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Purification, physicochemical and regulatory properties of serine hydroxymethyltransferase from sheep liver

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Abstract. Serine hydroxymethyltransferase (EC 2.1.2.1) was purified from the cytosolic fraction of sheep liver by ammonium sulphate fractionation, CM-Sephadex chromatography, gel filtration using Ultrogel ACA 34 and Blue Sepharose affinity chromatography. The homogeneity of the enzyme was rigorously established by Polyacrylamide gel and sodium dodecyl sulphate-polyacrylamide gel electrophoresis, isoelectrofocusing, ultracentrifugation, immunodiffusion and Immunoelectrophoresis. The enzyme was a homotetramer with a molecular weight of 210,000 ±5000. The enzyme showed homotropic cooperative interactions with tetrahydrofolate (n_H =2.8) and a hyperbolic saturation pattern with L-serine. At the lowest concentration of tetrahydrofolate used (0.2 mM), only 5% of the added folate was oxidized during preincubation and assay. The ^nH value was independent of the time of pre-incubation. Preincubation of the enzyme with serine resulted in a partial loss of the co-operative interactions (n_H =1.6) with tetrahydrofolate. The enzyme was regulated allosteri-cally by interaction with nicotinamide nucleotides; NADH was a positive effector while NAD+ was a negative allosteric effector. The subunit interactions were retained even at the temperature optimum of 60°C unlike in the case of the monkey liver enzyme, where these interactions were absent at higher temperatures. D-Cycloserine, a structural analogue of serine caused a sigmoid pattern of inhibition, in contrast with the observations on the monkey liver enzyme. Cibacron blue F3GA completely inhibited the enzyme and this inhibition could be reversed by tetrahydrofolate. Unlike in the monkey liver enzyme, NAD+ and NADH gave considerable protection against this inhibition. The sheep liver enzyme differs significantly in its kinetic and regulatory properties from the serine hydroxymethyltransferases isolated from other sources.

Keywords. Serine hydroxymethyltransferase; allostery; tetrahydrofolate.

Introduction

Although serine hydroxymethyltransferase (5,10-methylene-tetrahydrofolate-glycine hydroxymethyltransferase, EC 2.1.2.1) from bacterial (Mansouri *et al.,* 1972) and animal (Schirch, 1971; Nakano *et al.,* 1968; Fujioka, 1969; Jones and Priest, 1976; Ulevitch and Kallen, 1977a) sources has been studied extensively, it became known only recently that the enzyme from monkey liver (Ramesh and Appaji Rao, 1980a), isolated by a milder procedure, was a regulatory enzyme under the allosteric modulation of nicotinamide nucleotides (Ramesh and Appaji Rao, 1978). It was also observed that the partially purified enzyme from pig kidney

showed homotropic cooperative interactions with tetrahydrofolate and that this property was absent in the enzyme in cancerous tissues (Harish Kumar *et al.,* 1976). Since the enzyme occupies a key metabolic position in the supply of one-carbon fragments to the different coenzyme forms of folic acid, it could be predicted that this enzyme is regulatory in most organisms. Studies on the monkey liver enzyme also showed that Cibacron blue F3GA,which is used as an immobilized ligand in the purification of a large number of proteins containing the 'nucleotide fold', was a linear competitive inhibitor when tetrahydrofolate was the varied substrate, suggesting interaction at the tetrahydrofolate binding domain (Ramesh and Appaji Rao, 1980b). A recent study with dihydrofolate reductases isolated from chicken liver and *Lactobacillus casei* showed several interesting differences between the enzymes from these two sources in their mode of interaction with the dye (Subramanian and Kaufman, 1980). In view of the uniqueness of the initial discoveries with the monkey liver serine hydroxymethyltransferase (Ramesh and Appaji Rao, 1978. 1980a, b; Ramesh *et al.,* 1981a, b), it was of interest to isolate the enzyme from another mammalian source, such as sheep liver and examine its regulatory properties and its interaction with the dye Cibacron blue.

Materials and methods

Chemicals

All the chemicals used were of Analytical grade or purchased from Sigma Chemical Co., St. Louis, Missouri, USA, except CM-Sephadex C-50 and Blue Sepharose CL6B which were from Pharmacia Fine Chemicals, Uppsala, Sweden; Freund's complete adjuvant and agar from Difco Laboratories, Detroit, Michigan, USA; tetrahydrofolate was prepared by the method of Hatefi *et al.* (1959) and was also obtained as a kind gift from Dr. J. H. Mangum, Brigham Young University, Provo, Utah, USA; dichloromethotrexate and aminopterine were a kind gift from Dr. R. Silber, New York University Medical Centre, New York, USA; Ultrogel AcA-34 was a product of Industrie Biologique Franceise gifted by Mr. Henrik Perlmutter, LKB Products AB, Stockholm, Sweden; Cibacron blue F3GA was a kind gift from Ciba-Geigy, Basel, Switzerland and DL-[-^{14}C] serine (48.5 mCi/mmol) was from New England Nuclear, Boston, Massachusetts, USA.

Animals

Liver from healthy sheep was obtained from a local abattoir immediately after slaughter and chilled crushed ice.

Assay of serine hydroxymethyltransferase

The enzyme was assayed according to the method of Taylor and Weissbach (1965) with some modification. The usual components of the assay mixture (0.1 ml) were, 0.4 M potassium phosphate buffer, pH 7.4; 1 mM 2-mercaptoethanol; 1 mM EDTA; 0.1 mM pyridoxal 5'-phosphate; DL-[3-^{14}C] serine was diluted with 3·6 mM L-serine and 1.2×10^5 cpm were added; 1.8 mM tetrahydrofolate; 1.8 mM dithiothreitol and an appropriate amount of the enzyme. After preincubation for 5 min at 37^{o}C, the reaction was initiated by the addition of serine. After incubation for 15 mm at 37^{o}C the reaction was stopped with 0.1 ml of freshly prepared dimedon

(0.4 M in 50% (v/v) ethanol), the mixture heated for 5 min at 100°C and the [^{14}C]-formaldehyde-dimedon adduct extracted into toluene and the radioactivity measured in a Beckman LS-100C liquid scintillation spectrometer. One unit of enzyme activity was defined as the amount that catalyzed the formation of 1 µmol of formaldehyde/min. Specific activity was expressed as units/mg protein. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Tetrahydrofolate concentration was determined by spectral (Hatefi *et al.,* 1959); enzymatic (Ramesh *et al.,* 1978) and chemical methods. (Huennekens *et al.,* 1963).

Purification of sheep liver cytosolic serine hydroxymethyltransferase

The potassium phosphate buffer used in this study always contained 1 mM EDTA; 0.1 mM pyridoxal 5'-phosphate; and 1 mM 2-mercaptoethanol. Preliminary studies (data not given) showed that the enzyme was located in the cytosolic fraction of the sheep liver homogenate. The entire process of purification was carried out at 0-4°C.

