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Purification and kinetic mechanism of 5,10-metliyienetetra-hydrofolate reductase from sheep liver

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Abstract 5,10-Methylenetetrahydrofolate reductase (EC 1.1.1.68) was purified from the cytosolic fraction of sheep liver by $(NH_4)_2\ SO_4$ fractionation, acid precipitation, DEAE-Sephacel chromatography and Blue Sepharose affinity chromatography. The homogeneity of the enzyme was established by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, ultracentrifugation and Ouchterlony immunodiffusion test. The enzyme was a dimer of molecular weight 1,66,000 ± 5,000 with a subunit molecular weight of 87,000 ±5,000. The enzyme showed hyperbolic saturation pattern with 5-methyltetrahydrofolate. $K_{0.5}$ values for 5-methyltetrahydrofolate menadione and NADPH were determined to be 132 μM, 2.45 μM and 16 μM. The parallel set of lines in the Lineweaver-Burk plot, when either NADPH or menadione was varied at different fixed concentrations of the other substrate; non-competitive inhibition, when NADPH was varied at different fixed concentrations of NADP; competitive inhibition, when menadione was varied at different fixed concentrations of NADP and the absence of inhibition by NADP at saturating concentration of menadione, clearly established that the kinetic mechanism of the reaction catalyzed by this enzyme was ping-pong.

Keywords. 5,10-Methylenetetrahydrofolate reductase; purification; ping-pong mechanism.

Introduction

5,10-Methylenetetrahydrofolate reductase (EC 1.1 1.68), a flavoprotein, catalyses the conversion of 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4 folate) to 5-methyltetrahydrofolate (5-CH_3-H_4 folate), the major storage form of folic acid in bacteria, animals, and plants (Blakley, 1969). It is also the transport form in mammalian systems. The reaction catalysed by this enzyme is shown in equation (1).

$$5,10\text{–}CH_2\ \text{–}H_4\,\text{folate} + \text{NADPH} + H^+ \rightleftharpoons 5\text{–}CH_3H_4\,\text{folate} + \text{NADP}^+ \qquad (1)$$

In addition, 5-CH_3-H_4 folate transfers its methyl group to homocysteine to form methionine which is a precursor of S-adenosyl-methionine (SAM). SAM transfers its methyl groups to a variety of acceptors. 'The reaction catalysed by the reducetase can thus be considered as a first step in the branched pathway for the biosynthesis of several important end products containing C_1, units derived from folate metabolism. 5,-10-CH_2-H_4 folate occupies a central position in the pathway for the interconversion of folate coenzymes. It acts as a starting point for the biosyn-

Abbreviations used: H_2 folate,Dihydrofolate; H_4 folate, tetrahydrofolate; 5-CH_3-H_4 folate, 5-methyl tetrahydrofolate; 5,10-CH_2-H_4 folate, 5,10-methylenetetrahydrofolate; 5,10-CH^+-H_4 folate, 5,10-methenyltetrahydrofolate; SDS, sodium dodecyl sulphate; SAM, S-adenosylmethionine; DEAE, diethylaminoethyl.

thesis of thymidilate, purines and formylmethionine-tRNA. The reductase, thymidilate synthetase (EC 2.1.1.45) and 5,10-CH_2-H_4 folate dehydrogenase (EC 1.5.1.5) compete for this common intermediate and it is to be expected that mechanisms must be present to channel this intermediate among the competing pathways.

It was suggested that SAM and S-adenosyl homocysteine regulated the activity of this enzyme (Kutzbach and Stockstad, 1971; Daubner and Matthews, 1981). It is also possible that this enzyme activity can be regulated by protein protein interaction with the other enzymes of the pathway namely thymidilate synthetase, 5,10-CH_2-H_4 folate dehydrogenase, H_2 folate reductase (EC 1.5.1.3) and serine hydroxymethyltransferase (EC 2.1.2.1). These enzymes are present as protein complexes and also as multifunctional proteins (Paukert *et al.,* 1976; Caperelli *et al*.,1980).I n order to meaningfully understand the regulation of the pathway, it was necessary to isolate all these enzymes from a single source and study their protein-protein and protein-ligand interactions. The dehydrogenase had been isolated from sheep liver (Paukert *et al.,* 1976) and serine hydroxymethyltransferase was recently isolated from the same source, without loss of its regulatory properties (Manohar *et al.,* 1982). In this paper we describe a simple method for the isolation of 5,10-CH_2-H_4 folate reductase from sheep liver and some of its physicochemical and kinetic properties.

