1978

Determination of sulfhydryl groups and disulfide linkages of fumarylacetoacetate fumaryl hydrolase.

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DETERMINATION OF SULFHYDRYL GROUPS AND DISULFIDE LINKAGES OF FUMARYLACETOACETATE FUMARYL HYDROLASE

BY

BEILING CHENG

Thesis
Submitted to the Faculty of Graduate Studies through
the Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

Windsor, Ontario, Canada
1977

ABSTRACT

The sulfhydryl groups of native and denatured fumarylacetoacetate fumaryl hydrolase (E.C.3.7.1.2) (FAH) have been investigated with sulfhydryl specific reagents, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), and p-hydroxymercuribenzoate (PHB) by spectrophotometric titration. The disulfide linkages of FAH have been determined by Cleland's method (1968).

The results indicate that two of the three sulfhydryl groups per native FAH subunit are accessible. One of these two sulfhydryl groups is protectable by substrate. However the modification of these two accessible sulfhydryl groups results in an incomplete loss in enzymatic function. A residual sulfhydryl group and a disulfide linkage are considered as being masked.

The involvement of these sulfhydryl groups and the disulfide linkage in enzymatic function is discussed.

ACKNOWLEDGMENT

I would like to thank my advisor Dr. D. E. Schmidt Jr, and Dr. K. E. Taylor for their patient encouragement and guidence throughout this research work.

I wish to thank Dr. H. B. Packrell for his interest and comments; and L. Chen, W. Pu for their help in preparation of the enzyme.

I am forever indebted to my wife Ginger for her help not only with the thesis but also with all these years of "hard time". Also, I would like to thank my mother, my family and all my friends for their encouragement.

Finally, I would like to express my appreciation to Mr. Y. Chang and Mr. J. Stone (Mgr. Suntown & Co.) for their assistance in my study abroad.

To Professor K. M. Cheng (1891-1973)

- My father, the first one pleased with my intention to do graduate studies. I am sorry that he will never read this thesis.

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ABBREVIATION

	absorbance
DTE	dithioerythritol
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EDTA	ethylenediamine tetraacetate
FAH	fumarylacetoacetate fumaryl hydrolase
K_M	Michaelis constant
O.D.	optical density
PMB	p-hydroxymercuribenzoate
Tris	tris-(hydroxymethyl) aminomethane

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CHAPTER I

INTRODUCTION

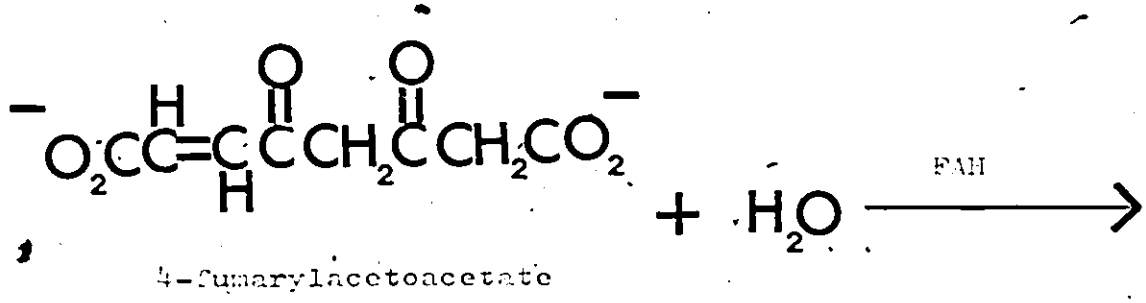
The sulfhydryl or thiol (-SH) groups of cysteine residues and the disulfide (-S-S-) groups of cystine have long attracted particular attention from enzymologists. This is due to the high reactivity and the specific function of these groups in a number of enzymes and other biologically active proteins. The role of sulfhydryl groups consists of binding substrates (alcohol dehydrogenase, Evans and Rabin, 1968), participating in the catalytic reaction (glyceraldehyde-3-phosphate dehydrogenase, Segal & Boyer, 1953), or maintaining the native and active conformation of the protein (lysine - transfer ribonucleic acid synthetase, Stern et al. 1966). The disulfide groups are undoubtedly of great importance in stabilizing the native conformations of many proteins (lysozyme, Neumann et al. 1964).

The involvement of sulfhydryl groups in the action of fumarylacetoacetate fumaryl hydrolase (E.C.3.7.1.2) (F H) was first described by Hsiang, Sim, Mahuran, and Schmidt (1972). They reported that all the sulfhydryl specific inhibitors except iodoacetate definitely inhibited the reactivity of FH. In contrast, serine esterase specific inhibitors had no effect on this enzyme.

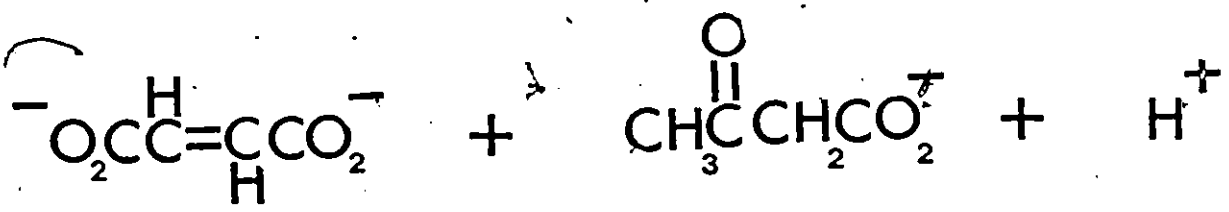
However, the way in which the sulfhydryl groups of FAH are involved in enzymatic function has not been determined. Consequently, this is an extremely interesting and challenging problem.

Previous workers have, however, contributed much useful information toward this target, since characterization and substrate specficity of FAH have been intensively examined with many different techniques. In development of a model of the active site to explain the catalytic mechanism, it is important to summarize the pertinent information from those experimental results.

FAH hydrolyzes a carbon-carbon bond of fumarylacetoacetate into fumarate and acetoacetate in the degradative pathway of tyrosine and phenylalanine in mammalian systems (Rawdin & Crandall 1951).



4-fumarylacetoacetate



fumarate acetoacetate

FAH appears to be a dimer with a monomer molecular weight of 40,000 g/mole. The subunits appear to be identical single polypeptide chains. No disulfide bonds join the monomers in the formation of dimer as evidenced by the observation that little difference is seen between the elution volume on Sepharose 6B in 6M guanidine-hydrochloride of reduced and alkylated FAH and untreated enzyme (Mahuran et al. 1977). It has been proposed, however, that zinc may act as a bridge between the two monomers (Ewing 1977).

FAH is an enzyme whose natural substrate is a dianion in the pH 6-9 region where the enzyme is active and it has been suggested that binding of 4-fumarylaceto-acetate is through both of the carboxyl groups of the substrate (Braun & Schmidt 1973). Thus the active site could have two positively charged groups to bind the two end carboxyl groups of the substrate. More importantly, FAH is subject to competitive inhibition by monovalent anions. One anion is bound per enzyme-inhibitor complex. This anion inhibition is interpreted as masking by the anions of a positive site on the enzyme (Braun & Schmidt 1973). Further studies showed that fluoride seems to be the best anion inhibitor of FAH activity and the binding affinity of FAH for substrate and inhibitor monovalent anions is

not affected significantly by pH over the range of 6.25-8.50. It was also suggested that both substrate and inhibitor bind the same site of FAH, and the binding group pKa's of FAH are outside this pH range (Pu 1977).

FAH has been found to catalyze the hydrolysis of various substrate analogs of the diketo acid type (2,4- and 3,5-diketo acid). It is interesting to note that as the group adjacent to the methylene carbon of 2,4-diketo acids gets bulkier, the compound becomes less able to serve as a substrate. However a methyl group is worse than an ethyl group, while a 3-phenylpropiono group is more favorable than a cinnamoyl group in the same case (Puren 1973). Another interesting feature of substrate specificity is that neither of 4-acetobutyrate (without diketone) nor fumarylacetone (without carboxylic function) can be hydrolyzed by FAH (Mahuran et al. 1977). Therefore, the specificity of FAH seems to require a diketone and a carboxylic acid function. However, a substrate analog acetopyruvate (a monoanion molecule), in high concentration (1.2 mM) can inhibit the reactivity of FAH by incorrect binding at one of the two positively charged areas (Hsiang et al. 1972).

It has been suggested that there is a free sulfhydryl group at or near the active site of FAH and that could be the nucleophile which attacks the C-4 or

2-3 carbonyl group of 2,3- or 3,3-diketo acid substrates respectively (Fanini 1975, Khuran 1975, and Swint 1977). This idea seems to be reinforced by the bell-shaped curves of log Vmax versus pI and log Vmax/Km versus pI from which apparent ionization constants pKa 6.9 and pKb 7.6 were determined. These ionization constants could correspond to imidazole and sulfhydryl groups respectively (Pu 1977). There is precedent for such a pair of active site groups since Olson and Park (1964) postulated that a sulfhydryl group situated in the active site of 3-phosphoglyceraldehyde dehydrogenase may be activated as a result of the formation of hydrogen bonds with neighboring functional groups such as imidazole. It leads to an increase of electron density at the sulfur atom of sulfhydryl group and consequently to an increase in the nucleophilic properties. Further, Lucas and Williams (1969) suggested that imidazole may act as general base removing the proton of the sulfhydryl group.