Ammonium sulphate fractionation

Liver tissue (150 g) was cut into small pieces and homogenized in 400 ml of 0.05 M phosphate buffer in an industrial Waring Blendor. The homogenate was centrifuged at 27,000 *g* for 20 min. The supernatant crude extract was fractionated with ammonium sulphate at 25 to 50% saturation and the precipitate obtained was suspended in a minimal amount of 0.05 M phosphate buffer and dialyzed against the same buffer (about 400 volumes).

CM-Sephadex C-50 chromatography

The dialyzed ammonium sulphate fraction was applied onto a CM-Sephadex C-50 column (2.5×36 cm), equilibrated with 0.05 M phosphate buffer. After washing the column with buffer until the absorbance of the eluate decreased to less than 0.05; the enzyme, bound as a yellow zone at the top of the column was eluted with a linear gradient (0.05 M to 0.5 M) of potassium phosphate buffer at a flow rate of about 20 ml/h. Fractions (2 ml) of the eluate, having considerable activity (>0.7 units/mg) were pooled and concentrated to about 1 ml by precipitation with ammonium sulphate (60% saturation) followed by dialysis against 0.05 M phosphate buffer.

Molecular exclusion chromatography on Ultrogel AcA-34

The concentrated enzyme solution (approx. 1 ml) was passed through an ultrogel AcA-34 column (110×1.5 cm) equilibrated with 0.2 M phosphate buffer, at a flow rate of about 10 ml/h. The fractions (2 ml) having more than 50% of the activity of the peak fraction were pooled.

Affinity chromatography on Blue-Sepharose CL-6B

The enzyme was loaded on a Blue-Sepharose CL-6B column (1 ×20 cm), equlibrated with 0.02 M phosphate buffer. After washing the column with about 75 ml of the same buffer, the enzyme was eluted with buffer containing 1 M KCl at a flow rate of about 20 ml/h. The fractions (2 ml) containing the enzymatic activity

were pooled and dialyzed against 0.05 M phosphate buffer. This preparation at a concentration of 1-1.5 mg/ml was divided into small volumes and stored at -40°C. Freshly thawed enzyme was used in each experiment. The results of a typical purification are summarized in table 1.

Table 1. Purification of sheep liver cytosolic serine hydroxymethyltransferase.

Step	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)	Fold purification	Per cent recovery
Crude	21609	986	0.046	1	100
Ammonium sulphate fractionation	12294	777	0.063	1.4	79
CM-Sephadex chromatography	128	181	1.42	31	19
Ultrogel filtration	34	196	5.75	125	20
Blue Sepharose affinity chromatography	27	162	6.03	131	16

Weight of liver tissue=150 g.
* μmol HCHO formed/min at 37°C and at pH 7.4.

Electrophoresis

Analytical Polyacrylamide gel disc electrophoresis was done at 4°C in both 5% and 7.5% gels in 0.5 M Tris-0.39 M glycine buffer, pH 8.6, at a current of 4 mA per gel (Davis, 1964). Protein bands in the gels were stained with Coomassie Brilliant Blue G (0.02% in 3.5% perchloric acid).

Sodium dodecylsulphate Polyacrylamide gel electrophoresis was done in 7.5 and 12.5% gels according to the method of Weber and Osborn (1969), both in the presence and in the absence of 2-mercaptoethanol. The marker proteins used to determine subunit molecular weight were, undissociated immunoglobulin, IgG (147,600), conalbumin (86,000), the H chain of IgG (49,000), ovalbumin (43,000), the L chain of IgG (24,000) and α-chymotrypsin (24,000). Isoelectric focusing in 5% gel was by the method of O'Farrel (1975).

Determination of molecular weight by gel filtration

The molecular weight of the native enzyme was determined by the gel filtration method of Andrews (1965) using a Sephadex G-200 column (1.5×50 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.4 and operated at a flow rate of 15 ml/h. The enzyme was monitored by measuring the activity. The standard protein markers used were bovine serum albumin (68,000), ovalbumin (43,000, lysozyme (14,400), chymotrypsin (24,000), myoglobin (17,000) and bovine type I thyroglobulin (660,000).

Ultracentrifugation

The sedimentation velocity of the purified enzyme (8 mg/ml) in 0.05 M potassium phosphate buffer, pH 7.4, was determined in a Spinco Model E Analytical Ultracentrifuge, using a single sector cell and Schlieren optics. The operating speed was 39,460 rpm and the temperature, 20°C. The $S_{20,w}$ was calculated (Schachman, 1957).

Immunological techniques

Antiserum to the purified enzyme was raised in the rabbit. The enzyme (750 μg) in Freund's complete adjuvant was injected intradermally into a rabbit. Four weeks later, about 350 μg of the protein in Freund's incomplete adjuvant was injected intradermally. After a further four weeks, a booster dose of 150 μg of the enzyme was administered in saline, intravenously. The animal was bled from the marginal ear vein on the seventh and ninth days after the last injection. The serum was separated from the blood clot and used as the antiserum.

Ouchterlony double diffusion analysis was done (Ouchterlony, 1958) in an agar plate. Antiserum (100 μl) was placed in the central well and different concentrations of the enzyme (5 μg to 30 μg/100 μl) were placed in the surrounding wells.

Immunoelectrophoresis was done in 1.25% agar with 0.05 M potassium phosphate buffer, pH 7.4 in the electrode chambers (Williams and Graber, 1955).

Spectroscopic methods

The absorption spectrum of the enzyme (1.28 mg/ml) both in the ultraviolet (uv) and in the visible ranges were obtained with a Beckman Model 26 spectrophotometer. Difference spectra were taken against 0.05 M potassium phosphate buffer, pH 7.4 containing 0.1 mM pyridoxal 5'-phosphate; 1 mM 2-mercaptoethanol and 1 mM EDTA.

The fluorescence spectra of the enzyme (50 μg/ml) were obtained in a Hitachi Perkin-Elmer spectrophotometer after dialyzing out pyridoxal 5'-phosphate against 0.05 M potassium phosphate buffer, pH 7.4 containing 1 mM 2-mercaptoethanol and 1 mM EDTA. The same buffer was used as blank.

The circular dichroic (c.d.) spectrum of the native enzyme was obtained in a Jasco 20A spectropolarimeter after dialyzing out the pyridoxal 5′-phosphate as before. The protein concentration and path length were 0.885 mg/ml and 10 mm for the visible and near uv regions and 0.3 mg/ml and 1 mm for the far uv region, respectively. The helical content was calculated according to Greenfield and Fasman (1969).

Results

Purification of the enzymes

Table 1 summarizes the results of a typical purification procedure for the isolation of the sheep liver serine hydroxymethytransferase. The enzyme fraction obtained from the ultrogel column was only slightly contaminated and this contaminating

protein was eliminated by using the Blue Sepharose affinity matrix (figure la). The enzyme elutes from the Blue Sepharose column as a single symmetrical protein peak corresponding to the activity peak (data not given). With a starting material of 150 g of tissue, the method described above yields 22-28 mg of the enzyme of specific activity 6 ± 0.2 units/mg protein, purified about 130-fold, with a recovery of 15-20%. Stored at –40° C, the enzyme showed no appreciable loss of activity for over two months.