Materials and methods

Chemicals

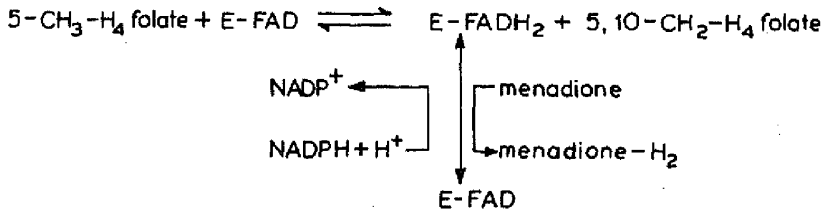
All the chemicals used were of Analytical grade or purchased from Sigma Chemical Co., St. Louis, Missouri, USA, except Blue Sepharose CL6B and DEAE-Sephacel, which were from Pharmacia Fine Chemicals, Uppsala, Sweden. Freund's complete adjuvant and agar were from Difco Laboratories, Detroit, Michigan, USA; H_4 folate was prepared by the method of Hatefi *et al.* (1959). [^{14}C]-5-CH_3-H_4 folate was from Amersham International Plc, Amersham, UK.

Enzyme assay

The enzyme activity was assayed by three different procedures.

Assay 1: The incubation mixture contained in a final volume of 0.1 ml: 20 µmol of ascorbate, 0.1 µmol FAD, 50 nmol [^{14}C]-5-CH_3-H_4folate (1000 cpm/nmol), 20 µmol potassium phosphate buffer (pH 7.2), 160 nmol EDTA, approximately 70 nmol menadione and an aliquot of the enzyme. The reaction mixture was preincubated at 37° C for 5 min and [^{14}C]-5-CH_3-H_4 folate was added with mild agitation to start the reaction. Assay tubes were incubated for 10 min and the reaction was stopped by the addition of 60 µl of freshly prepared dimedone reagent (3 mg/ml dimedone in 1 M acetate buffer, pH 4.5) and heating in a boiling water bath for 5 min. After cooling the assay tubes in ice, the [^{14}C]-labelled formaldehydedimedone adduct was extracted into toluene and the radioactivity measured in a LKB Rack-Beta-Spectrometer. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the formation of 1 µmol of formaldehyde per min. This assay depends on the reoxidation of reduced enzyme generated by

the interaction with 5-CH_3-H_4 folate by the externally added electron acceptor, menadione according to the following scheme (equation 2).



(2)

This procedure is essentially that described by Donaldson and Keresztesy (1962).

Assay 2: This assay was carried out at 25° C under an atmosphere of nitrogen according to the method of Kutzbach and Stokstad (1971) . The assay mixture contained in a total volume of 1 ml: 50 µmol potassium phosphate buffer, pH 7.2; 0.002 µmol FAD; 0.15 µmol NADPH; 10 µmol 2-mercaptoethanol; 1 µmol 5,10-CH_2-H_4 folate and enzyme. The reaction mixture was bubbled with nitrogen, the cuvettes covered with parafilm and the reaction was started after a preincubation period of 5 min, by the addition of either folate substrate, or NADPH with Hamilton gas tight syringe. The initial rates were recorded at 366 nm in Cary 219 recording spectrophotometer.

The activity was expressed as µmol of NADPH oxidized per min. This assay is represented in equation 2.

Assay 3: This assay represents the diaphorase activity of the enzyme. The assay mixture contained in a total volume of 1 ml: 50 µmol potassium phosphate buffer, pH7.2; 0.002 nmol FAD; 0.15 µmol NADPH and 120 nmol menadione to initiate the reaction. The absorbance change with time was recorded at 343 nm in a Cary 219 recording spectrophotometer.

Electrophoresis

Sodium dodecyl sulphate (SDS)-Polyacrylamide gel electrophoresis was carried out at room temperature (23 ±3° C) in 7.5% gels in Tris-glycine buffer, pH 8.3 containing 0.1% SDS according to the method of Laemmli (1970). The marker proteins used to determine subunit molecular weight were bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000) and β-lactoglobulin (18,400). The numbers in paranthesis indicate molecular weights (M_r)

Determination of molecular weight by gel filtration

The molecular weight of the native enzyme was determined by the gel filtration (Andrews, 1965) using a Sepharose-4B column (1×60 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.2, $S_{20,w}$ value was determined in a Spinco Model E Analytical Ultracentrifuge using a single sector cell equipped with Schlieren optics and the centrifugation was done at 180,000 *g* at 20° C. The $S_{20,w}$ was calculated according to the method of Schachman (1957).