However, from inspection of Pu's pH-rate profiles, it seems that reduction in enzymatic activity at high pH is in conflict with the expected greater nucleophilicity of the sulfhydryl group conjugate base, if the sulfhydryl residue were acting as nucleophile in the enzymatic function.

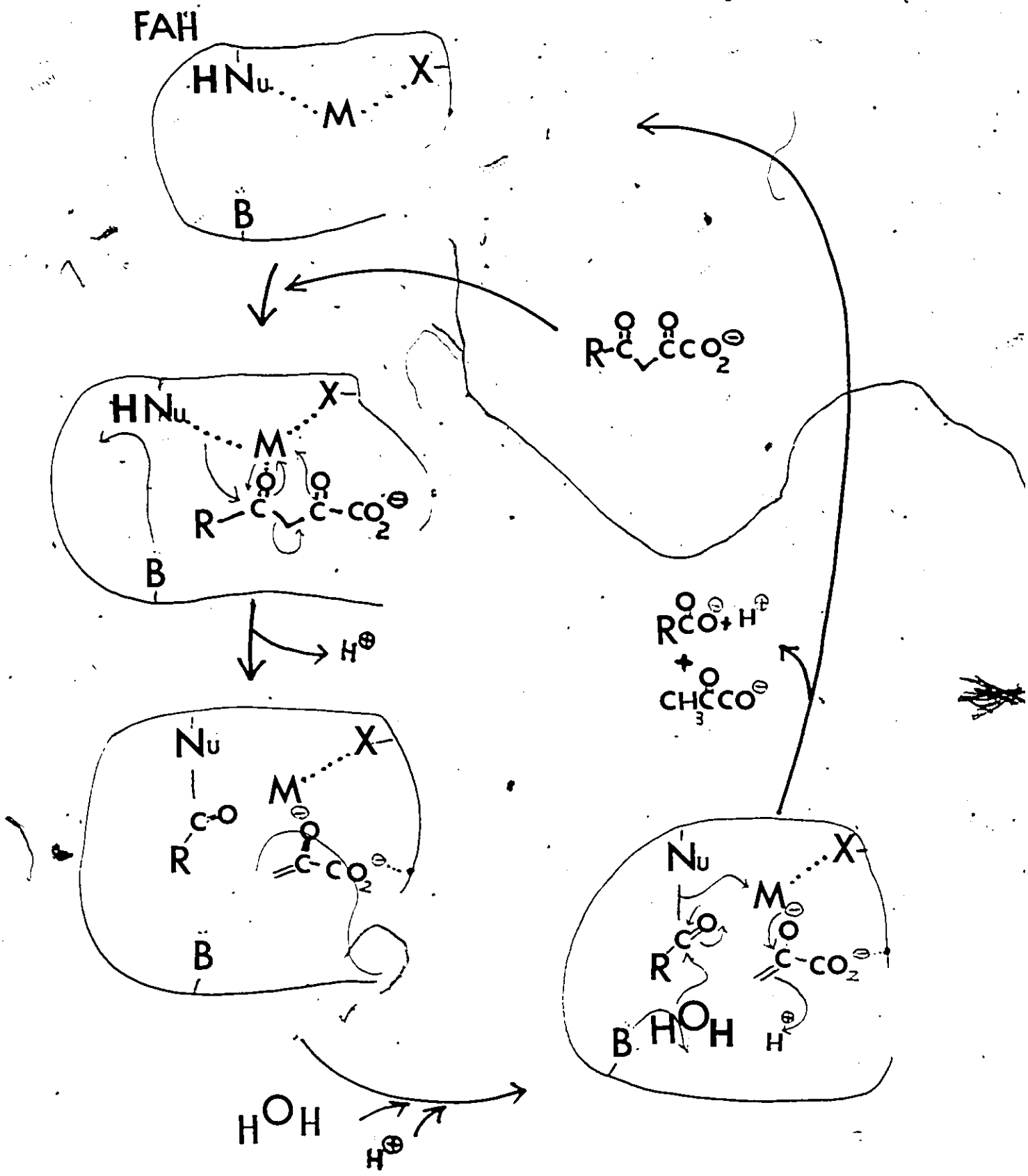
Thus Pu postulated that the loss of enzymatic activity at high pH is due to electrostatic repulsion between the thiolate anion and the negatively charged substrate molecule.

Recently Ewing (1977) speculated that a magnesium ion may hold and activate the 2,4-diketo acid (substrate) so that the enzyme's nucleophilic attack on the 4-keto carbon could take place. In proposing this mechanism, Ewing (1977) presented evidence that FAH does not catalyze cleavage via Schiff's base formation. Therefore, the plausible mechanism of FAH action based on all of the above mentioned suggestions is depicted in Scheme 1.

Up to the present time, only preliminary work on the determination of sulfhydryl groups of FAH has been done by Mahuran (1975). He indicated that three sulfhydryl groups per denatured monomer were found by DTNB titration, while in the active enzyme only two of these sulfhydryl groups were free to react with this specific reagent. In addition, Mahuran showed that a total of five half cystine residues per FAH subunit were found from amino acid analysis.

However, many reports have shown that the sulfhydryl groups of some enzymes could not react with [illegible], but were situated [illegible]. For instance, Lee et al.

SCHEME 1



X: binding group WITH metal M: metal NuH: nucleophile B: base

(1977) indicated that three of the six sulfhydryl groups of [illegible] which reacted with PMB did not react with [illegible]. Therefore, it remains possible that PAH possesses hydryl groups inaccessible to [illegible] or that it has only one disulfide linkage per subunit.

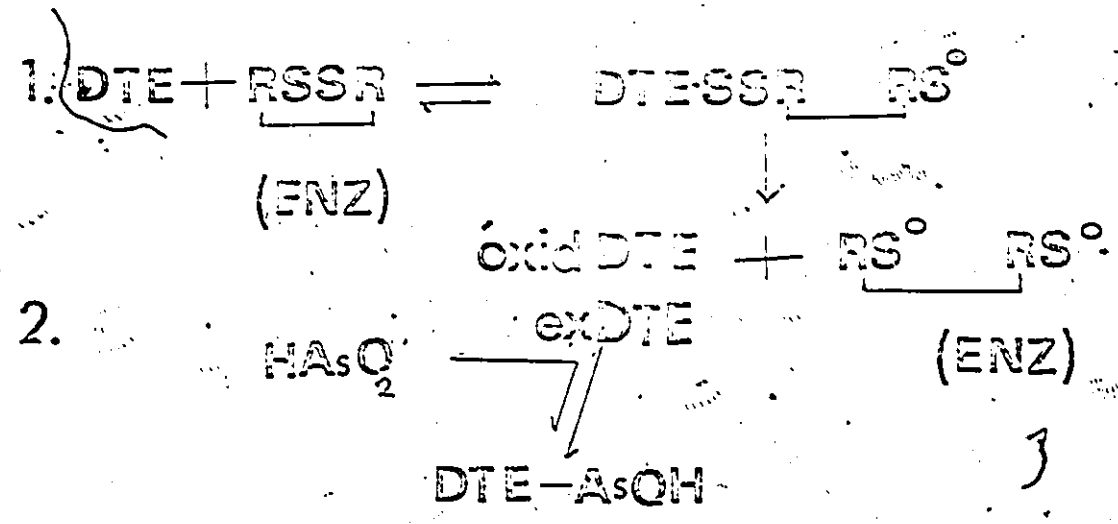
[illegible]Lain (1975) reported that PMB is a highly specific inactivating reagent of PAH since kinetic studies indicated that the inactivation step was preceded by formation of a dissociable complex with K_I = 0.041 mM. More importantly, gross conformational change or dissociation into subunits did not occur after PMB treatment of PAH as evidenced by estimation of the elution volumes of native and PMB-treated PAH on gel filtration. For the reasons given above, PMB will be employed in this work as an alternative method for determination of sulfhydryl groups. Secondly, the presence of substrate (propionopyruvate, 0.4 mM) or competitive inhibitor (fluoride, 20 mM), protects the enzyme from PMB inhibition. However substrates can prevent loss of enzymatic activity not only by direct protection of functional groups but also as a consequence of their stabilization of the structure of the enzyme. Hence to establish that a substrate protects sulfhydryl groups it is not enough to carry out measurements of enzymatic activity alone; a direct measurement of the number of these groups before and after [illegible] of the specific reagent is needed.

Many have also found that PCMB acts on PAL as a reversible and competitive inhibitor (K_i = 20 μM) at pH 7.3. However, one might expect that PCMB employed at higher pH would show an irreversible effect on the activity of the enzyme, since it has been indicated that reaction with PCMB is much faster at pH 8-9 than in weakly acidic solution (Ellman 1958, Rocklemurst and Little 1973).

