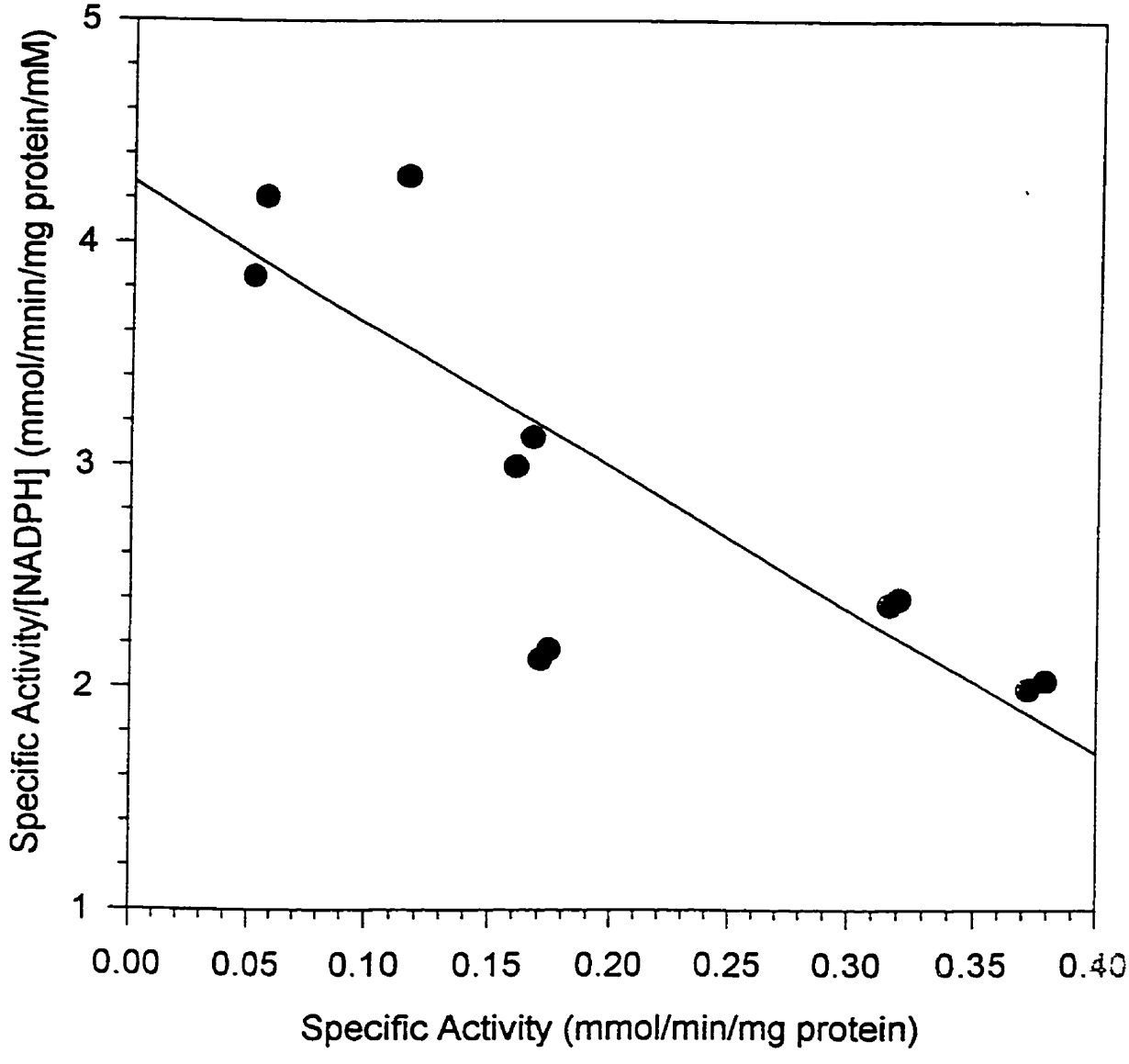
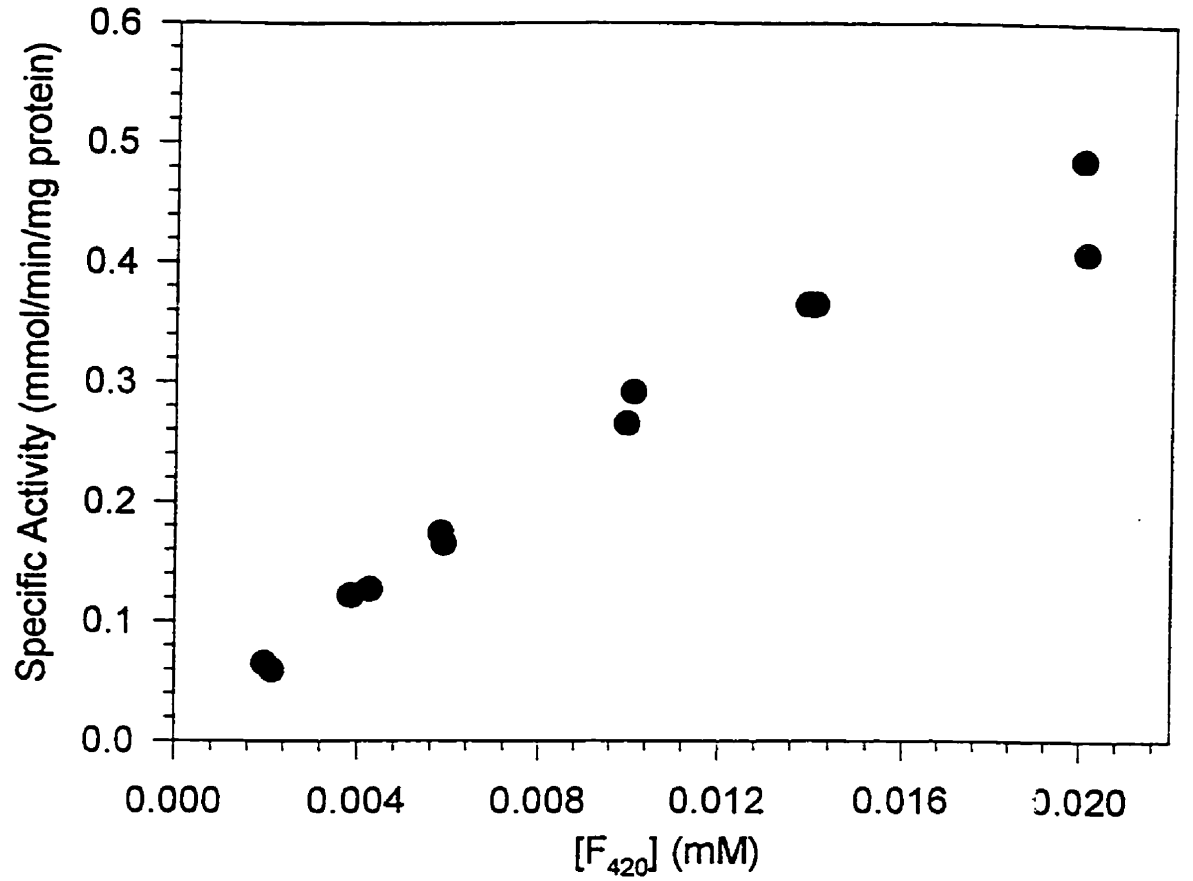