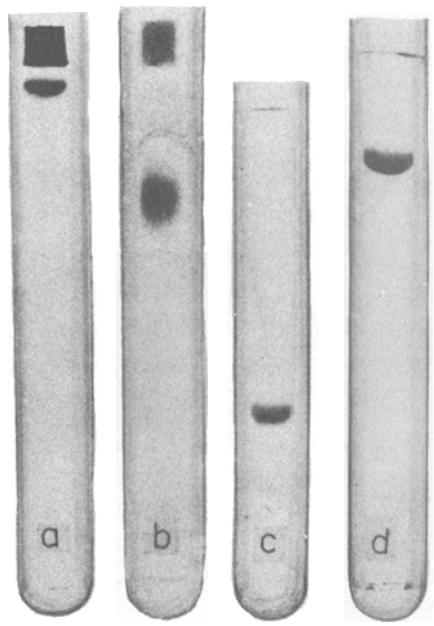

Figure 1. Analytical Polyacrylamide and SDS-polyacrylamide gel electrophoresis.

Figure la and 1b represent electrophoresis of the purified enzyme (50 µg) on 7.5% and 5% Polyacrylamide gels, respectively. Electrophoresis was done at 4°C in 0.5 M Tris—0.39 M glycine buffer, pH 8.6, at a current of 4 mA/gel. The protein bands were stained with Coomassie Brilliant BlueG. Figure lc and 1d represent sodium dodecyl sylphate-polyacrylamide gel electrophoresis of the purified enzyme in 7% and 12.5% gels respectively. The enzyme (200 ug) was treated with sodium dodecyl sulphate (2%) and heated for 3 min at 100°C. An aliquot corresponding to 30 µg protein was subjected to electrophoresis at a current of 4 mA/tube. The gels were stained with Coomassie Brilliant Blue R and destained with 10% acetic acid.

Criteria of homogenity

Upon electrophoresis in 7.5% Polyacrylamide gel, the enzyme preparation (30-50 µg) gave a single protein band of very low mobility (figure la); on a 5% gel the protein migrated more freely but gave a diffused band (figure lb). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of the enzyme also resulted in a single protein band in both 7% and 12.5% gels (figure lc and 1d).Isoelectric focusing of the enzyme preparation resulted in a single protein band corresponding to a pI of 4.2.

Upon ultracentrifugation, the enzyme sedimented as a single sharp symmetrical peak (figure 2a). The sedimentation coefficient value ($S_{20,w}$) was calculated to be 6.8.

A single precipitin line was obtained in the Ouchterlony immunodiffusion test at all the concentrations (5-30 µg/100 µl) of the enzyme. The ends of the precipitin lines joined to form an uniform hexagon (figure 2b). Immunoelectrophoresis of the enzyme also resulted in a single precipitin line (data not given).

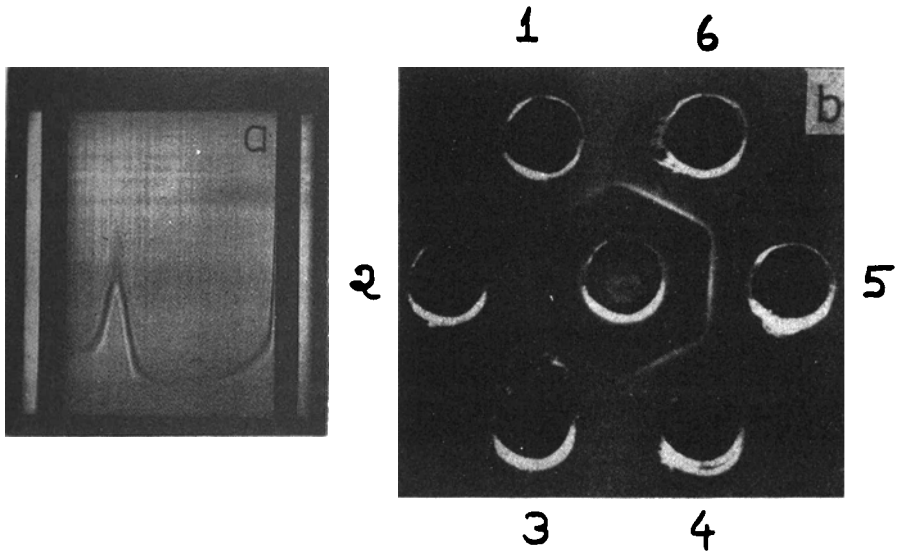

Figure 2a. Sedimentation pattern of sheep liver serine hydroxymethyltransferase.

The ultracentrifugation analysis was done with a purified preparation of the enzyme (8 mg/ml) in 0.05 M potassium phosphate buffer, pH 7.4, in a Spinco Model E Analytical Ultracentrifuge provided with Schleiren optics. The operating speed was 39,460 rpm and the temperature, 20°C. The photograph was taken 75 min after the attainment of this speed.

Figure 2b. Ouchterlony immunodiffusion analysis of sheep liver serine hydroxymethyltransferase.

Antiserum (100 µl) was placed in the central well and different concentrations of the purified enzvme (5, 10, 15, 20, 25 and 30 µg /100 µl) were placed in the surrounding wells (1 — 6).

Determination of molecular weight and subunit composition of the enzyme

The molecular weight of the native enzyme estimated by gel filtration using a calibrated Sephadex G-200 column was found to be 210,000 ±5000 (figure 3).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, both in the presence and in the absence of 2-mercaptoethanol, gave rise to a single protein band corresponding to a molecular weight of 55,000 ±2500 (figure 3).

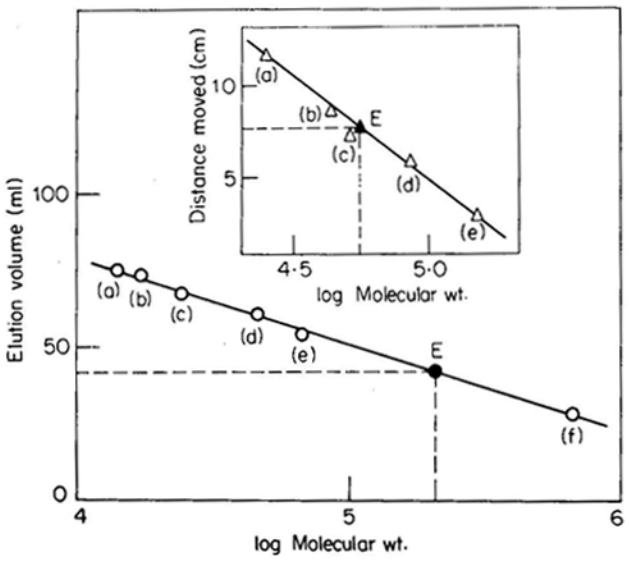

Figure 3. Determination of molecular weight and subunit composition of sheep liver serine hydroxymethyltransferase. For the determination of the molecular weight, the enzyme (E) was passed through a Sephadex G-200 column. The standard protein markers used were lysozyme, (a); myoglobin, (b); chymotrypsin, (c); ovalbumin, (d); bovine serum albumin, (e); and bovine type I thyroglobulin, (f). The elution volumes were plotted as a function of log molecular weight. The molecular weight of serine hydroxymethyltransferase was determined by extrapolation to be 210,000 ±5000. Inset represents sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis in 7.5% gel, The sheep liver enzyme (200 µg) and the marker proteins (200 µg) were separately subjected to denaturation by sodium dodecylsulphate as described above. Another sample of the enzyme and IgG were separately denatured in the presence of sodium dodecylsulphate and 2-mercaptoethanol (2%). The electrophoresis, staining and destaining were done as described in figure 1. The mobilities of α-chymotrypsin, (a); L-chain of IgG, (a); ovalbumin, (b); H-chain of IgG, (c); conalbumin, (d); and undissociated IgG, (e); were plotted as a function of log molecular weight. The subunit molecular weight of the enzyme (E) was determined by extrapolation to be 55,000 ±5000.