Many reports have suggested (Schramm 1964, Ingram 1969, and Symes & Sourkes 1975) that EDTA (10 mM) can enhance the ability of PCMB to detect more buried sulfhydryl groups which are liganded with metal ions. However, PAL has been tentatively classified as a metalloenzyme with the possibilities that a magnesium ion and a sulfhydryl group exist at or near the active site (Zwiar 1977). Therefore, as an additional parameter, 10 mM EDTA will be used in any buffer for PCMB titration. Since DNPA interferes with the absorbance of PCMB (Boyer 1954 and Bettell et al. 1968), it can not be applied in PMB titration.

Furthermore, it is desirable that a direct method for determination of disulfide linkages should be employed. The method adopted was that described by Cleland (1968) based on the reduction of disulfide linkages with DTT and determination of the resulting monothiols with PCMB in the presence of a reagent capable

to the reaction sequence:



(oxid DTE : oxidized DTE
ex DTE : excess DTE)

CHAPTER II

EXPERIMENTAL

A. Methods and Materials

1) General

(i) Materials

The following materials were commercially available; 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), from Aldrich Chemical Company; acetopyruvic acid, p-hydroxy mercuribenzoate (PMB), and dithioerythritol (DTE), from Sigma Chemical Company.

(ii) Enzyme Preparation

Fumarylacetoacetate fumarylhydrolase was isolated and purified from beef liver in the cold room by the method of Hsiang et al.(1971) with the modifications according to Mahuran et al. (1977). The enzyme was stored in sodium phosphate buffer (25 mM, pH 7.3) at -20°C until used. No glycerol or DTE was added to the stock solution.

(iii) Instrumentation

A Beckman ACTA MARK VI was used in all assays. By circulating water from a thermostated bath through the cell compartment the temperature was maintained at 30.0 ± 0.5°C and 24.0 ± 0.5°C in enzymatic activity assay and titration respectively. Cuvettes (1 cm light

path) were carefully matched and were always placed with the same face towards the light source.

(iv) Reagents and Solutions

All reagents and substrate solutions were made fresh each day. The buffers were made up every four weeks. Before each assay, the reagents and solutions were incubated and shaken with a incubation shaker at 24°C.

DTNB, 1.2 mg, was dissolved in 1 ml 50 mM acetate buffer, pH 5.0 (Zahler and Cleland 1968), under a nitrogen atmosphere and sealed with parafilm. The concentration of this solution was 3 mM. This method of preparation ensured the stability of DTNB. PMB (1.9 mg, 5.3 μmole) was dissolved in water plus 0.05 ml of 0.1 N sodium hydroxide to give final volume of 10 ml. After centrifugation the concentration was determined at 232 nm by dilution in 50 mM sodium phosphate buffer, pH 7.0. A molar extinction coefficient $1.69 \times 10^4 . M^{-1} . cm^{-1}$ was used to calculate the concentration of PMB in solution (Carne et al. 1977).

2) Enzymatic activity assays

Into a 3 ml cuvette were pipetted 2.6 ml of 25 mM sodium phosphate buffer, pH 7.3, and 0.3 ml of a 1.22 mM acetopyruvic acid solution. To this solution was added 50 μl of enzyme by microsyringe. The

disappearance of substrate was monitored at 295 nm for 5 min.

One unit of enzyme activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 µmole of substrate per minute under the standard conditions defined above. In each case acetopyruvate was used as substrate (ε acetopyruvate = 7.8 ml/µmole·cm). Therefore : 1 unit of enzyme = amount of enzyme that hydrolyses 1 µmole of acetopyruvate per minute.

$$\text{No. of units} = \frac{\Delta \text{OD/min}}{7.8 \text{ ml/µmole}} \times \text{dilution factor} \times \text{vol. of enzyme solution}$$

The specific activity is defined as units of enzyme activity divided by the amount of total protein in solution. To estimate the protein content of the enzyme solution, the absorbance reading at 280 nm and ε_{PNH} = 1.3 ml/mg·cm was used (Mahuran et al. 1977).

3) Accessible Sulfhydryl Group Determination

(i) Reaction with DTNB

a. PNH was reacted with DTNB by the method of Ellman (1958 & 1959). To the reaction mixture containing 3.0 ml of sodium phosphate buffer (100 mM, pH 8.0, EDTA 10 mM) and 0.1 ml of DTNB (3 mM), 0.15 ml of enzyme (1.765 mg/ml) was added to give a final volume of 3.25 ml.

A reference cell containing all the reagents but enzyme was used. The absorbance was read at 412 nm with a recorder as a function of time.

b. A duplicate DTNB titration was carried out following the appropiate condition as a. After 2 and 10 min, 1 ml of reaction mixture was removed to measure the remaining enzymatic activity. A reference mixture containing enzyme and buffer (without DTNB) was used to measure the original activity at the appropiate time.

c. In order to determine the substrate protection effect, a duplicate DTNB titration with same quantity of enzyme was also carried out as a, but a modified buffer containing acetopyruvate at a concentration equal to five times its K_M value (K_M = 1.366 mM, Sim, 1971) was used. It was important to note that high concentration of acetopyruvate could inhibit the enzymatic activity (Hsiang et al. 1972).

(ii) Reaction with PMB

a. PMH was reacted with PMB using the method of Boyer (1959) and the modification by Carne et al. (1977). Aliquots of PMB solution were injected into a cuvette containing a mixture of 0.2 ml of PMH (0.451 mg/ml), and 2.8 ml sodium phosphate buffer (50 mM, pH 7.0). Then the reaction was followed by the change in absorbance

at 250 nm. (A reference cell containing enzyme was titrated with water at the same time.) To determine the absorbance of PMB alone, a parallel titration was done without enzyme. The difference between the two titrations was used to give the titration curve.

b. For determination of residual activity of the modified enzyme, independent assays in duplicate were carried out with addition of the same amount of PMB of each stepwise titration as a to a known amount of enzyme. After an appropiate time 1 ml was removed from each reaction mixture and monitored for remaining activity. A reference mixture containing enzyme and buffer (without PMB) was used as a control in the activity assays. Since the substrate, acetopyruvate, strongly absorbed at 295 nm, it was impossible to do PMB titration with native enzyme under protection of substrate at a concentration equal to five times its K_m.

4) Total Sulfhydryl Group Determination

The sulfhydryl groups in proteins exhibit variable reactivity towrad titrant due to steric factors. Therefore, determination of total sulfhydryl content requires that the protein be denatured.

(i) Reaction with DTNB

PMB (0.2 ml, 2.20 mg/ml) and 2.8 ml of 100 mM

sodium phosphate buffer (containing EDTA 10 mM, urea 8 M) of pH 8.0, were pipetted into a 12 ml screw-cap vial. A nitrogen atmosphere was maintained for 5 min and the mixture was then stirred gently at room temperature for 10 min. After denaturation was complete, 0.1 ml of 3 mM DTNB was added into this sample and a blank and the increase in absorbance at 412 nm was recorded.

(ii) Reaction with PMB

The same as method (3-ii a), but the buffer, containing 8 M urea (final concentration), was added into a 12 ml screw-cap vial under a nitrogen atmosphere. The sample was stirred for 10 min at lowest speed. Then it was titrated spectrophotometrically at 250 nm with fresh aliquots of PMB (FMU 0.15 ml, 0.628 mg/ml).

5) Disulfide Bond Determination

(i) Reaction with DTNB after Reduction

Before the Cleland method was applied to determine accessible disulfides of the reduced enzyme, FMU activity was measured before and after incubation (15 min.) with sodium arsenite (2.5 mM final concentration) in order to detect vicinal sulfhydryl groups (Symes & Sourkes, 1975), since the presence of vicinal sulfhydryl groups affects the results of Cleland method (Habeeb 1972).

Then, FAH was reacted with DTNB according to the method of Zahler and Cleland (1968). FAH, 0.2 ml, (0.767 mg/ml) was mixed with 0.1 ml of 50 mM Tris-HCl buffer of pH 10.5 and 0.1 ml of 3 mM DTE. The reduction of disulfides was allowed to proceed for 20 min.

When reduction was complete 1 ml of Tris buffer (pH 7.75, 200 mM, containing 10 mM EDTA), and 1.5 ml of 5 mM $NaAsO_2$ were added to give a final volume of 2.9 ml. The solutions were mixed and allowed to stand for at least 2 min.

To start the reaction in the cuvette, 33 µl of 3 mM DTNB was added to 1 ml of sample mixture. The absorbance at 412 nm was recorded for a half hour. A blank containing all the reactants except the protein solution was treated in the same way simultaneously.

(ii) Reaction with DTNB after Denaturation and Reduction

FAH was titrated with DTNB by the same method as in (5-i) slightly modified according to Ando and Steiner (1973). FAH (0.16 ml - 0.20 ml) was mixed with Tris buffer and DTE as in (5-i) but 0.2 ml of 8 M urea or 15 M urea was added separately. (Thus, urea concentration of this incubation step was 3 M and 5.4 M respectively.)