Specific Activity/[NADPH] (mmol/mnin/mg protein/mM)
5
4
3
2
1
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
Specific Activity (mmol/min/mg protein)

Figure IV.12. (top) Michealis-Menton and (bottom) Lineweaver-Burke Plots for F_{420} in the purified $NADP^+$:F_{420} Oxidoreductase of *Msph. stadtmanae*. The Lineweaver-Burke plot showed a K_m of 62.5 μM. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.000
0.004
0.008
0.012
0.016
0.020
[F420] (mM)

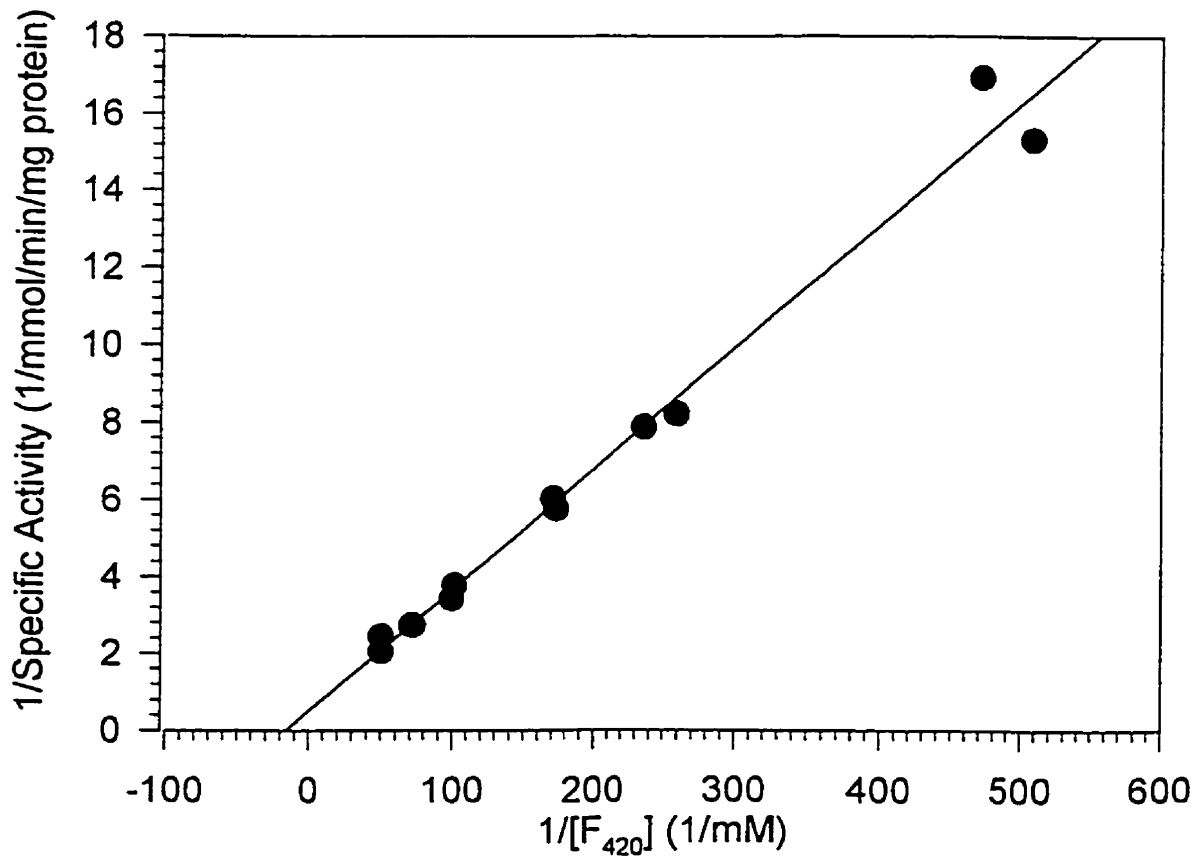

1/Specific Activity (1/mmol/min/mg protein)
18
16
14
12
10
8
6
4
2
0
-100
0
100
200
300
400
500
600
1/[F420] (1/mM)

Figure IV.13. Eadie-Hofstee Plot for F_{420} in the purified $NADP^+$:F_{420} Oxidoreductase of *Msph. stadtmanae*. The plot showed a K_m of 52.3 μM. The experiment was performed twice in duplicate.