Catalytic properties of the enzyme

The specific activity of the enzyme isolated by the above procedure was 6 units/mg protein. Assuming the existence of four identical catalytic sites, the catalytic centre activity was calculated to be 315 min^{-1}. The enzymatic activity increased linearly with the concentration of the enzyme upto about 18 µg/ml reaction mixture. The time course of the enzymatic reaction was linear for over 30 min. The activity of the enzyme increased with temperature upto 60°C and decreased sharply between 60 and 70°C; the activation energy was calculated to be 9.64 Kcal/ mol.

Regulatory properties

The enzyme showed positive homotropic cooperative interaction with tetrahydro-folate, giving rise to a sigmoid saturation pattern when preincubated with different concentrations of H_4-folate (figure 4); a Hill coefficient (n_H) value of 2.8 and a $K_{0.5}$

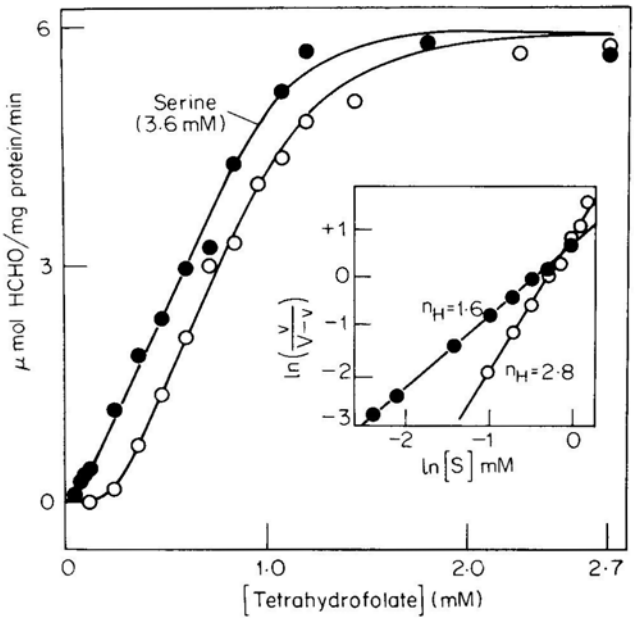

Figure 4. The tetrahydrofolate saturation pattern of the enzyme.

The enzyme (0.6 μg/l00 μl) was preincubated for 5 min at 37°C with various concentrations of H_4-folate (o) or L-serine (3.6 mM) (●) and the reaction was started by the addition of serine or tetrahydrofolate. The inset shows a Hill plot, from the slop of which n_H values of 2.8 and 1.6 for the enzyme preincubated with H_4-folate and serine, respectively were obtained.

value of 0.7 mM were calculated from the Hill plot (inset figure 4). The n_H value of 2.8 was seen at all temperatures between 37°C and 60°C. However, this sigmoidicity was reduced to n_H=1.6–1.8 when the enzyme was preincubated for 5 min with a saturating concentration (3.6 mM) of L-serine and the reaction started with varying concentrations of tetrahydrofolate (figure 4). The value did not reach 1.0 as expected for complete loss of homotropic cooperativity in the presence of a positive allosteric effector. This effect of serine was reversed upon dialyzing out the serine. The saturation pattern of L-serine was hyperbolic with a K_m value of 0.9 mM (figure not given). NAD^+ and NADH were negative and positive allosteric effectors with respect to tetrahydrofolate saturation (figures 5 and 6). NAD^+ (2 mM) increased the sigmoidicity to an n_H of 3.3 and at 10 mM the n_H value reached 3.8, very close to the theoretical maximum (n_H=4) for an enzyme with four subunits (inset figure 5). NADH decreased the cooperativity to n_H=2 at 5 mM (not shown in the figure) and to n_H l. 8 at 50 mM (inset figure 6).

In a recent communication Schirch and Quashnock (1981) have attributed the cooperativity observed by us earlier with the monkey liver and mung bean enzymes (Ramesh and Appaji Rao, 1978; 1980a, b; Rao and Appaji Rao, 1980) to the oxidation of tetrahydrofolate, during preincubation and assay, and have remarked that the sigmoidicity could be dependent on the time of preincubation of the enzyme with H_4-folate and the assay conditions. In order to resolve this problem, the oxidation of tetrahydrofolate was examined by spectral, chemical and

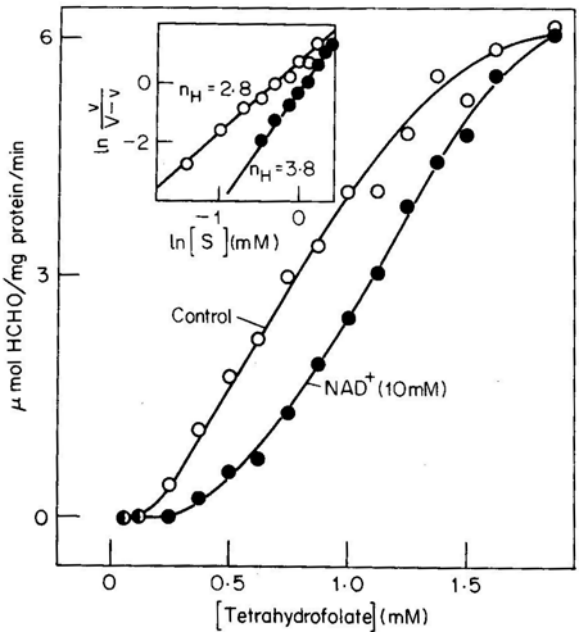

Figure 5. The effect of NAD^+ (10 mM) on the H_4 folate saturation pattern.