Reduction of the protein disulfide was allowed

to proceed separately for 30 min and 60 min at room temperature. Then 1.0 ml of 200 mM Tris buffer, pH 7.75, and 1.5 ml of 5 mM $NaAsO_2$ were added, and the mixture was left standing for another 5 min. Into a 1.5 ml cuvette, 1 ml of this mixture was pipetted. Then 33 µl of 3 mM DTNB was added to the reaction mixture and the absorbance at 412 nm was recorded at intervals of 2 min for a period of 1/2 - 1 hour. A reference cell containing all the reactants except protein was used.

B. Results

1) Calculation of Number of Sulfhydryl Groups and Disulfide Bonds

In all essays, samples and titrants were added to a mixture in a photometer cell. Therefore the data should be corrected by the dilution factor.

$$C_o = \frac{A}{\varepsilon \times 1} \times D$$

where

C_o = concentration of original solution

A = absorbance data
(at 412 nm if DTNB was titrant; or
at 250 nm if PMB was titrant)

ε = extinction coefficient
13,600/M/cm for nitromercaptobenzoate anion formation (Ellman 1958 and Silverstein 1975) and 7,600/M/cm for mercury mercaptide formation (Boyer 1954)

D = dilution factor

1 = 1 cm in all assays

For example :

The concentration of enzyme containing 0.770 mg protein/ml is 19.2 μM of subunit. If 0.2 ml of this sample solution was diluted to 2.8 ml and then a sulfhydryl specific titrant (0.1 ml) was added in a spectrophotometer cell, the absorbance at a specific wavelength, (say 412 nm

for DTNB assays), was 0.055.

In this case

$$C_o = \frac{0.055}{13{,}600 \frac{1}{M \cdot cm} \times cm} \times \frac{3.1\ ml}{3\ ml} \times \frac{3\ ml}{0.2\ ml}$$

$$= 62.7\ \mu M$$

$$\text{No. of sulfhydryl groups} = \frac{\text{original sulfhydryl conc.}}{\text{enzyme conc.}}$$

$$= 3.3$$

No. of disulfide bonds = (No. of sulfhydryl groups after reduction - No. of total sulfhydryl groups)/2

2) Accessible Sulfhydryl Group Determination

(i) Reaction with DTNB

Two (2.0 ± 0.2) sulfhydryl groups per subunit of native PFK are accessible to DTNB. While only one (1.0 ± 0.1) group can be accessible to DTNB in the presence of substrate at a concentration equal to five times its K_m value (Fig. 1). This suggests that there are two free sulfhydryl groups on the hydrophilic surface of PFK subunit. One of the two sulfhydryl groups, however, is protectable by substrate.

No precipitation occurred in these assays in contrast to those in Mahuran's report (Mahuran 1975). This might be due to the low concentration of enzyme used in the present assays which kept intermolecular reaction to a minimum.

After approximately 2 - 8 min and 10 - 16 min of reaction with DTNB 24%, 16% of the original activity were detected respectively. It is important to note that during activity assay, the reaction of DTNB with enzyme still continued to proceed due to presence of huge excess DTNB.

(ii) Reaction with PMB

The plot (Fig. 2) of the change in absorbance at 250 nm versus PMB concentration has two linear regions. These linear regions intersect at two sulfhydryl groups per mole of PMH subunit approximately. To reach this point in the titration a 4.2-fold molar excess of PMB was needed. No more sulfhydryl groups were found by further addition of PMB.

The reaction of PMH with 1.5-fold excess of PMB resulted in a decrease of 29% in the original activity. When each of 2.3, 4.2 and 6-fold excess of PMB was added, 87%, 73 and 79% of the original activity was lost respectively.

Further addition of 7.9-fold PMB resulted in almost complete inactivation of enzymatic activity (trace (< 5%) or no activity). After F II was incubated with 6-fold PMB for the same time as the 7.9-fold PMB treatment, 87% of the original activity was lost.

Both DTNB and PMB titrations with the native enzyme show there are two sulfhydryl groups accessible on the hydrophilic surface of the enzyme. This is in agreement with Mahuran's work (1975). At least one of these two sulfhydryl groups is probably located at or near the active site.

3) Total Sulfhydryl Group Determination

(i) DTNB Titration with Denatured Enzyme

After incubation (10 min) with denaturant (8 M urea, final concentration) under a nitrogen atmosphere, 3.0 ± 0.2 sulfhydryl groups were titrated per mole of FAH subunit by DTNB (0.1 mM final concentration) (Fig. 3). This is in agreement with Mahuran (1975).

(ii) PMB Titration with Denatured Enzyme

3.1 ± 0.1 sulfhydryl groups were titrated per mole of FAH subunit. The plot of the change in absorbance has three linear portions which intersect at 1 and 3 sulfhydryl groups per mole of enzyme subunit (Fig. 4). It seems that one of the three sulfhydryl

groups per subunit after denaturation is very reactive to PMB. (Only 1.0-fold PMB was needed.)

In summary, these results confirm that one of three sulfhydryl groups is masked and is not accessible to either PMB and DTNB, without addition of a denaturant.

Fig. 1 Determination of Accessible Sulfhydryl Groups by DTNB Titration

DTNB : 3 mM (stock solution), 0.1 mM (final concentration)

O : native PDH (without substrate protection) buffer - sodium phosphate 100 mM, pH 8.0, (EDTA 10 mM)

● : native PDH (with substrate protection) buffer - sodium phosphate 100 mM, pH 8.0, 5 K_M substrate (EDTA 10 mM)