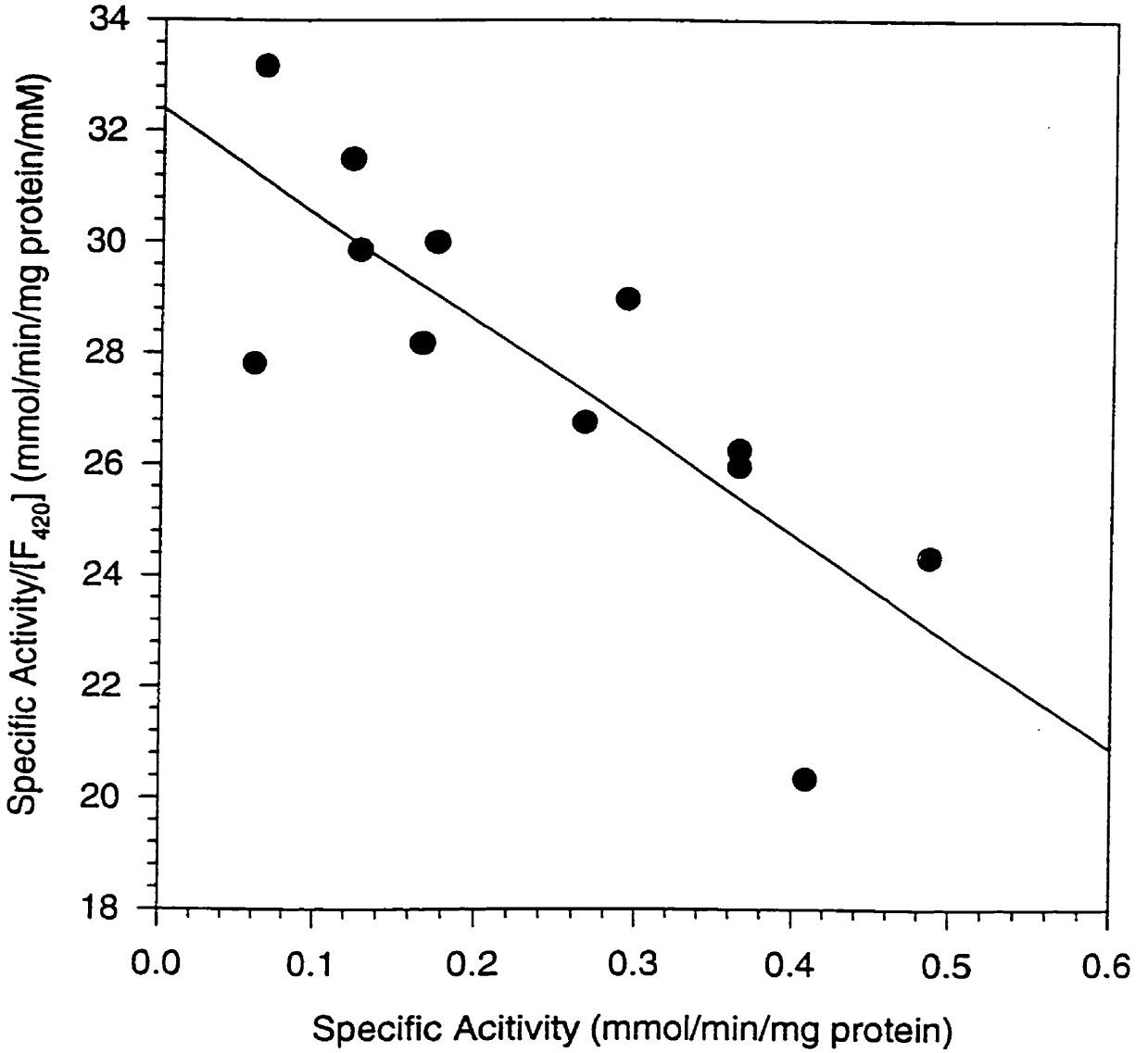
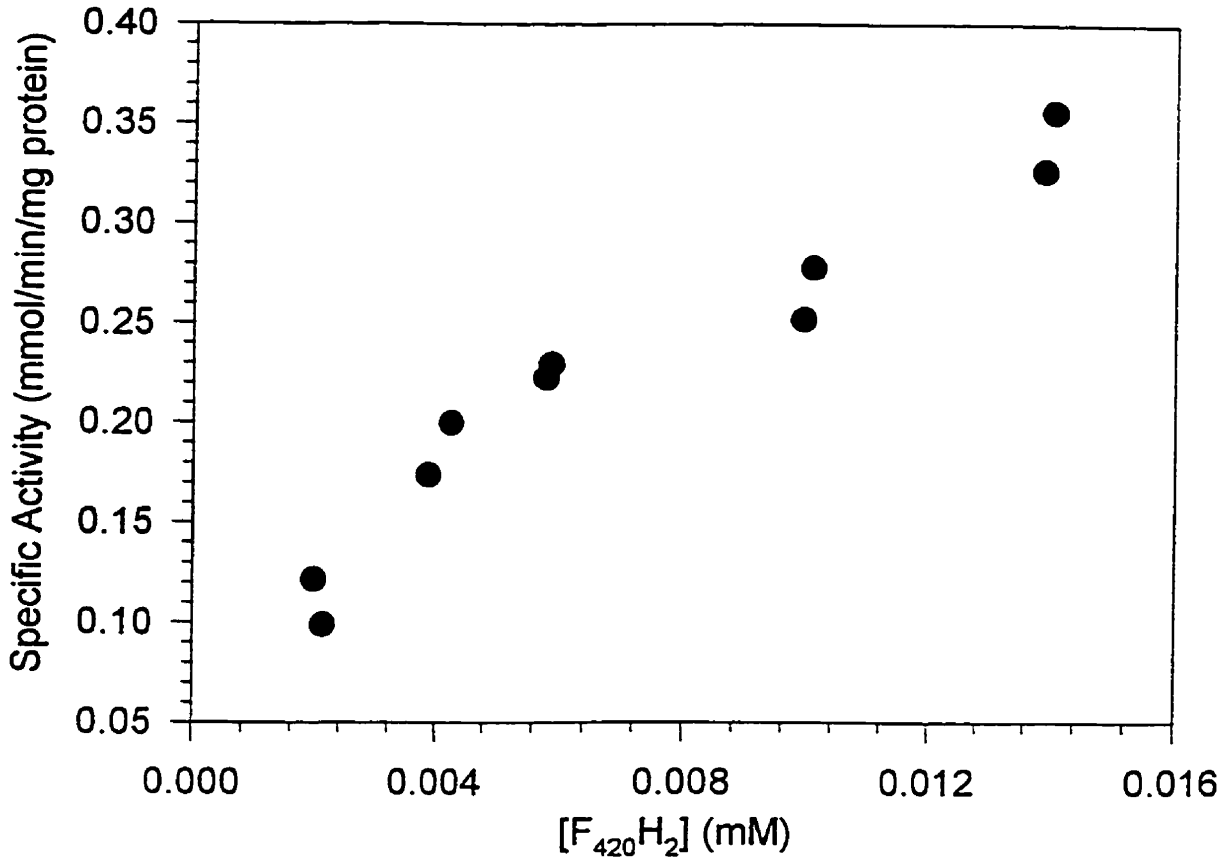