The enzyme (0.6 μg/100 μl) was preincubated with various concentrations of H_4-folate (O) and also with H_4-folate and NAD^+ (10 mM) (●) for 5 min at 37°C and the reaction was started by the addition of L-serine (3.6 mM). The inset shows a Hill plot, from the slope of which n_H values of 2.8 and 3.8 were obtained in the absence and presence, respectively of NAD^+

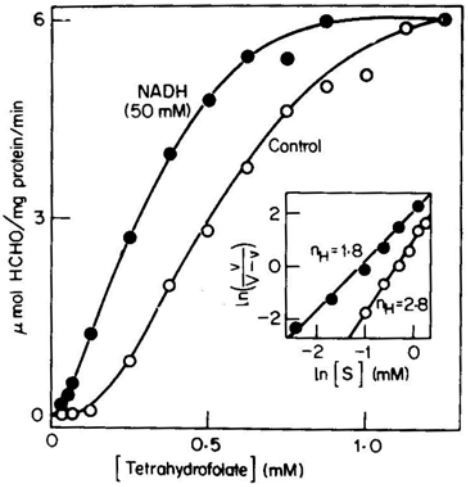

Figure 6. The effect of NADH (50 mM) on the H_4-folate saturation pattern.

The enzyme (0.6 μg/100 μl) was preincubated with various concentrations of H_4-folate (O) and also with H_4-folate and NADH (50 mM) (●) for 5 min at 37°C and the reaction was started by the addition of L-serine (3.6 mM). The inset shows a Hill plot, from the slope of which n_H values of 2.8 and 1.8 were obtained for the absence and presence, respectively of NADH.

enzymatic methods. The tetrahydrofolate prepared by us by the procedure of Hatefi *et al.* (1959) and the commercially available samples from Sigma Chemical Co. were incubated at 37°C in phosphate buffer (pH 7.4) containing 1.8 mM dithiothreitol for time periods of 0, 10, 20, 40 and 80 min. Even at the lowest concentration of H_4-folate used by us for the assay of serine hydroxymethyltransferase, only 5% oxidation was observed. The spectral change was minimal as recorded at the time intervals mentioned in a Gilford recording spectrophotometer. It can be seen from table 2 that very little oxidation seems to have occurred when H_4-folate was incubated at 37°C for periods of time ranging from 0 to 20 min and estimated by a chemical method (Huennekens *et al.,* 1963).

Table 2. Stability of H_4-folate during preincubation and assay of serine hydroxymethyltransferase.

Time (min)	Chemical assay (μmol N^5, N^{10}-methenyl-H_4-folate)	Enzymatic assay (μmol HCHO)
0	0.025(100)	0.025(100)
10	0.025(100)	0.024(96)
20	0.024(96)	0.023(92)
40	0.022(88)	0.021(84)

* Numbers in parentheses indicate per cent of control (0 min) value.

H_4-folate (0.25 mM) was incubated at 37°C and aliquots were drawn at 0, 10, 20, 40 and 80 min, and the amount of H_4-folate present was estimated by converting it completely to N^5, N^{10}-CH_2-H_4-folate by adding excess of enzyme (60 μg, compared to 0.6 μg normally used in the assay) and labelled serine. It can be seen from table 2 that very little H_4-folate was oxidized in 20 min, the time taken for preincubation and assay of the serine hydroxymethyltransferase.

The substrate saturation patterns at varying concentrations of H_4-folate and at a saturating concentration of serine (3.6 mM) were carried out by preincubating the enzyme with tetrahydrofolate and assaying the enzyme activity for 1, 5 and 15 min. The ^nH values determined from the Hill plots were 2.2, 2.8 and 2.8 respectively. The requirement for preincubation could be due to the slow conformational change induced by the binding of these ligands. However, this needs to be carefully examined.

Inhibition by folate and serine analogues

Table 3 gives the per cent inhibition of the activity of the enzyme at various concentrations of folate and serine analogues added to the standard reaction mixture. Dichloromethotrexate was a potent inhibitor of the enzyme activity, inhibiting it 87% at a concentration of 20 mM, whereas aminopterin only produced 35% inhibition at this concentration. Methotrexate was without effect at this

Table 3. Inhibition of sheep liver serine hydroxymethyltransferase by folate and serine analogues.

Inhibitor	Concentration (mM)			
	1	5	10	20
	PER CENT INHIBITION			
Folate	10	38	53	63
Dihydrofolate	7	24	35	45
Aminopterin	5	18	27	35
Dichloromethotrexate	35	59	74	87
O-Acetyl-L-serine	3	11	21	38
Phospho-L-serine	10	26	37	43
L-O-Methylserine	23	50	63	71

Concentration. Folate and dihydrofolate were not very strong inhibitors of the sheep liver serine hydroxymethyltransferse. The inhibition produced by serine analogues were in the following order of effectiveness: O-methylserine >phospho-serine >O-acetylserine. Figures 7a and 7b show double reciprocal plots of

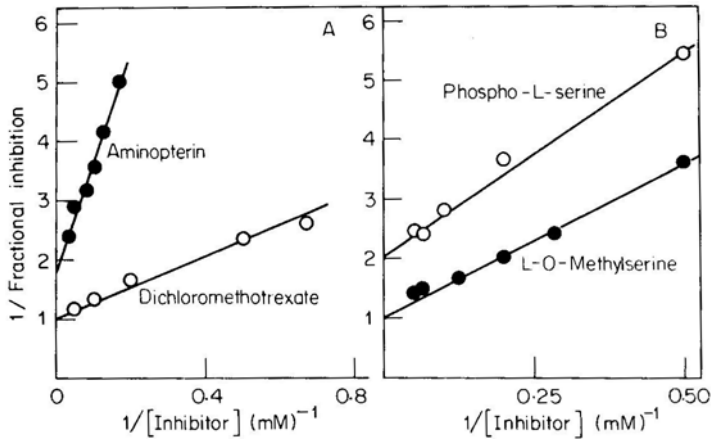

Figure 7. Partial inhibition analysis of the interaction of sheep liver serinehydroxymethyl-transferase with aminopterin (●), dichloromethotrexate (o) (figure 7a) and phospho-L-serine (o), L-o-methylserine (●) (figure 7b). The enzyme (0.6 µg/100 µl) was preincubated for 5 min at 37°C with H_4-folate (1.8 mM) and the inhibitor. The enzyme activity in the absence of inhibitor was normalized to 1. The theoretical basis for this analysis is as follows:

$$\frac{1}{i} = \frac{aK_i(K_m+[S])}{K_m(a-1)} \cdot \frac{1}{[I]} + a \cdot \frac{aK_m+[S]}{K_m(a-1)} \quad (1)$$

where i=1 –V_i/V, V_i being the velocity of the reaction in the presence of inhibitor and V being

the velocity in the absence of the inhibitor; α is the factor by which K_i and K_s are altered when EI and ES react with the substrate or inhibitor. The ordinate intercept in a plot of 1/i vs 1/[I] is given by:

$$\frac{\alpha K_m + [S]}{K_m(\alpha - 1)} \qquad \text{When } [I]=\infty,\ 1/i = [K_i(1 + \frac{[S]}{K_m})\ [1/[I]+1.]$$

Thus, for a complete competitive inhibitor the ordinate intercept is 1 in the above plot and >1 for a partial competitive inhibitor.

fractional inhibition versus the concentration of inhibitor. Folic acid and dichloromethotrexate were complete inhibitors while dihydrofolate and amino-pterin were partial inhibitors. Phospho L-serine was a partial inhibitor while DL-O-methylserine and O-acetyl L-serine were complete inhibitors. β-Phenylserine and threonine had no effect on the enzyme activity at these concentrations. Figure 8 shows the inhibition by D-cycloserine; the inhibition was sigmoid and the inhibition at 1.0,1.5 and 1.75 mM were significantly less (0,20 and 47% compared to 12, 43 and 65%) when the enzyme was preincubated with L-serine instead of with tetrahydrofolate (figure 8).