Immunological techniques

Antiserum to the purified enzyme was raised in a male rabbit weighing approximately 2.5 Kg obtained from the Central Animal Facility of the Institute. The enzyme (500 µg) in Freund's complete adjuvant was injected intradermally at weekly intervals. After 4 weeks, a booster dose of 1 mg of enzyme was directly administered into the thigh muscle. After 9 days, the rabbits were bled through the marginal ear vein and the serum obtained was tested for the presence of antibody. After administering a second booster dose of the enzyme, the animal was bled 9 days later and the serum separated from the blood clot was used as the antiserum after partial purification by $(NH_4)_2SO_4$ precipitation and passage through diethylaminoethyl (DEAE)-cellulose chromatography.

Ouchterlony double diffusion analysis was carried out in an agar plate (Ouchterlony, 1958). Antiserum (100 µl) was placed in the central well and 50 µg of enzyme was placed in the surrounding two wells.

Results

Isolation and purification of the5,10-CH_2-H_4folate reductase from sheep liver

Potassium phosphate buffer, 0.05 M, pH 7.2 containing 0.3 mM EDTA (designated as Buffer A) was used extensively in the study and all the steps of purification were carried out at 0 5° C.

Fresh liver obtained from healthy sheep immediately after slaughter was chilled in ice and transported quickly from the local abattoir to the laboratory. The liver (125 g) was trimmed of excess fat, connective tissue and gall bladder. The liver was cut into slices and homogenized in 400 ml of Buffer A using a Waring blendor at high speed for 1.5 to 2 min. The pH of the homogenate was adjusted to 6.0 by adding 4 M acetic acid and centrifuged at 27,000 *g* for 10 min. To the supernatant, solid ammonium sulphate was added to 25% saturation, stirred for 10 min and centrifuged at 27,000 *g* for 10 min. The supernatant fraction was raised to 50% saturation by a further addition of solid ammonium sulphate. The precipitate obtained after centrifugation at 27,000 *g* was suspended in cold distilled water in a Potter-Elvehjem homogenizer. The pH of this suspension was adjusted to 4.5 using 4 M acetic acid. The precipitate was dissolved in Buffer A and the pH adjusted to 7.2 with 1 MNH_4OH and was left overnight. After centrifugation, the supernatant was designated as "acid precipitate" (table 1). This fraction was diluted with an equal volume of Buffer A and loaded at a flow rate of 30 ml/h on to a DEAE-Sephacel column (2.5×40 cm) equilibrated with Buffer A containing 10% glycerol. The column was washed with 4 bed volumes of Buffer A containing 0.1 M KCl and 10% glycerol until the absorbance of the eluate at 280 nm was less than 0.05. The column was washed further with one bed volume of Buffer A containing 0.3 M KCl and 10% glycerol and the enzyme was eluted with 2 bed volumes of 0.6 M KCl in Buffer A. Active fractions were pooled, diluted to twice the original volume and loaded at a flow rate of 15 ml/h on to a Blue Sepharose column (2.5 × 10 cm), equilibrated with Buffer A containing 0.3.M KCl and 10% glycerol. The column was washed with 2–3 bed volumes of equilibrating buffer and the enzyme was eluted from the column with 2 bed volumes of 2 M KCl in Buffer A containing 10% glycerol. Active fractions were pooled and concentrated

in an Amican ultrafiltration cell using XM-50 membrane. Results of a typical purification procedure are summarised in table 1.

Table 1. Summary of purification procedure for 5,10-CH2-H4 folate reductase from sheep liver.

Step	Protein (mg)	Activity units*	Specific activity $\times 10^3$	Fold purification	Recovery
$(NH_4)_2SO_4$ fractionation	3,000	39.0	13	1	100
Acid precipitation	834	38.2	45	4	98
DEAE-Sephacel chromatography	53	8.8	166	13	22
Blue Sepharose affinity chromatography	6	7.8	1305	100	20

* µmol HCHO formed/min at 37° C.