34
32
30
28
26
24
22
20
18
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Specific Activity/[F420] (mmol/min/mg protein/mM)
Specific Acitivity (mmol/min/mg protein)

Figure IV.14. Michealis-Menton and Lineweaver-Burke Plots for $F_{420}H_2$ in the purified $NADP^+$:F_{420} Oxidoreductase of *Msph. stadtmanae*. The Lineweaver-Burke plot showed a K_m of 7.7 μM. The experiment was performed twice in duplicate.


Specific Activity (mmol/min/mg protein)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.000
0.004
0.008
0.012
0.016
[F420H2] (mM)

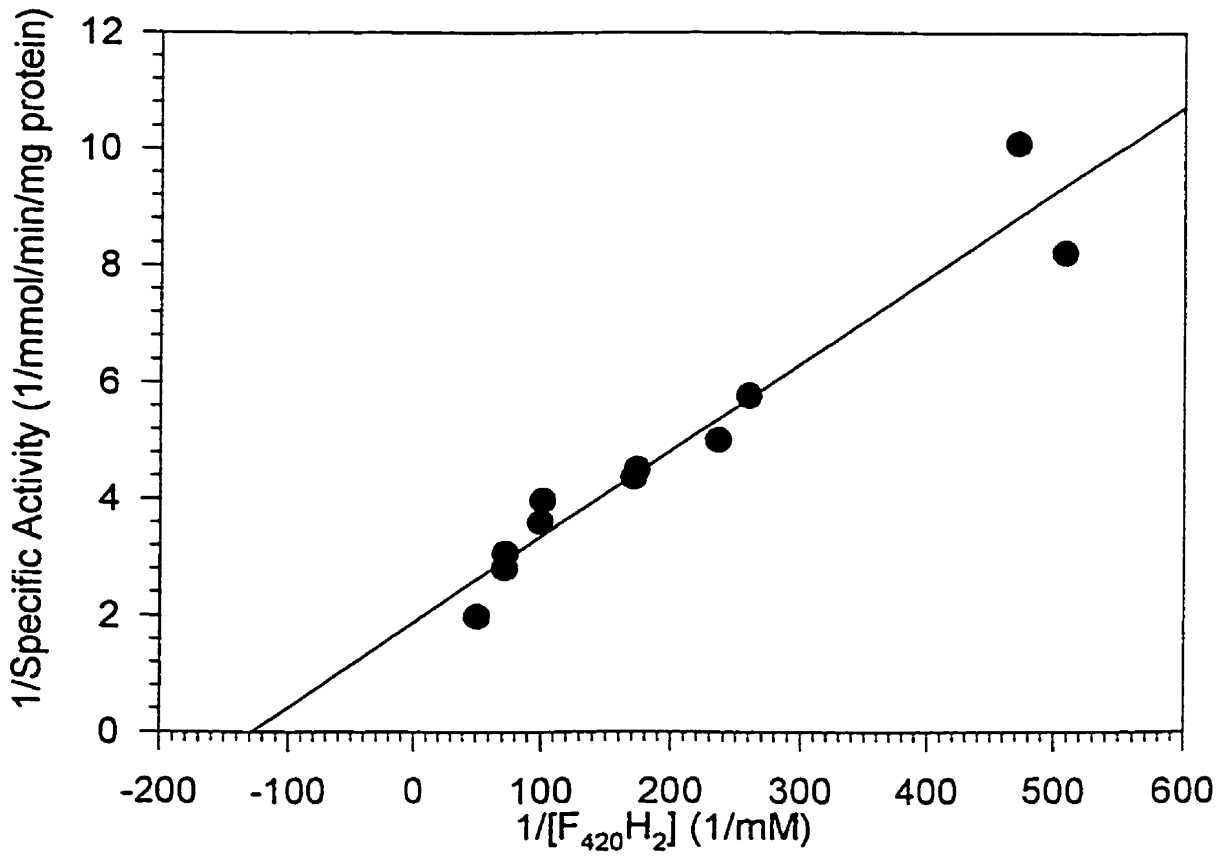
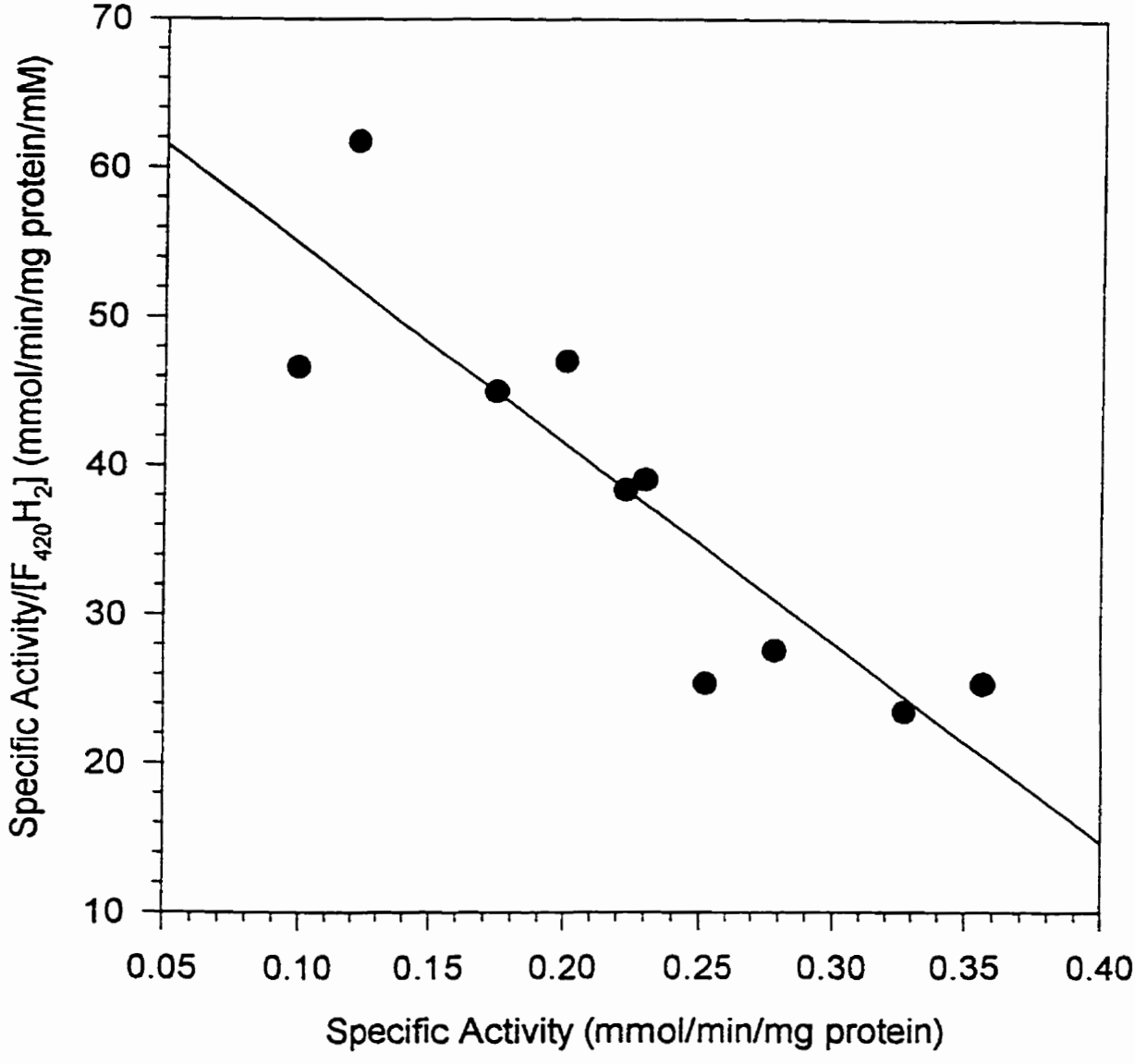

1/Specific Activity (1/mmol/min/mg protein)
12
10
8
6
4
2
0
-200
-100
0
100
200
300
400
500
600
1/[F420H2] (1/mM)

Figure IV.15. Eadie-Hofstee Plot for $F_{420}H_2$ in the purified $NADP^+$:F_{420} Oxidoreductase of *Msph. stadtmanae*. The plot showed a K_m of 7.5 μM. The experiment was performed twice in duplicate.