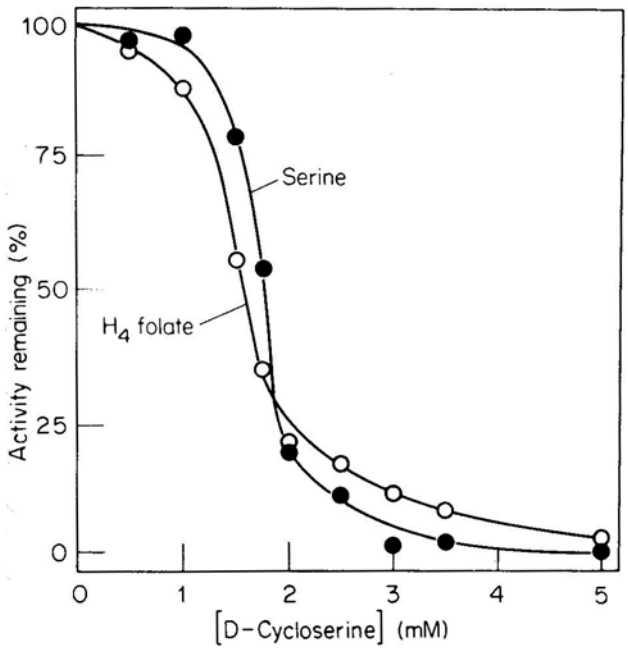

Figure 8. Inhibition of sheep liver serine hydroxymethyltransferase by D-cycloserine.

The enzyme (0.6 µg/100 µl) was preincubated for 5 min at 37°C with D-cycloserine and H_4-folate(1.8mM) (o) or L-serine (3.6 mM) (●) before initiating the reaction by the addition of the second substrate.

Inhibition by Cibacron Blue

Cibacron blue inhibited the enzyme activity completely (figure 9). NAD (30 mM) partially reversed the inhibition caused by Cibacron blue at all concentrations of the dye as shown in figure 9. Increasing concentrations of NAD^+ at a fixed

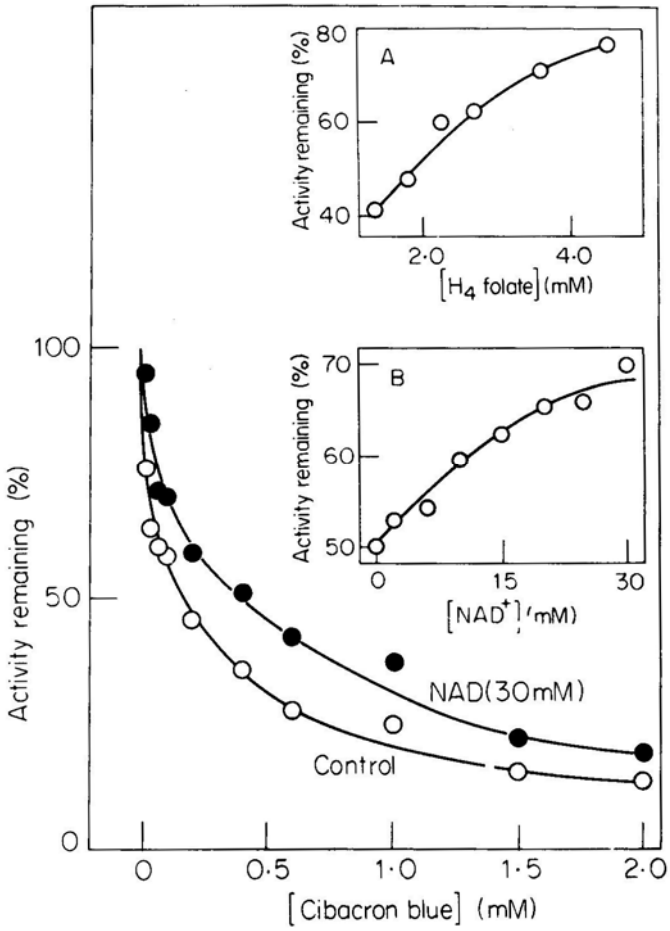

Figure 9. The inhibition pattern of sheep liver serinehydroxymethyltransferase by Cibacron blue.

The inhibition is measured in the absence (o) and in the presence (●) of NAD+. The enzyme (0.8 µg/100 µl) was preincubated for 5 min at 37°C with different concentrations of the dye and with H_4-folate (1.8 mM) alone or with H_4-folate (1.8 mM) and NAD^+ (30 mM) and the reaction started by the addition to L-serine (3.6 mM). Inset A shows a plot of the enzyme activity as per cent of control (activity in the absence of any inhibitor and at saturating concentrations of substrates 3.6 mM serine and 1.8 mM H_4-folate) vs varying concentration of H_4-folate (1.8 to 5.5mM) (o). Inset B shows a plot of the enzyme activity as per cent of control (in the presence of 0.2 mM Cibacron blue and at saturating concentrations of H_4-folate and L-serine) vs concentration of NAD^+ (0 to 30 mM). NAD^+ (30 mM) did not alter the activity of the control. The concentration of Cibacron blue was fixed at 0.2 mM in all these experiments and inhibited the enzyme activity to 40% of the control value.

concentration of Cibacron blue (0.2 mM) caused increasing reversal of inhibition (figure 9, inset B). Similarly, tetrahydrofolate could also overcome the inhibition by Cibacron blue (figure 9, inset A). Double reciprocal plots of the data showed that at infinite concentration of NAD^+, reversal of the inhibition to 80% of the uninhibited activity was obtained whereas with H_4-folate complete reversal of the inhibition was reached (figures not given).