The procedure yielded 6 mg of the enzyme with a specific activity of 1–1.3 units/mg protein. From the $(NH_4)_2SO_4$ fractionation step, the fold purification of the enzyme was 100 and the recovery was 15–20%. The activity could not be detected in the crude extract possibly due to the presence of the enzyme in low amounts or the occurrence of an endogenous inhibitor. The enzyme in the acid precipitation steps yielded considerable degree of purification. The enzyme was strongly bound to Blue Sepharose, suggesting the presence of nucleotide binding site (Stellwagen, 1977). Dihydrofolate reductase (Subramanian and Kaufman, 1980) and serine hydroxymethyltransferase (Ramesh and Appaji Rao, 1980; Manohar *et al.,* 1982) also contain binding sites for this dye at or near their active sites. These observations suggest similarities in the topology of the active sites of enzymes of folate metabolism and is not surprising in view of the structural similarities between this triazine portion of the dye and the pteridine moiety of folic acid.

Stability of the enzyme

The enzyme prepared without glycerol was very unstable and lost all the activity within 5–6 h. However, inclusion of 10% glycerol in all buffers used during and after acid precipitation step stabilised the enzyme for 2 weeks when stored at –40°C.

Criteria of homogeneity

(1) SDS-Polyacrylamide gel electrophoresis of the enzyme preparation (50 µg) gave a single band on 7.5% gel (figure 1A); (2) Upon ultracentrifugation, the enzyme sedimented as a single sharp symmetrical peak. The sedimentation coefficient value ($S_{20,W}$) was calculated to be 11; (3) Ouchterlony immunodiffusion test gave a single precipitin line and the ends of the precipitin lines joined without spur formation (figure 1B); (4) Enzyme was eluted as a single symmetrical peak on gel filtration in a calibrated Sepharose-4B column (figure not given).

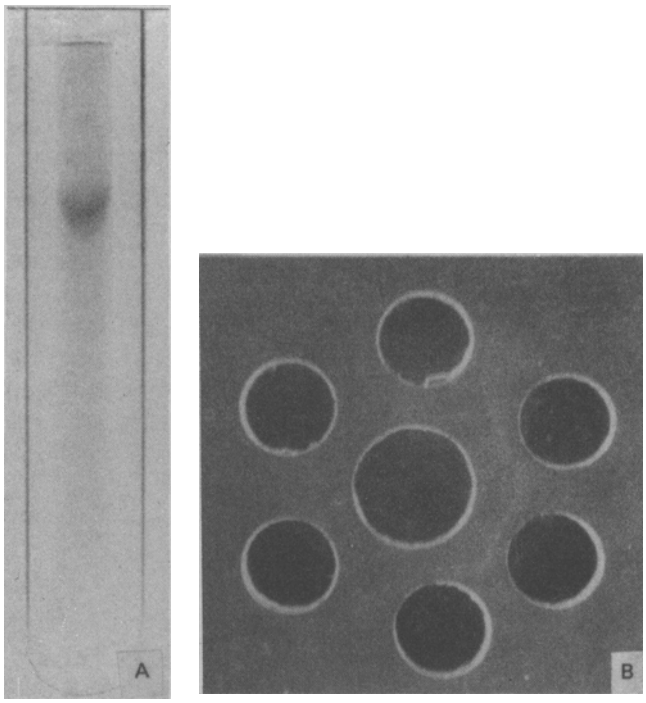


Figure 1. A. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the purified enzyme in 7.5% gel. The enzyme (250 µg) was treated with sodium dodecyl sulphate (2%) and heated for 3 min at 100°C. An aliquot corresponding to 40 µg protein was subjected to electrophoresis at a current of 4 mA/tube. The gel was stained with Coomassie Brilliant Blue R and destained with a mixture of methanol and acetic acid and water. **B.** Ouchterlony immunodiffusion analysis of sheep liver 5,10-CH_2-H_4 folate reductase. Antiserum was placed in the central well and 50 µg of the enzyme was placed in the surrounding two wells.

Determination of molecular weight and subunit composition of the enzyme

The apparent M_r of the native enzyme estimated by gel filtration using a calibrated Sepharose-4B column was 1,66,000 ± 5,000 (figure 2). Subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis to be 87,000 ±5,000 (figure 2 inset) suggesting that the enzyme was probably a dimer.