70
60
50
40
30
20
10
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
Specific Activity/[F420H2] (mmol/min/mg protein/mM)
Specific Activity (mmol/min/mg protein)

of the enzyme. This would indicate that the $NADP^+$:F_{420} oxidoreductase of *Msph. stadtmanae* is in rather small amounts compared to other important enzymes such as the hydrogenase.

It is extremely interesting that there is such a huge increase in specific activity from the CFE to the 75-90% $(NH_4)_2SO_4$ pellet. This is consistent with observations in Chapter II. The reason for this is still not clear, see Chapter II. No such increase in specific activity and no increase in total activity was seen in *Mc. vannielii* or *Mb. thermoautotrophicum* when their respective enzymes were purified (Yamazaki et. al., 1980, 1982, Jacobsen and Walsh, 1984).

The Lineweaver-Burke plots for the purified enzyme appeared to be linear and therefore first-order. On the basis of the Lineweaver-Burke plots, the K_m for NADPH was 142 μM, 370 μM for $NADP^+$, 62.5 μM for F_{420}, and 7.7 μM for $F_{420}H_2$. It is interesting that the K_m's were first order in CFE, and for NADPH and F_{420} in the 65-90% $(NH_4)_2SO_4$ pellet, and for all four substrates in the purified enzyme. This may indicate that the $(NH_4)_2SO_4$ treatment had some effect on the enzyme. However, although the K_m's are first order in both the CFE and the purified enzyme, they are different (see Table V.1.). Another reason for the difference between the CFE and the 65-90% $(NH_4)_2SO_4$ pellet may be a difference in the number of enzymes present in solution that use $NADP^+$, NADPH, F_{420} or $F_{420}H_2$. It would be assumed that there would be a smaller number of enzymes overall and enzymes using these substrates in the 65-90% $(NH_4)_2SO_4$ pellet than in the CFE. There are also some interesting trends with respect to the K_m's. It is curious that the substrates for the reduction of F_{420} remain first-order, while the substrates for the reduction of $NADP^+$ change from first- to non-first order. This may further indicate a

difference in the enzyme for a preferred direction *in vivo*. Also, for the reduced forms of the substrates (NADPH and $F_{420}H_2$), the K_m's decrease from CFE to pure enzyme indicating an increase in affinity for the substrates. The K_m's for the oxidized forms of the substrates however, increase, indicating a decrease in affinity for these substrates.

These changes in affinity are curious. Do they indicate a preference in direction, simply a change in the number of enzymes utilizing these substrates, or perhaps an alteration in the affinity or binding nature of the active site of the enzyme due to the loss of the non-covalently bound, proteinaceous unit? These are all rather acceptable possibilities. However, none can be ascertained for certain at this time.

Chapter V: General Discussion and Conclusions

The work presented in this thesis essentially incorporates two similar, but interrelated projects. The first being the purification of the $NADP^+$:F_{420} oxidoreductase of *Msph. stadtmanae,* and the second being the comparison of the properties associated with $NADP^+$:F_{420} oxidation/reduction of several methanogens.

The study of different methanogens was originally conceived to determine if the unexpected increase in total and specific activity seen in *Msph. stadtmanae* could be found in other methanogens or if it was unique to *Msph. stadtmanae*. As data were collected it appeared that there were some similarities or trends. With the exception of *Msph. stadtmanae* growing with 2-propanol, all methanogens tested appeared to prefer the reduction of $NADP^+$ rather than the oxidation of NADPH. Also, this work has shown that there is some degree of grouping as far as family divisions are concerned. Family Methanococcales appeared to use 2 separate enzymes for $NADP^+$:F_{420} oxidation/reduction, while family Methanomicrobiales appeared to use only one. This is in disagreement with the purification of an $NADP^+$:F_{420} reductase from *Mc. vannielli*. As such it would markedly help the validity of the survey to conduct these experiments on Mc. *vannielli*. However, the data given in the paper concerning *Mc. vannielli* only reports the pH optimum for NADPH oxidation and does not indicate that any further work was done concerning NADPH oxidation. This may indicate that although NADPH oxidation activity was present, it was negligable compared to $NADP^+$ reduction activity (Yasmazaki and Tsai, 1980).

Family Methanobacteriales was a mixture as *Mb. thermoautotrophicum* and *Msph. stadtmanae* possessed 2 separate enzymes, while *Msph. stadtmanae* without 2-propanol, *Msph. caniculi*, and *Mb.* G2R possessed only one enzyme. Genus *Methanosphaera* was also unique unto itself in that *Msph. stadtmanae* and *caniculi* were the only organisms to show precipitation of the bulk of activity in the 65-90% $(NH_4)_2SO_4$ pellet. As well, *Msph. stadtmanae* was shown to express a difference in its' means of NADPH formation depending on its' nutrition; i.e. the presence or absence of 2-propanol. This is a significant finding as not only is this the first report of its kind in so far as a comparison among methanogens, but it is also the first report of the observation of a biosynthetic enzyme of a methanogen under different nutritional constraints. In the presence of 2-propanol there was a reversal of the preferred direction, a disappearance of the previously seen increase in total and specific activity, and it appeared that there were 2 $NADP^+/F_{420}$ enzymes produced instead of one. This would be a valuable study to complete with purification and characterization of the suspected enzymes in this species from some of the more environmentally prevalent methanogens tested here such as *Msp. hungatei* and *Mb. thermoautotrophicum*.