Heat stability of the enzyme

It was earlier observed that heat inactivation in the presence of L-serine was a convenient method to purify the enzyme (Schirch, 1971) but it was shown by us that this procedure resulted in the desensitization of the monkey liver enzyme (Ramesh and Appaji Rao, l980a). It was, therefore, of interest to study the effect of heat on the enzyme in the presence of substrates. Figure 10 shows the heat inactivation

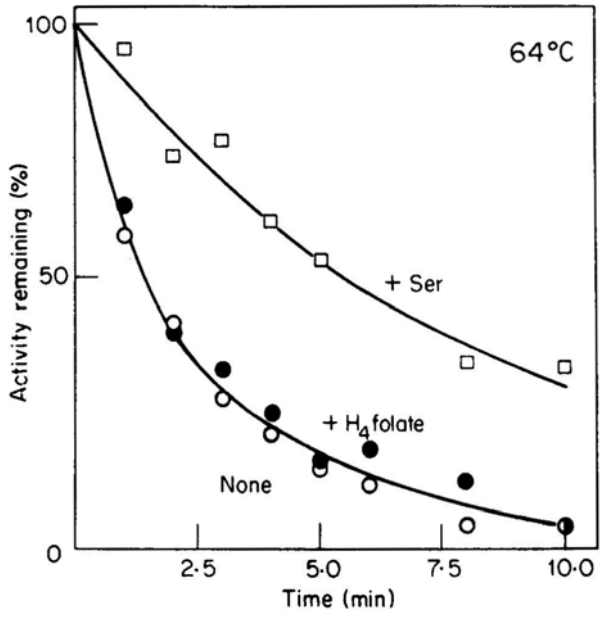

Figure 10. The thermal denaturation profiles of sheep liver serinehydroxymethytransferase. (a) In the presence of L-serine (3.6 mM) (□), (b) H4folate (3.6mM) (●) and (c) in the absence of any substrate (O). The enzyme was incubated (with or without substracte) at 64°C and a constant volume pipetted out at every time point into an assay tube which was immediately chilled in ice. At the end of 10 min the aliquots of the enzyme were assayed at 37°C at saturating concentrations of H_4-folate and L-serine Since H_4-folate was easily oxidized, 3.6 mM H_4-folate alone was taken in a separate experiment, kept at 64°C for 10 min and assayed enzymatically at 37°C in order to ensure that it did not decrease below the saturating concentration in the above experiment.

patterns at 64°C of the native enzyme, the enzyme in the presence of a saturating concentration (3.6 mM) of L-serine and the enzyme in the presence of a saturating concentration of H_4-folate. It is seen from the figure that the heat inactivation patterns of the native enzyme and the enzyme in the presence of H_4-folate are similar but differ significantly from that of the enzyme in the presence of serine.

Spectroscopic characterization

The enzyme had two absorption maxima at 277 and 428 nm. The enzyme showed two fluorescence emission maxima at 340 nm and 337 nm when excited at 285 nm and 295 nm, respectively (figures not given). The far u. v., c.d. spectrum (figure 11) was characteristic of a protein containing a large amount of α-helix (33%); the $[\theta]_{mrw}$ values at 200 and 208 nm were 11.45 and ll.66×l0^3 deg cm^2 d mol^{-1}. The visible and near u.v., c.d. spectrum (figure 12) showed a peak at 430 nm, characteristic of bound pyridoxal 5′ phosphate and negative peaks at 268 and 278 nm, characteristics of aromatic amino acid residues.

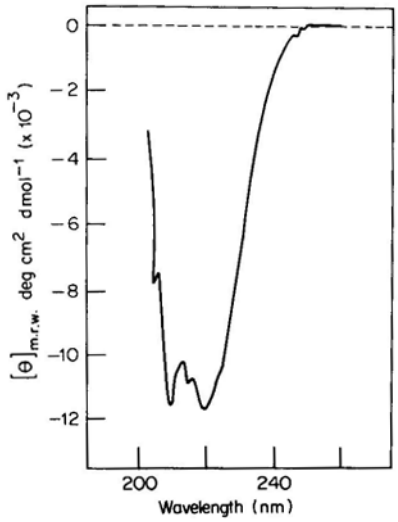

Figure 11. The far uv c:d. spectrum of sheep liver serine hydroxymethyltransferase.

The spectrum was taken at an enzyme concentration of 0.3 mg/ml in a 1 mm cell, after dialysing out free pyridoxal 5' phosphate.

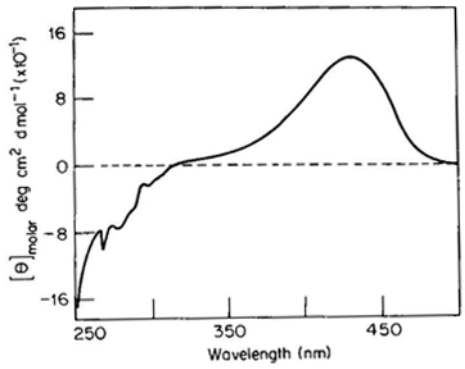

Figure 12. The near u.v. and visible c.d. spectrum of sheep liver serine hydroxymethyltransferase.

The spectrum was taken at a protein concentration of 0.885 mg/ml in a 10 mm cell, after dialysing out free pyridoxal 5′ phosphate.

Discussion

The kinetic and catalytic reaction mechanisms of serine hydroxymethyltransferase have been studied extensively albeit not elucidated completely with the enzyme obtained from several mammalian and microbial sources (Schirch *et al.,* 1973; Ching and Kallen, 1979; Hanson and Davis, 1979; Ulevitch and Kallen, 1977a, b). Except for the observations on the enzyme from pig kidney (Harish Kumar *et al,* 1976) and monkey liver (Ramesh and Appaji Rao, 1978, 1980a), there is very little information on its regulation. The sheep liver enzyme, isolated in a homogeneous form (figures 1,2 and 3) by avoiding the use of heat denaturation in the presence of L-serine during the purification resulted in the retention of its allosteric properties. The sheep liver enzyme purified by us was different from the monkey, rabbit, lamb and rat enzymes (Schirch, 1971; Nakano *et al.,* 1968; Ulevitch and Kallen, 1977a; Ramesh and Appaji Rao, 1980a) with respect to several important features, although there was essential similarity in the physicochemical properties of these enzymes.

The sheep liver enzyme, like the enzyme isolated from other sources was adsorbed strongly to CM-Sephadex and to folate-Sepharose. Another characteristic feature shared by this enzyme with the pig kidney (Braman *et al.,* 1981) and monkey liver enzymes (Ramesh and Appaji Rao, 1980a) is its ability to interact with Cibacron.Blue-Sepharose. The pH and temperature optima of this enzyme were similar to those of the other enzymes. All the serine hydroxymethyltransferases isolated so far have a molecular weight of about 200,000 and are made up of four identical single polypeptide subunits (Fujioka, 1969; Ulevitch and Kallen, 1977a; Ramesh and Appaji Rao, 1980a; Rao and Appaji Rao, 1980). All these enzymes except that from the mung bean seedlings (Rao and Appaji Rao, 1981) require pyridoxal 5′ phosphate for catalysis and sulphydryl groups for activity.

Unlike the enzyme from rabbit, lamb, ox etc. (Jones and Priest, 1976; Ulevitch and Kallen, 1977a, b), the sheep liver serine hydroxymethyltransferase exhibits homotropic cooperative interactions with the substrate, H_4-folate, as evidenced by a sigmoid saturation pattern (figure 4) and an n_H value of 2.8. The pig kidney (Harish Kumar *et al.,* 1976) and monkey liver (Ramesh and Appaji Rao, 1978) enzymes also show this cooperativity with n_H values of 3.9 and 2.5, respectively. The cooperativity of H_4-folate interactions was abolished in the monkey liver enzyme by preincubation with a saturating concentration (3.6 nM) of L-serine (Ramesh and Appaji Rao, 1978), whereas this treatment only decreased the n_H value of the sheep liver enzyme to 1.6-1.8 (figure 4), suggesting that the nature of cooperative interactions in this enzyme was different. NADH, a positive allosteric effector for the monkey liver enzyme (Ramesh and Appaji Rao, 1978) converted the sigmoid saturation pattern to hyperbolic (n_H=l) whereas in the sheep liver enzyme, NADH could only reduce the n_H value to 1.8 (figure 6). NAD^+ was a negative allosteric effector in both the cases (figure 5). In the monkey liver enzyme, cooperativity was abolished at 60°C whereas an n_H value of 2.8 was observed in the sheep liver enzyme even at 60°C. On the contrary, and unlike the monkey liver enzyme, after several months of storage at –40°C, the sheep liver enzyme showed decreased cooperativity (n_H=1.8) along with decreased (about 30% less) activity (results not shown).