Catalytic properties of the enzyme

The specific activity of the enzyme isolated by the above procedure was 1.2 units/mg protein (one unit activity is defined as the amount of enzyme required to form 1 µmol formaldehyde per mg protein per min). Assuming the existence of two identical sites, the turnover number per catalytic site was calculated to be 208 min^{-1}. When the enzyme was preincubated with menadione and the reaction was started with 5-CH_3-H_4 folate, activity increased with increasing concentration

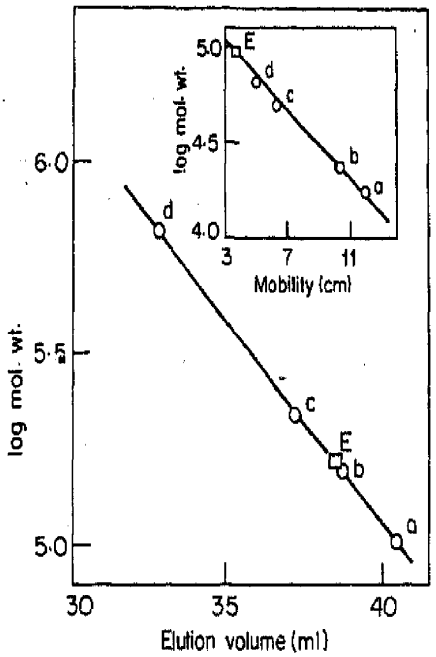


Figure 2. Determination of molecular weight and subunit composition of sheep liver 5,10-CH_2 -H_4 folate reductase.

For the determination of molecular weight, the enzyme (E) was passed through a Sepharose-4B column. The standard protein markers used were (a) hexokinase; (b) glucose oxidase; (c) catalase; and (d) thyroglobulin. The elution volumes were plotted as a function of log molecular weight. The molecular weight of 5,10-CH_2-H_4 folate reductase was determined to be 1,66,000 ±5,000. Inset represents sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis in 7.5% gel. The enzyme (250 µg) and the marker proteins (250 pg) were separately subjected to denaturation by sodium dodecyl sulphate as described in figure 1A. The electrophoresis, staining and destaining were done as described in figure 1A. The mobilities of (a) β-lactoglobulin; (b) trypsinogen; (c) ovalbumin; (d) bovine serum albumin were plotted as a function of log molecular weight. The subunit molecular weight of the enzyme (E) was determined by extrapolation to be 87,000 ±5,000.

of enzyme upto 7 µg/ml (figure 3) . The time course of this reaction showed an initial burst when it was started by the addition of menadione after preincubating the enzyme with 5-CH_3-H_4 folate for 5 min. However, the reaction rate was linear upto 10 min when the reaction was started with 5-CH_3-H_4 folate after preincubation with menadione (figure 4A, B). When the temperature of the reaction was varied from 20° C to 90° C, an optimum temperature of 37° C was observed with a complete loss of activity beyond 50° C. The maximum activity was observed at pH 6.5, which is in good agreement with the pH optimum for the enzyme reported from other sources (Kutzbach and Stokstad, 1971). The enzyme exhibited three activities: (a) 5-CH_3-H_4 folate—menadione oxidoreductase (equation 2); (b) NADPH menadione oxidoreductase; (c) NADPH–5,10 CH_2-H_4 folate oxido-reductase activities (equation 2). Menadione was a better electron acceptor than dichlorophenol indophenol and cytochrome-C (table 2).

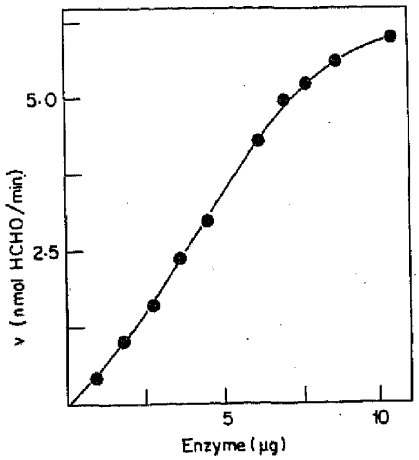


Figure 3. Enzyme concentration curve. Different concentrations of enzyme were preincubated for 5 min at 37° C with menadione (1 mM), and other assay components and the reaction was started with 5-CH_3-H_4 folate (0.5 mM). The activity was measured as described in assay 1.