SH GROUPS/MOLE OF FAH SUBUNIT

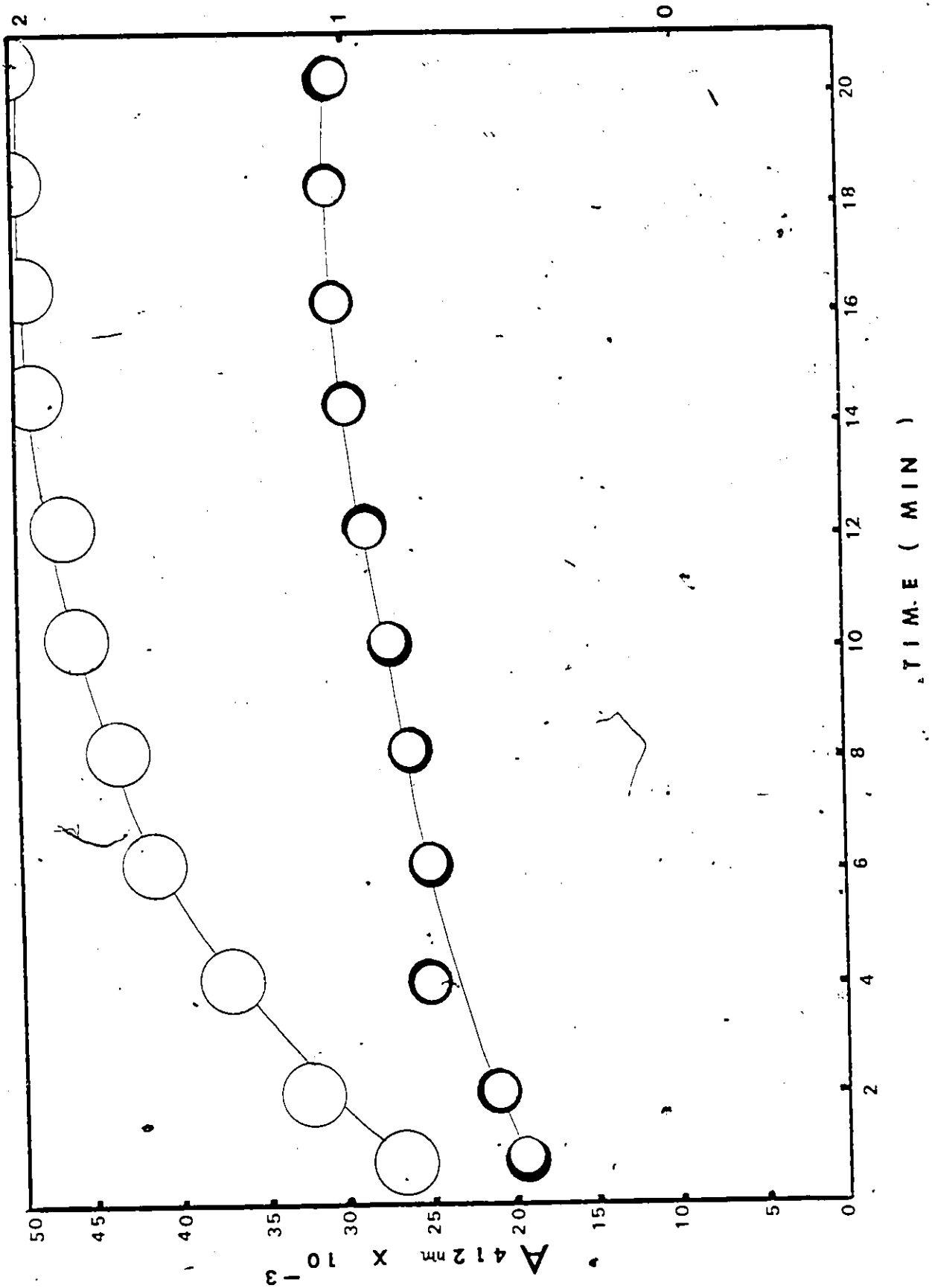


Fig. 2 Determination of Accessible Sulfhydryl Group By PMB Titration

● Native FAH A_{250}

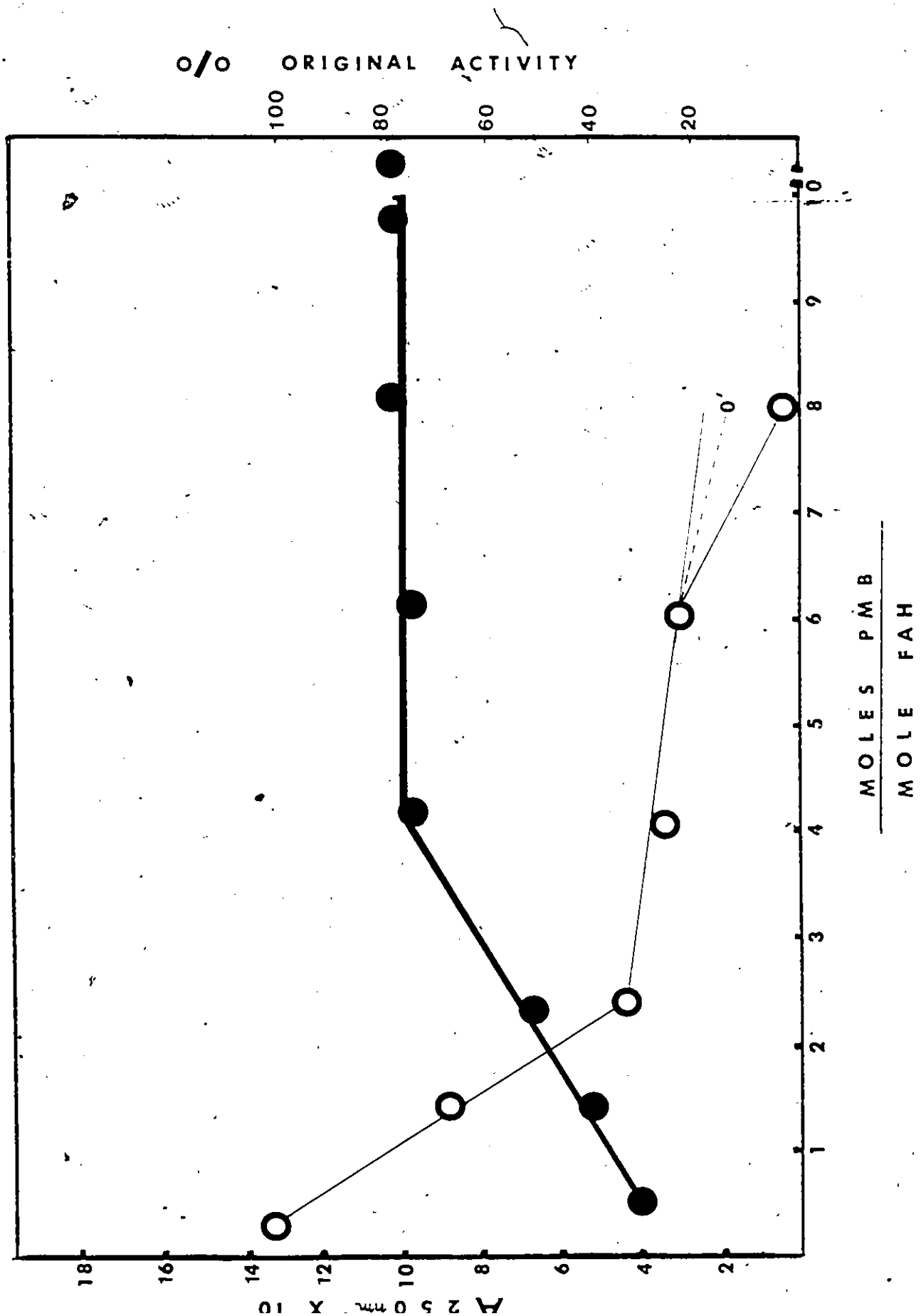
○ % of original activity remain after varied amount of PMB addition

○' % of original activity remain (Prolonged incubation with 6 fold PMB)

PMB : 0.52 mM (stock solution)

Buffer : sodium phosphate 50 mM, pH 7.0 without EDTA

The original activity of FAH is expressed as an activity of a control sample containing everything except PMB under the same conditions.


O/O ORIGINAL ACTIVITY
100
80
60
40
20
MOLES PMB / MOLE FAH
1
2
3
4
5
6
7
8
9
10
O'
A 250mμ X 10
2
4
6
8
10
12
14
16
18

3 Determination of Total Sulfhydryl Groups by DTNB Titration

DTNB : 3 mM (stock solution)

0.1 mM (final solution)