Schirch and Quashnock (1981) have concluded that there is no cooperativity in the interactions of tetrahydrofolate with serinehydroxymethyltransferase based on the observation that tetrahydrofolate is oxidized at low concentrations (below 20 μM) by a complex second order process dependent on the concentrations of tetrahydrofolate and oxygen. They have also observed that sigmoidicity was dependent on the time of preincubation and assay. Using a coupled assay and measuring the velocity within 20 seconds, they have obtained a hyperbolic saturation pattern which was different from the sigmoid pattern obtained using the assay procedure employed by us. They were also unable to obtain any NADH effects and failed to observe binding of NADH to the enzyme by equilibrium dialysis.

Our results with the sheep liver enzyme reported in this communication are contrary to these observations. The discrepancy is apparently due to their using tetrahydrofolate concentrations which were an order of magnitude less than those used by us (0.04-0.25 mM by Schirch and Quashnock (1981), and 0.25-1.8 mM by us in this study and by Ramesh and Appaji Rao, 1978, (1980a, b) suggesting that two tetrahydrofolate saturation patterns may exist in this enzyme. The discrepancy between our results and those of Schirch and Quashnock (1981) could not be due to oxidation of tetrahydrofolate as at the concentrations used by us, no oxidation of tetrahydrofolate was observed (table 2). The hypothesis put forth by Schirch and Quashnock (1981) that the observed cooperativity is due to an artifact of the assay and not an inherent property of the enzyme, appears invalid, as the n_H values for different enzyme preparations (3.9, 1.0, 2.5, 2.2 and 2.8, respectively for the pig kidney, mouse L1210 tumor, monkey liver, mung bean and sheep liver enzymes) (Harish Kumar *et al.,* 1976, Ramesh and Appaji Rao, 1978; Rao and Appaji Rao, 1980) vary considerably and denaturation with heat results in the alteration of n_H values for the monkey liver enzyme (Ramesh *et al.,* 1981b) but not for the mung bean (Rao and Appaji Rao, 1980) and sheep liver enzymes. In addition, there is specificity in the interaction of effector molecules; some, like NADH, decrease the cooperativity and others, like NAD^+, increase the cooperativity. These observations clearly point out that cooperativity of H_4-folate interactions is an inherent property of the enzyme rather than an artifact of assay.

Although Schirch and Quashnock (1981) report their inability to observe NADH effects of the kinetics as well as binding of this ligand to the monkey liver enzyme, we have observed in this study that pyridine nucleotides alter the cooperativity of the tetrahydrofolate interactions with the sheep liver enzyme and also affect the inhibition of the enzyme by Cibacron Blue. These workers, based on the binding studies of the monkey liver enzyme with NADH, remark that 'if binding is occurring, it may be taking place with a Kd value greater than 2 mM'. It can be seen from the results presented in this study that the minimum concentration of NADH required to cause a significant change in the n_H value is approximately 5 mM. Therefore, it is not surprising that binding was not observed when the equilibrating chambers contained 0.5 mM NADH (Schirch and Quashnock, 1981).

The antibiotic, D-cycloserine, was a potent linear competitive inhibitor of the monkey liver enzyme (Ramesh, 1980), whereas it inhibited the sheep liver enzyme

in a sigmoid pattern (figure 8). The above observations point to significant differences in the quaternary structure and subunit interactions of the monkey and sheep liver enzymes. Antibodies raised against the monkey liver enzyme partially cross-reacted with the sheep liver enzyme and *vice versa* (Ramesh and Appaji Rao, 1980a), suggesting some similarity in the structures of these two proteins but also indicating that they were not identical. This preliminary investigation seems to suggest that a comparative study of the enzyme from these two sources involving an analysis of their desensitization and denaturation profiles, is essential for the understanding of a common basis for subunit interactions among the serine hydroxymethyltransferases.

A comparison of the catalytic properties of the sheep liver enzyme with that from other sources shows some common features and several characteristic differences. The V_{max} of the monkey and sheep liver enzymes were 3.3 and 6 units, respectively; the $K_{0.5}$ values of these two enzymes were 0.54 and 0.7 mM, respectively for tetrahydrofolate and 0.74 mM and 0.9 mM, respectively for L-serine. Unlike the enzyme isolated from other sources (Ulevitch and Kallen, 1977a, b, c), β-phenylserine (20 mM) and threonine (100 mM) did not inhibit significantly the sheep liver enzyme. A possible reason for this difference could be the use of a desensitized enzyme by other workers.

Dichloromethotrexate inhibited the sheep liver enzyme completely, whereas aminopterin was a partial inhibitor (figure 7); both these antifolates were complete inhibitors of the monkey liver enzyme (Ramesh and Appaji Rao, 1980a). Methotrexate, on the other hand, was a partial inhibitor of the monkey liver enzyme while it did not produce significant inhibition of the sheep liver enzyme. Folate was a complete inhibitor of both the enzymes while dihydrofolate was a partial inhibitor.

Cibacron Blue has been used to probe into the 'nucleotide-fold' of dehydrogenases, kinases and several binding proteins (Rossman *et al.,* 1974; Thompson *et al.,*1975; Thompson and Stellwagen, 1976; Stellwagen, 1977; *Lepo et al.,* 1979), but recent evidence seems to suggest that it might interact at other sites on the enzyme, especially at hydrophobic pockets (Subramanian and Kaufman, 1980; Grazi *et al.,* 1978; Beissner *et al.,* 1979; Barden *et al.,* 1980). Interaction of the dye with the monkey liver serine hydroxymethyltransferase is a clearcut instance of binding at the folate domain due to structural similarity in the triazine moiety of the dye and folic acid (Ramesh and Appaji Rao, 1980b). The interaction of triazines at the active site of this enzyme is indicated by the inhibition of the activity of the pig kidney enzyme by the Baker antifolates (Harish Kumar, P. M., Appaji Rao, N. and Mangum, J. H. —personal communication). In the case of the sheep liver enzyme, however, NAD^+ and NADH as also tetrahydrofolate, are able to overcome significantly the inhibition by the dye, indicating multiple interacting sites for the dye with this enzyme.

The results presented in this paper clearly demonstrate that serine hydroxylmethyltransferase is a regulatory protein and that the sheep liver enzyme could be an important system to probe into the structure, function and regulation of this first enzyme of the folate pathway.

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