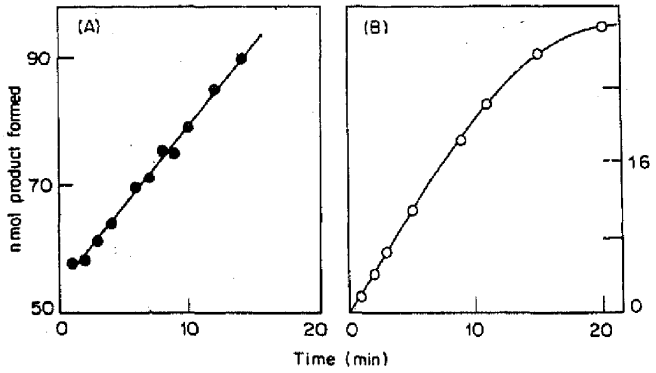


Figure 4. Time course of the reaction catalysed by 5-10-CH_2-H_4 folate reductase monitored using assay 1. **A.** Enzyme was preincubated with 5-CH_3-H_4 folate and other assay components for 5 min at 37° C. The reaction was started with menadione and was stopped at various time intervals .(●) **B.** Enzyme was preincubated with menadione and other assay components for 5 min at 37° C. The reaction was started with 5-CH_3-H_4 folate and was stopped at different time intervals (O).

Table 2. Relative electron acceptor activities in the radioactive assay of 5,10-CH_2-H_4 folate reductase.

Electron acceptor	Activity (nmol)
Menadione	2.8
O_2	2.1
Dichlorophenol indophenol	1.0
Cytochrome-C	1.6

The enzyme showed a hyperbolic saturation pattern for the 5-CH_3-H_4folate-menadione oxidoreductase activity when initial velocity was plotted against varying concentrations (0.05–0.5 mM) of 5-CH_3-H_4folate (figure 5). Lineweaver-Burk plot of this saturation data was linear (figure 5 inset). $K_{0.5}$ value of 0.13 mM

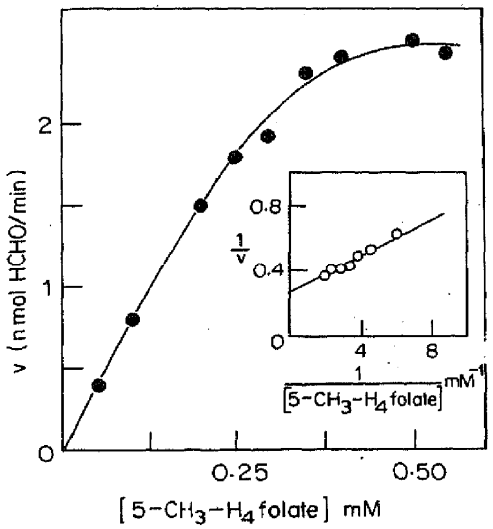


Figure 5. The 5-CH_3-H_4 folate saturation pattern of the enzyme in assay 1. Enzyme was preincubated with menadione and other assay components for 5 min at 37° C and the reaction was started with various concentrations of 5-CH_3-H_4 folate (0.05-05 mM) as a mixture of cold and hot 5-CH_3-H_4folate. The inset shows the Lineweater-Burk plot.

was calculated from the Hill plot. When menadione (5–200 μM) and NADPH (10-150 μM) were varied at saturating concentrations of the other substrate in the NADPH-menadione reductase assay, a hyperbolic saturation pattern was obtained (figure 6A, B). The $K_{0.5}$ values for menadione and NADPH were calculated to be 2.45 μM and 16 μM, respectively from the Hill plots.

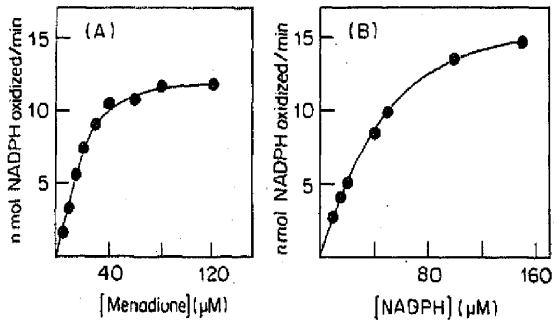


Figure 6.A. Saturation pattern for menadione in menadione reductase assay. After preincubating the enzyme with 150 μM NADPH at room temperature, the reaction was started with varying concentrations of menadione (5–200 μM). **B.** Saturation pattern for NADPH in menadione reductase assay. After preincubating the enzyme with varying concentrations of NADPH (10–150 μM), the reaction was started with saturating concentration of menadione (125 μM).