Buffer : sodium phosphate 100 mM, pH 8.0

EDTA 10 mM, urea 8 M

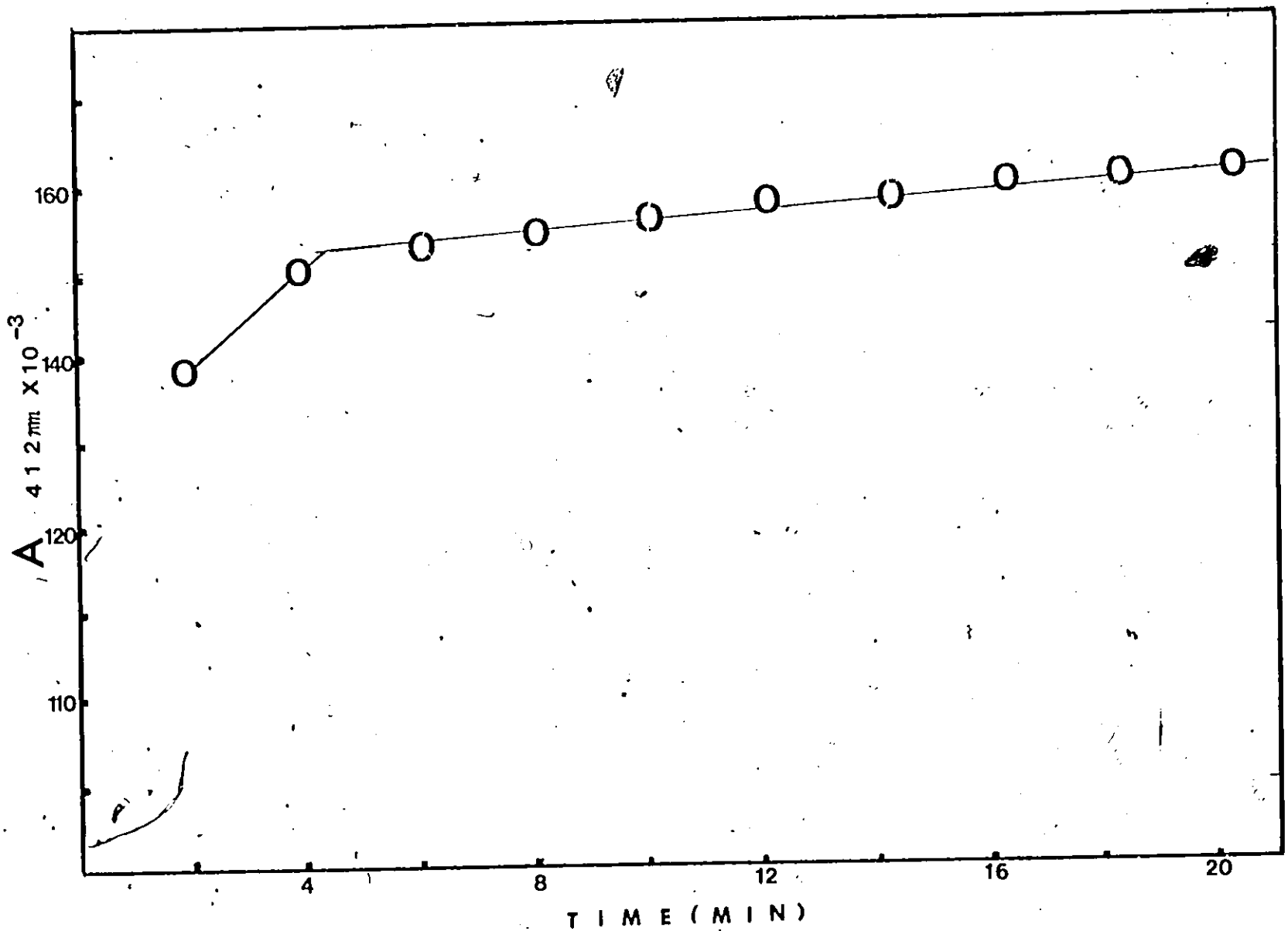
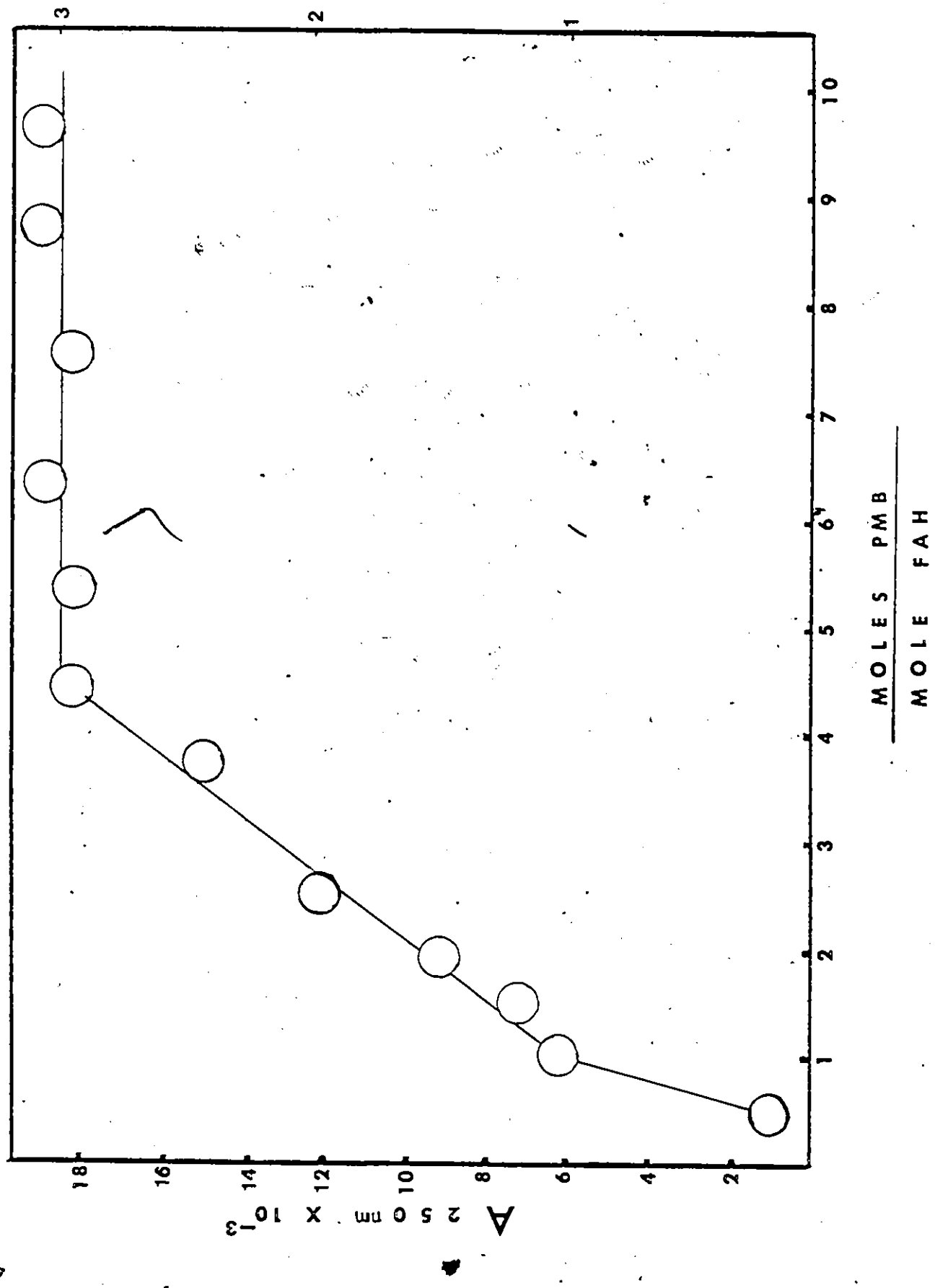

160
140
120
110
A 412nm X10^{-3}
4
8
12
16
20
TIME (MIN)
3
2
SU GROUPS/MOLE OF FAH SUBUNIT

Fig. 4 Determination of Total Sulfhydryl Groups by PMB Titration

PMB : 0.52 mM (stock solution)

Buffer : sodium phosphate, 50 mM, pH 7.0

urea 8 M, <u>without</u> EDTA


SH GROUPS/MOLE OF FAH SUBUNIT
3
2
1
A 250 nm . X 10⁻³
18
16
14
12
10
8
6
4
2
MOLES PMB / MOLE FAH
1
2
3
4
5
6
7
8
9
10

4) Disulfide Bond Determination

(i) DTNB Titration with Reduced Enzyme

Sodium arsenite (2.5 mM) does not inhibit the reactivity of FAH. This indicates that FAH lacks vicinal sulfhydryl groups. After 30 min reduction with DTE, only 1.7 ± 0.1 sulfhydryl groups per mole of FAH subunit were accessible to the DTNB (Fig. 5). The result is within experimental error of that for unreduced enzyme and this indicates that no disulfide bonds are accessilbe to the reducing agent in the native enzyme.

(ii) DTNB Titration with Reduced and Denatured Enzyme

Incubation times of less than one hour and low urea concentrations in this step were found insufficient for complete reduction of disulfide bonds. For example, the sample after 30 min of incubation (with 3 M urea) for reduction gave incomplete reaction with titrant. Even with a reaction time of 76 min, only three sulfhydryl groups could be found (Fig. 6). On the contrary, for a sample after one hour of incubation (with 5.4 M urea) 4.9 ± 0.1 sulfhydryl groups were almost immediately (3 min) accessible to the titrant and the titration curve seemed very stable for at least 20 min. Therefore,

$$\text{No. of -S-S- per FAH subunit} = \frac{(4.9 \pm 0.1) - (3.0 \pm 0.2)}{2} = 0.95 \pm 0.15$$

$$= 1$$

only one disulfide bond per mole of F1 subunit was detected. This experiment also confirmed the total half cystine content of 4.8 moles per mole of F1 subunit from amino acid composition analysis (Mahuran 1975).

In summary, it has been found that two sulfhydryl groups are accessible and not vicinal in native enzyme. One of them can be protected by the substrate and presumably is located at or near the active site. In addition one sulfhydryl group and one disulfide linkage are inaccessible to the reagents used in the native enzyme (Table 1).

Fig. 5 Determination of Accessible Disulfide Linkage by DTNB Titration

DTNB : 3 mM (stock solution), 0.1 mM (final concentration)

DTE : 3 mM (stock solution), 0.1 mM (final concentration)

$NaAsO_2$: 5 mM (stock solution), 2.5 mM (final concentration)

Buffer: $Tris_1$ 50 mM, pH 10.5 (incubation step)

$Tris_2$ 100 mM, pH 7.75 (EDTA 10 mM)