The purification of the $NADP^+$:F_{420} oxidoreductase from *Msph. stadtmanae* proved to be quite a challenge. The first step was the kinetics as described in Chapter II of this thesis. The first difficulty proved to be stabilizing the 65-90% $(NH_4)_2SO_4$ pellet. This in itself took approximately 3 months of growing and testing cells. It is curious how two buffers that are so different (Trizma base pH=8.1, MES pH=6.1) were the only buffers that were able to stabilize the 65-90% $(NH_4)_2SO_4$ pellet. It is also interesting that stabilization occured at the same pH ranges as was found for other similar enzymes

previously purified (Eker et. al., 1989; Jacobsen and Walsh, 1984; Yamazaki et. al., 1980, 1982; Kunow et. al., 1993). Perhaps it was more a question of the pK_a of the buffer rather than the buffer itself. 20 mM Tris, pH=8.1 stabilized the 65-90% $(NH_4)_2SO_4$ pellet, while harvesting buffer (20 mM Tris, 2 mM DTT, pH=8.1) did not.

The 65-90% $(NH_4)_2SO_4$ pellet proved to possess some qualities not observed or tested in the other purified enzymes. First, there was the increase in total and specific activity as compared to the CFE, along with the loss of this increase when CFE was added back to the 65-90% $(NH_4)_2SO_4$ pellet but not bCFE. This is not to mention the fact that the enzyme appears to be allosteric.

Elucidation of the purification protocol was also extremely frustrating. The determination of the protocol used took approximately 2 years. This was not due to a lack of diligence, but rather the lack of positive results. The final protocol required approximately 5 days.

The K_m's of the enzyme in the CFE, 65-90% $(NH_4)_2SO_4$ pellet, and the purified enzyme were initially rather confusing. All four substrates showed first-order kinetics in the CFE, while $F_{420}H_2$ and $NADP^+$ had non-first order kinetics in the 65-90% $(NH_4)_2SO_4$ pellet, and all returned to first-order kinetics in the purified enzyme (Table V.1.). In the 65-90% $(NH_4)_2SO_4$ pellet, possible explainations for the non-first order K_m's could include an effect on the enzyme from the 65-90% $(NH_4)_2SO_4$ treatment, the influence of other $NADP^+$:F_{420}-dependent enzymes, or both. It is possible that the 65-90% $(NH_4)_2SO_4$ treatment was the cause if the removal of the non-covalently bound proteinaceous unit is considered. If the $(NH_4)_2SO_4$ was the cause of the removal of the unit, the $(NH_4)_2SO_4$ may have had an effect on the active site of the enzyme.

Table V.1.: Comparison of the Kinetics of the $NADP^+$:F_{420} Oxidoreductase in CFE, 65-90% $(NH_4)_2SO_4$ pellet, and Pure Enzyme

	CFE	65-90% $(NH_4)_2SO_4$ pellet	Pure Enzyme
K_m's (μM)			
$NADP^+$	130.0	N.F.O.	370.0
NADPH	256.0	149.0	142.0
F_{420}	16.1	37.0	62.5
$F_{420}H_2$	41.7	N.F.O.	7.7

*-K_m's are based on Lineweaver-Burke regression values.

N.F.O.- non-first order.

enzyme. The only way to confirm this would be to desalt the enzyme with ultrafiltration after the 65-90% $(NH_4)_2SO_4$ treatment and perform the K_m's.

The purified enzyme itself has proven to be unique. *Mb. thermoautotrophicum* is a homotetramer of 112 kDa, while *Archeoglobus fulgidus* (55 kDa), *Streptomyces griseus* (42 kDa), and *Mc. vanielii* (85 kDa) are all homodimers. *Msph. stadtmanae* is an α, β, γ of 60, 50, and 45 kDa giving it a functional molecular weight between 148 and 155 kDa. The evidence as to the uniqueness of the $NADP^+$:F_{420} oxidoreductase of *Msph. stadtmanae* is quite convincing. Not only were there unusual kinetics involved, but the structure of the enzyme is far different from that of any others seen.

All of this evidence only serves to strengthen the original idea that there is some sort of a unit being lost during the $(NH_4)_2SO_4$ treatment. This has now been extended to be able to say that a non-covalently bound, proteinaceous subunit is being lost during $(NH_4)_2SO_4$ treatment. This is not a completely unique prospect.

An NADPH-flavin (FAD or FMN) oxidoreductase in *Vibrio fischeri* was found to have a non-covalently bound prosthetic compound which dissociated from the protein during SDS-PAGE and when the protein was treated with hot methanol. The compound was yellowish in color and showed absorption peaks indicating it was an oxidized flavin. As well, the addition of FMN to the apoprotein markedly increased the activity of the protein (Inouye, 1994).

One of the difficulties has been attempting to compare the purified enzymes *of Mb. thermoautotrophicum* and *Mc. vanielii*. In the case of *Mc. vanielii*, there was data for the optimal pH for both $NADP^+$ reduction and NADPH oxidation, however the enzyme was termed an $NADP^+$ reductase and no other work on the oxidation of NADPH done. If there

is evidence of activity for both directions, then why was only one direction considered in the work? Granted it is assumed that the reduction of $NADP^+$ is the *in vivo* direction, but a complete characterization of the enzyme providing characteristics of both directions would have provided greater insight into its physiological role.

Further investigation would prove useful. Since there appears to be a difference in the type(s) of enzymes used in *Msph. stadtmanae* for NADPH formation in the presence of an alternate electron donor, purification of the enzyme(s) would help to validate this result. Also, an N-terminal amino acid determination of the purified enzyme could be useful in comparison with that of *Mc. vanielii*. This would allow a more detailed comparison of the two enzymes, and may help to explain some of the differences seen here.