Kinetic mechanisms

To establish the kinetic, mechanism, NADPH-menadione reductase assay was used. Evidence for a ping-pong mechanism was obtained from initial velocity and product inhibition studies. Double reciprocal plots of the initial velocities when menadione concentration was varied at different fixed concentrations of NADPH (16,50 and 150 μM) gave parallel set of lines as shown in figure 7A. Similar pattern was observed when NADPH was varied at different fixed levels of menadione (60, 120 μM) (figure 7B). The parallel set of lines obtained in both the cases suggest that a ping-pong mechanism may be operating. This was further supported by three experiments using product as an inhibitor. In the first experiment, the

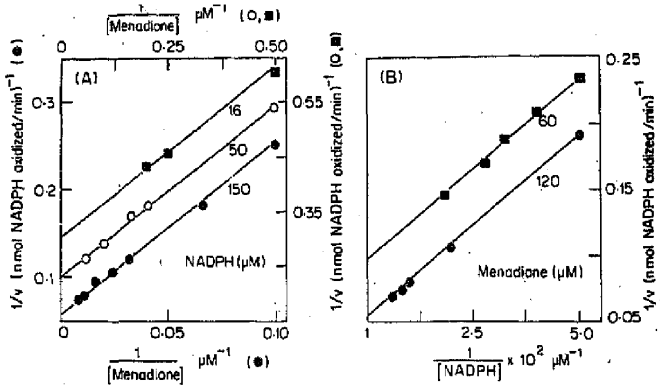


Figure 7. A. Double reciprocal plots for menadione saturation at different fixed concentrations of NADPH. Menadione saturation patterns were done at different concentrations of NADPH i.e., 16 μM (■), 50 μM (O), 150 μM (●). **B.** Double reciprocal plots for NADPH saturation at different fixed levels of menadione. NADPH saturations were done at two different concentrations of menadione i.e, 60 μM(■)120 μM (●).

NADPH concentration was varied (8–40 μM) at different fixed concentrations (4, 6 and 8 mM) of the product NADP at unsaturating (30 μM) menadione concentration and the double reciprocal plots gave a series of intersecting lines indicating non-competitve type of inhibition (figure 8A). In the second experiment, when

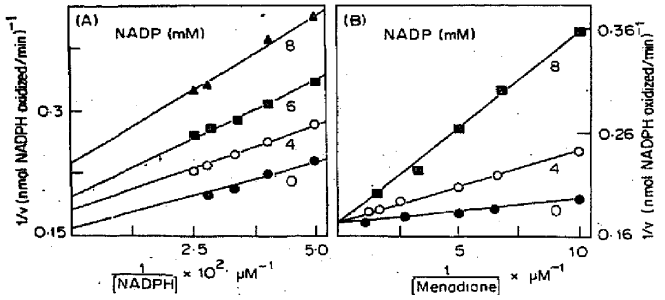


Figure 8 Product inhibition analysis by NADP. **A.** Non-competitive inhibition by NADP with NADPH. NADPH saturation patterns were done at different fixed concentrations of NADP, i.e, 0 mM (●),4 mM (O), 6 mM (■), 8 mM (▲), and at 30 μM menadione. **B.** Competitive inhibition by NADP with respect to menadione. Menadione saturation patterns were done at 50 μM NADPH and at different concentrations of NADP i.e.,0 mM (●), 4 mM (O), 8 mM (■).

NADPH was varied at 10 mM NADP (the concentration at which 75% of inhibition was observed at unsaturating (60 μM) menadione concentration) and at a saturating (125 μM) menadione concentration, no inhibition was observed. In the third experiment, menadione was varied (10–120 μM) at different fixed concentrations (4,8 mM) of NADP and at unsaturating (50 μM) NADPH concentration. A set of lines intersecting on the ordinate was observed indicating competitive inhibition by NADP (figure 8B).