SH GROUPS/MOLE OF FUH SUBUNIT

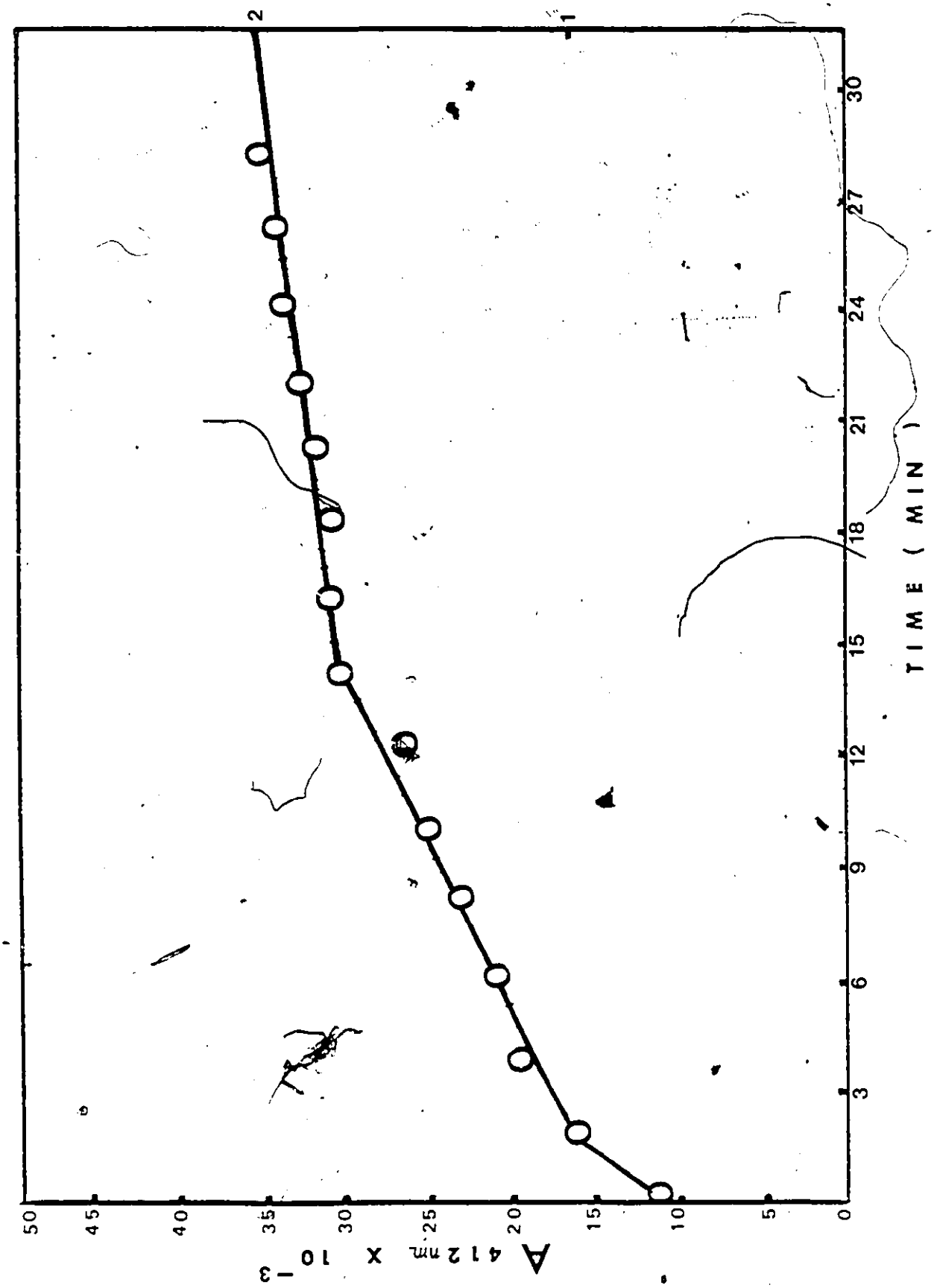


Fig. 6 Determination of Total Disulfide Linkage by DTNB Titration

● incubation one hour (with urea 5.4 M)

-•-• incubation half hour (with urea 3 M)

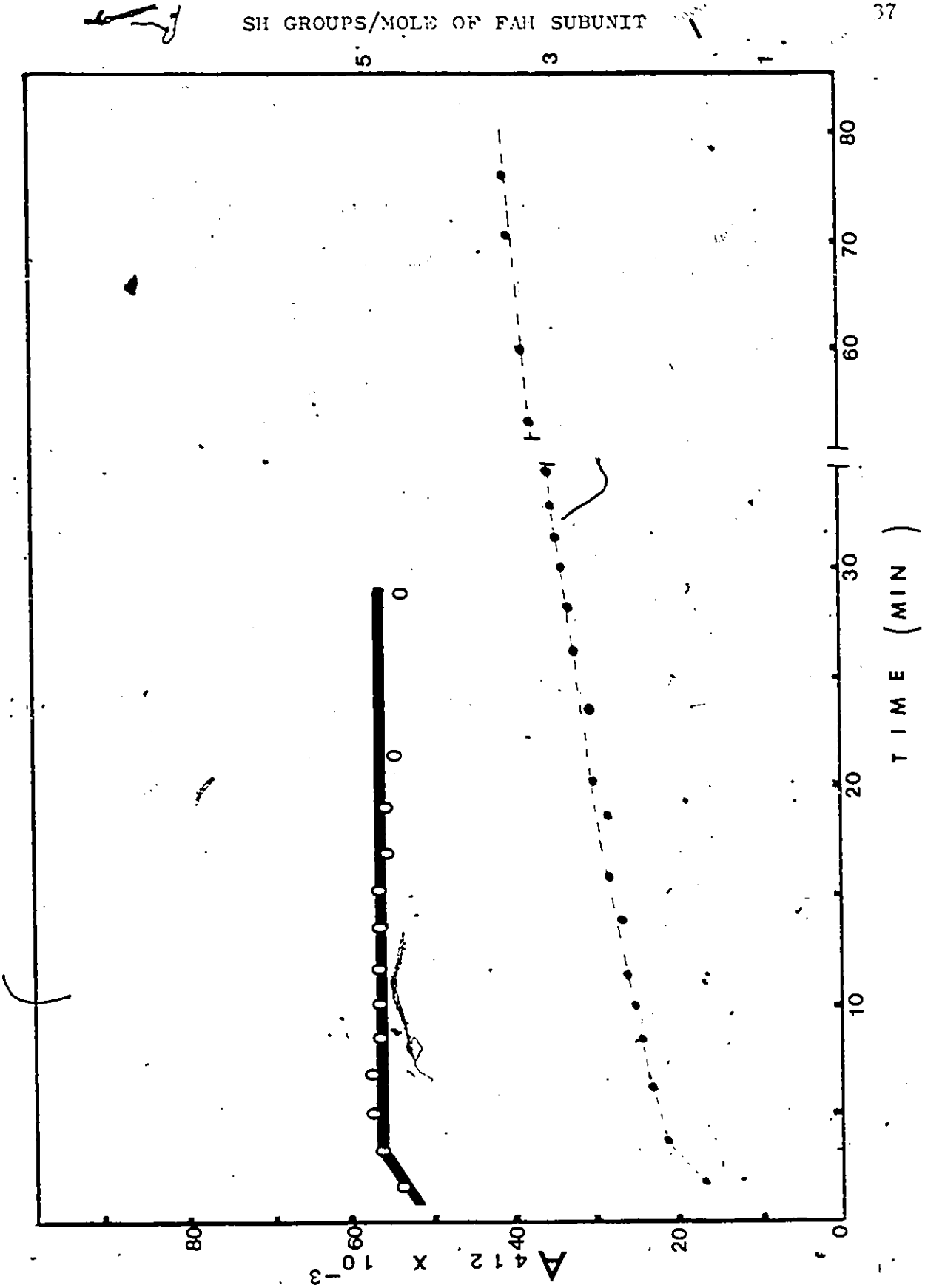

SH GROUPS/MOLE OF FAH SUBUNIT
5
3
1
0
0
80
70
60
30
20
10
0
TIME (MIN)
80
60
40
20
0
$A_{412} \times 10^{-3}$

TABLE 1

Number of -SH & -S-S- Groups of PMI Per Monomer
(MW 40,000 g/mole)

		PMB Titration	DTNB Titration
1. native enzyme	-SH	1.9 ± 0.1	2.0 ± 0.2 (1.8*)
2. substrate protected enzyme			1.0 ± 0.1
3. denatured enzyme		3.1 ± 0.1	3.0 ± 0.2 (3.2*)
4. reduced enzyme			1.7 ± 0.1
5. reduced/denatured enzyme			4.9 ± 0.1 (4.8 : amino acid anal.*)
	-S-S-		1

*Maburan's Ph. D. dissertation (1975)

CHAPTER III

DISCUSSION

The numbers of sulfhydryl groups per subunit in native and denatured FMH, two and three respectively, determined in this report are in agreement with the preliminary result of Mahuran (1975). In addition it was found that 10 mM EDTA does not act to enable detection of more sulfhydryl groups in this case.

In the presence of urea, the total number of half-cystine residues determined by Cleland's method (1 hr. incubation) is the same as that from amino acid analysis (Mahuran 1975). Apparently, the lone disulfide linkage is buried in the interior of the protein molecule of the enzyme. This disulfide linkage is postulated to maintain the tertiary conformation of protein structure. By comparison it may be noted that one of the three disulfide linkages of native bovine trypsin inhibitor is extremely exposed at the top of a pear-shaped molecule and is easily reduced (Liu *et al.* 1971).

Although Nagainis (1975) indicated that DTNB acts as a reversible competitive inhibitor of FMH, her work was carried out at relatively lower concentration of DTNB (0 - 0.08 mM) and lower pH (pH 7.3). In contrast

it has been shown here that DTNB inhibits the enzymatic activity irreversibly at pH 8.0 and a concentration of 0.1 mM. It has been emphasized that DTNB is fully reactive at pH 8.0 (Ellman 1958). Thus it may be suggested that the combination of lower reagent concentration and reactivity in Nagainis' experiments resulted in a situation where the second order inactivation rate was just too low to allow significant amounts of reaction in the chosen incubation period. On these bases PMI can be considered a sulfhydryl enzyme according to the criteria of Boyer (1959).

It has been shown that acetopyruvate at a concentration five times its K_M can protect one sulfhydryl group from DTNB titration. By comparison Nagainis (1975) reported that propionopyruvate at a concentration equal to its K_M could also protect 30% of the activity of PMI from inhibition by PMB. These results suggest that the sulfhydryl group unreactive in the presence of substrate may be at or near the active site. This is reinforced by the fact that fluoride anion, an active-site-directed inhibitor, can slow the PMB inhibition (Nagainis 1975). Furthermore, kinetic evidence was accumulated indicating that both DTNB and PMB formed reversible complexes competitive with substrate binding prior to modification

of the enzyme. However, it is possible that substrate stablizes the enzyme in such a way as to alter the conformation and thus masks a sulfhydryl group from DTNB or PMB modification. In this case, the protectable sulfhydryl group need not be considered in the active site.