The survey of the methanogens should be expanded to include others, especially *Mc. vanielii*. Since the enzymes of *Msph. stadtmanae* and *Mc. vanielii* have been compared throughout this thesis, it would prove useful and help to validate the work completed here. As well, purification of the enzyme(s) in some of the organisms tested would either prove or disprove both the findings and the methods used to determine the presence of one or two enzymes in each of the organisms. Although the methods used for this determination are not conflicting, it would help to complete the picture and overall validate the findings reported here.

Both the purification of the the $NADP^+$:F_{420} oxidoreductase of *Msph. stadtmanae* and the comparison of this activity in several methanogens are original findings. The comparison of $NADP^+$:F_{420} oxidation/reduction of several methanogens is important

work for further understanding and use of these unique and economically important microorganisms.

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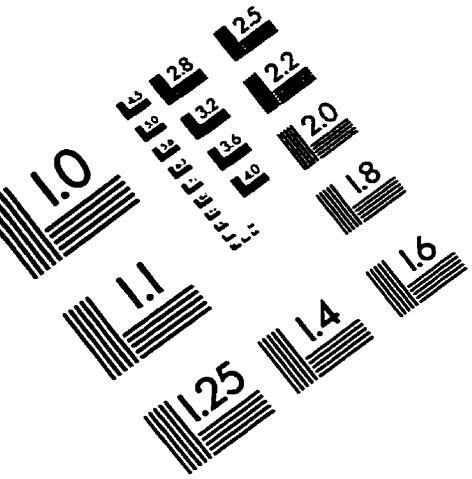
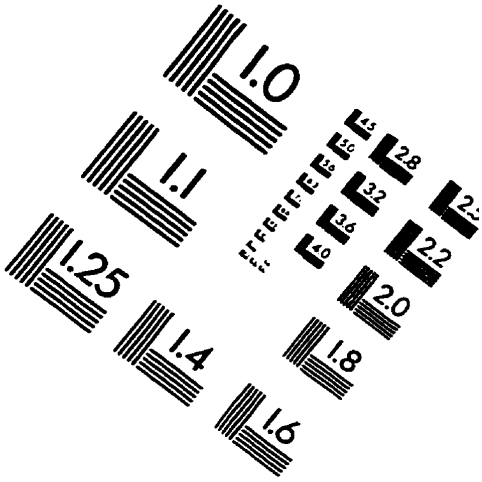
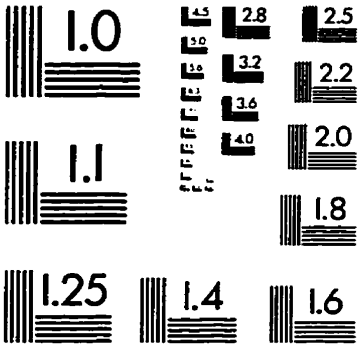
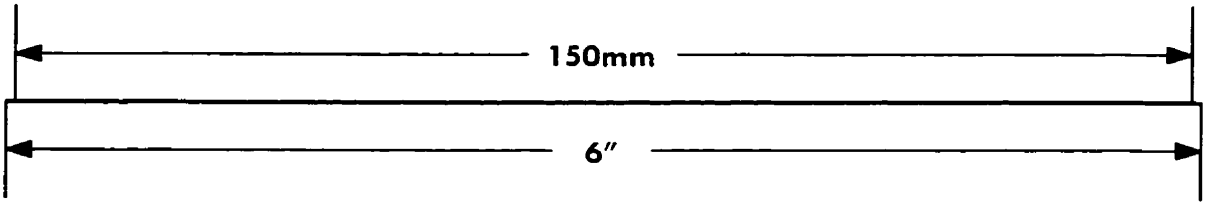
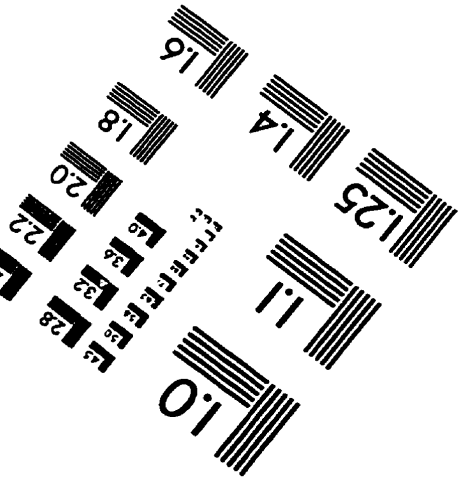
IMAGE EVALUATION TEST TARGET (QA-3)

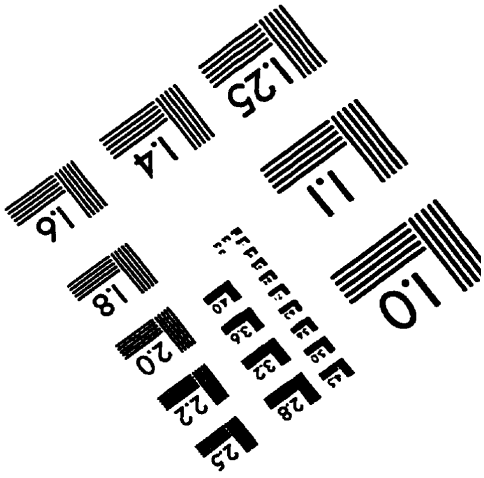












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989