Discussion

It was earlier reported that SAM regulated the activity of 5,10-CH_2-H_4 folate reductase from pig liver (Kutzbach and Stokstad, 1971; Daubner and Matthews, 1981) . However, other mechanisms of regulation of this enzyme and its kinetic mechanism were not investigated. Based on the results presented in this paper, the following kinetic mechanism for the reaction catalyzed by 5,10-CH_2-H_4 folate reductase can be postulated (figure 9).

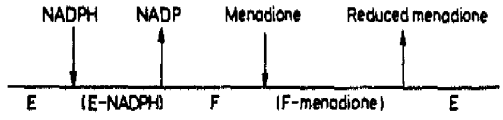


Figure 9. Ping-pong mechanism for the diaphorase activity catalyzed by the 5,10-CH_2-H_4 folate reductase.

The parallel set of lines obtained when menadione or NADPH concentrations were varied at different fixed concentrations of the second substrate suggested that a ping-pong mechanism could be operative for the reaction catalysed by this enzyme (figure 7 A, B). In product inhibition studies, when NADPH was varied at a fixed subsaturating concentration of menadione and at different fixed concentrations of NADP (figure 8A), a set of intersecting lines were obtained showing that NADP was a non-competitive inhibitor with respect to NADPH. Such a pattern could arise if NADPH and NADP are binding to two different forms of the enzyme (E and F, respectively) that are linked by reversible steps. In the second experiment, when NADPH was varied at saturating concentration of menadione and at 10 mM NADP, no inhibition was observed. The absence of inhibition is due to menadione and NADP binding to the same form of enzyme and enzyme is not available for NADP interaction at saturating menadione concentration. The competitive inhibition observed, when menadione was varied at subsaturating concentration of NADPH and at different fixed concentrations of NADP supports this observation. Using the rules enunciated by Cleland (1970), the substrate saturation and product inhibition pattern for the mechanism given in figure 9, were predicted and compared with the observed pattern (table 3). All these results are compatible with the ping-pong mechanism postulated in figure 9.

Table 3. Comparison of the observed initial velocity and product inhibition patterns for the reaction catalysed by 5,10-CH_2 -H_4 folate reductase.

Property	Predicted patterns			Observed patterns
	Ordered	Random	Ping-pong	
Initial velocity: Menodine varied. NADPH different fixed concentrations.	Intersecting lines	Intersecting lines	Parallel lines	Parallel lines
NADPH varied. Menadione different fixed concentrations	Intersecting lines	Intersecting lines	Parallel lines	Parallel lines
Product inhibition: NADPH varied. Menadione unsaturated	Non-competitive	Non-competitive	Non-competitive	Non-competitive
NADPH varied. Menadione saturated	Un-competitive	Non-competitive	No inhibition	No inhibition
Menadione varied. NADPH unsaturated.	Non-competitive	Non-competitive	Competitive	Competitive

The reaction shown above is a diaphorase assay to measure the activity of the enzyme and the physiological reaction is the pyridine nucleotide dependent reduction of 5,10-CH_2-H_4 folate. By analogy with diaphorase reaction the reducetion of 5,l0-CH_2-H_4 folate can be postulated to occur by the following kinetic mechanism (figure 10).

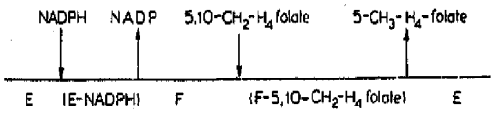


Figure 10. Kinetic mechanism for the reduction of 5,10-CH_2-H_4 folate by NADPH.

Evidence to suggest that 5-CH_3-H_4 folate reacts with the E form of enzyme was obtained by preincubating the enzyme with 5-CH_3-H_4 folate prior to starting the reaction by the addition of menadione in the radioactive assay. A burst in the reaction velocity was observed (figure 4A). The burst could be attributed to the reducetion of the enzyme by 5-CH_3-H_4 folate to form reduced enzyme and 5,10-CH_2-H_4 folate which dissociated to liberate formaldehyde. Further evidence to support this mechanism can be obtained by spectrally monitoring the reduction of this enzyme by NADPH and the 5-CH_3-H_4 folate and determining the product inhibition and the substrate saturation patterns of the physiological reaction.

The elucidation of the kinetic mechanism of the reaction catalysed by this enzyme and the availability of the enzyme in a purified form now enables a detailed study of the regulation of this enzyme activity by interaction with nucleotides and other enzymes of the pathway as well as the changes in its activity in neoplastic tissues.

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