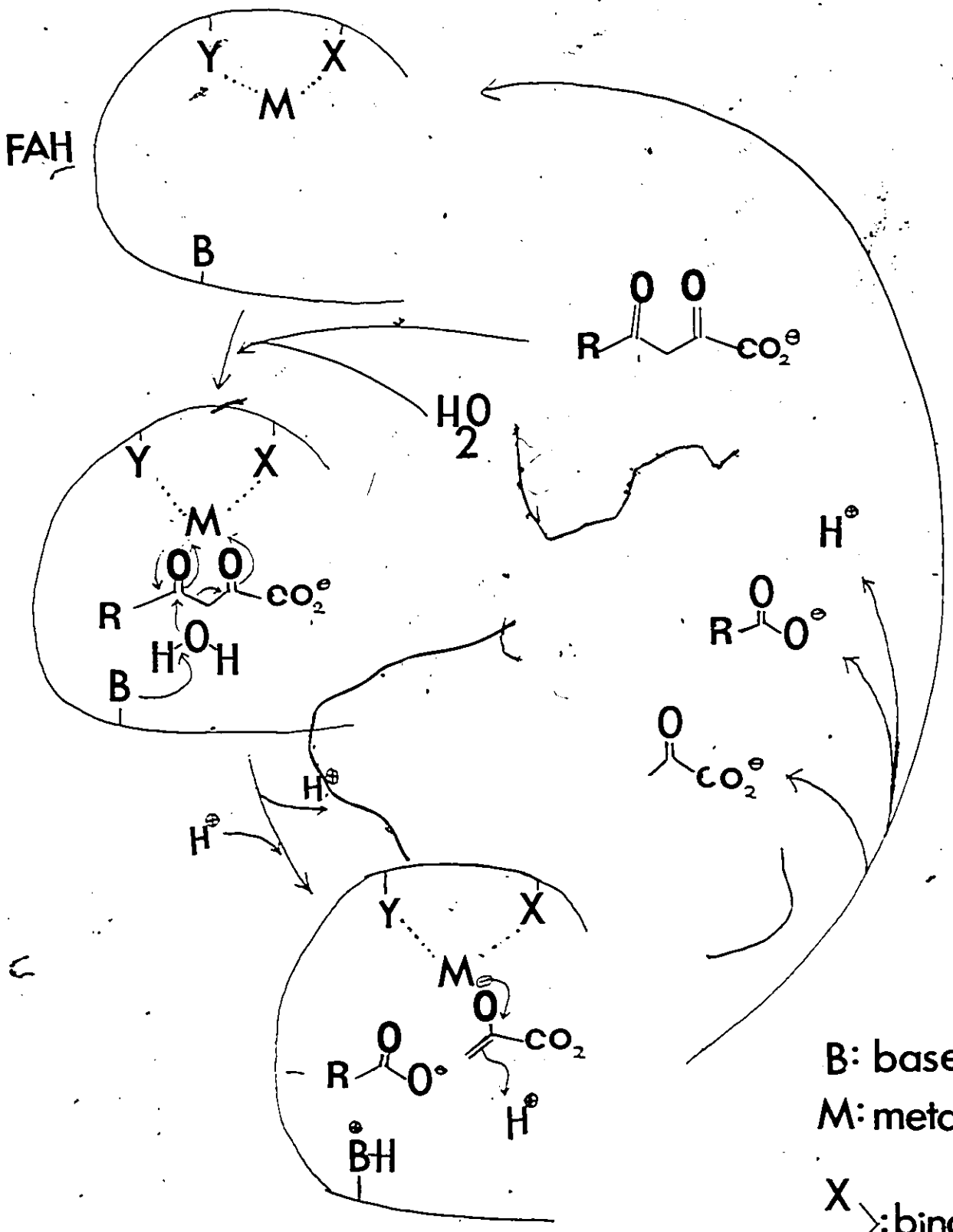
However, FMH still has 15-20% residual activity toward the substrate even after those two sulfhydryl groups have been modified with DTNB or PMB. Also to be considered is the fact that the enzyme contains one inaccessible sulfhydryl group per subunit. One may postulate many different explanations for these phenomena.

Apparently, FMH is not influenced by EDTA (Durani & Schmidt, 1973, and this report). However, Ewing (1977) indicated that a magnesium ion may be involved as more than a structural entity and proposed an enzyme-metal-enzyme intramolecular bridge at the active site. If this magnesium exists at the active site but is inaccessible to EDTA, this metal should be buried somewhere. In other words a chelating sulfhydryl group may also be burried in the same place and consequently inaccessible to thiol titrants. This appears to be the case with prolidase (E.C.3.4.3.7) where a Mn^{2+} ion combines with an enzyme sulfhydryl group and protects it from the sulfhydryl reagent, iodoacetamide (Smith et al. 1954). From this

postulate, it is suggested that the buried sulfhydryl group in FAH may chelate a magnesium ion and act as a nucleophile. Therefore, even after the two free sulfhydryl groups are modified, this buried sulfhydryl is still capable of reacting with substrate. The substrate protectable sulfhydryl may act in binding substrate or merely act to maintain the sensitive conformation of the active site and modification of this latter group in the absence of substrate only acts to decrease the efficiency of catalysis but not to prohibit it.

Another possibility is based on a proposed mechanism that the catalytic function of FAH comes from an attack initiated by water (Scheme 2), or a buried serine residue (same as Scheme 1 with NuH = OH). In this case the substrate protectable sulfhydryl group located in the active site acts to maintain functional structure or to stablize the binding to the substrate, and if this sulfhydryl group is modified, it leads to decrease the activity of the enzyme. In this case the third sulfhydryl group would be considered to be buried in a hydrophobic region to maintain the protein complex. Symes and Soukes (1975), for example, emphasized that most of the essential sulfhydryl groups of beef and rat liver enzymes (monoamine oxidase etc.) may be more involved in maintaining

sheme 2.



B: base

M: metal

X, Y: binding group with metal

the stable conformation instead of being directly involved in catalysis.

Another explanation may be that the third sulfhydryl group is chelating the zinc as a mercaptide bridge between the two monomers (Ewing 1977) by analogy with the case of yeast alcohol dehydrogenase where it is thought that zinc atoms stabilize the quaternary structure through formation of mercaptide bridges between the monomers to form the active tetramer (Kägi & Vallee 1960).

However, it is not possible to distinguish among these postulates from the present experimental results. Further analysis of the kinetics of the modified enzyme may give more information about this essential sulfhydryl group. More importantly, a number of direct methods of identifying a sulfhydryl group in the active site should be employed. For instance, identification of the sulfhydryl group in the active site of acetoacetyl CoA thiolase (E.C.2.3.1.9) was achieved by incubation of the enzyme with excess labeled substrate $\left(^{14}C\right)$ -acetyl-CoA. Then the modified peptide was isolated from its tryptic hydrolysate and its reactive sulfhydryl group identified (Gehring and Harris 1968). Pinkus and Meister (1972) have used a analog of glutamine, L-2-amino-4-oxo-5 chloro $\left(5-^{14}C\right)$ pentanoic acid for identifying a nucleophilic group at the active site of carbamoyl phosphate

synthetase. They found that this analog occupies the site of glutamine and forms a covalent bound with a sulfhydryl group. Peptide mapping, radioautography and electrophoresis techniques along with sequence studies permit determination of the location of modified residues as suggested by Anderson and Perham (1969). Furthermore, X-ray crystallography is the only method that can presently yield the relative positions of all, or nearly all, the atoms in an enzyme with the aid of additional information such as amino acid sequence of the enzyme. For instance, in horse liver alcohol dehydrogenase a zinc ion is liganded in a distorted tetrahedral arrangement by four sulfur atoms from cysteinyl residues 97, 100, 103, and 111 as determined by X-ray diffraction and amino acid sequence studies (Brändén et al. 1975).

CHAPTER IV

SUMMARY

Quantitative sulfhydryl group titration of the native enzyme indicates that two sulfhydryl groups are accessible for reaction with either DTNB or PMB. One of these two sulfhydryl groups is protectable by substrate. With additional information of kinetic studies from Nagainis (1975), one might postulate that protectable sulfhydryl group is probably at or near the active site. The addition of 8 M urea increased the number of accessible sulfhydryl groups to three.

Incubation of the enzyme with DTE and denaturant showed five sulfhydryl group residues to be present. This value approaches the number of half cystine residues determined following amino acid analysis (Mahuran 1975). This indicates only one disulfide linkage is buried in the interior of the protein sturcture. Presumably, this lone disulfide linkage plays a major role in stabilizing of protein conformation.

10 mM EDTA does not act to enable detection of more sulfhydryl group in this case.

Native FAH still has residual activity even after those two sulfhydryl groups are modified with DTNB

or PMB. These results are consistent with the inaccessible sulfhydryl group's involvement in enzyme catalysis as a nucleophile towards substrate. Alternatively, nucleophile attacks on the substrate by an enzyme-assisted water molecule or by an inaccessible serine residue hydroxyl group.

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