Allosteric serine hydroxymethyltransferase from monkey liver: Correlation of conformational changes caused by denaturants with the alterations in catalytic activity

KASHI S. RAMESH,* V. S. ANANTANARAYANAN** and N. APPAJI RAO***

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012 and

**Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada AIB 3×9

*** To whom all correspondence should be addressed

Abstract. The far-ultraviolet region circular dichroic spectrumof serine hydroxymethyltransferase from monkey liver showed that the protein is in an α-helical conformation. The near ultraviolet circular dichoric spectrum revealed two negative bands originating from the tertiary conformational environment of the aromatic amino acid residues. Addition of urea or guanidinium chloride perturbed the characteristic fluorescence and far ultraviolet circular dichroic spectrum of the enzyme. The decrease in $(\theta)_{222}$ and enzyme activity followed identical patterns with increasing concentrations of urea, whereas with guanidinium chloride, the loss of enzyme activity preceded the loss of secondary structure. 2-Chloroethanol, trifluoroethanol and sodium dodecyl sulphate enhanced the mean residue ellipticity values. In addition, sodium dodecyl sulphate also caused a perturbation of the fluorescence emission spectrum of the enzyme. Extremes of pH decreased the $-(\theta)_{222}$ value. Plots of $-(\theta)_{222}$ and enzyme activity as a function of pH showed maximal values at pH 7.4-7.5. These results suggested the prevalence of "conformational flexibility" in the structure of serine hydroxymethyltransferase.

Keywords. Serine hydroxymethyltransferase; conformational changes; denaturants; fluorescence; circular dichroism; structure-activity relationship.

Introduction

Earlier reports from this laboratory demonstrated that the homogeneous monkey liver serine hydroxymethyltransferase isolated by omitting a heat denaturation step, used by earlier workers (Nakano *et al.,* 1968; Fujioka, 1969; Akhtar & El-Obeid, 1972; Cheng & Haslam, 1972; Rowe & Lewis, 1973; Palekar *et al.,* 1973; Jones & Priest, 1976; Ulevitch & Kallen, 1977) had a molecular weight of 208,000

Present Address* Hematology Research Laboratory, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114, USA.

with four identical subunits (Ramesh & Appaji Rao, 1980b) and exhibited both homotropic and heterotropic allosteric interactions (Ramesh & Appaji Rao, 1978). It was also demonstrated that the interaction of Cibacron Blue 3G-A with the monkey liver enzyme (Ramesh & Appaji Rao, 1980a) and also the binding of anti-body to it (Ramesh, 1980) produced conformational changes in the enzyme. These observations and the lack of any information on the solution conformation (*i.e.*, the secondary structure) of this protein, prompted the present investigation on the structure-activity relationship of the enzyme.

The present paper reports the results of studies on the changes in the conformation and activity of the monkey liver serine hydroxymethyltransferase on the addition of urea, guanidinium hydrochloride (GdmCl), sodium dodecyl sulphate (SDS), haloalcohols, acid and alkali, using circular dichroic (c.d.) and fluorescence probes.

Materials and methods

Materials

The following biochemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, USA: 2-mercaptoethanol, pyridoxal 5'-phosphate, EDTA and SDS. DL-[3$-^{14}$C] serine (48.5 mCi/mmol) was obtained from New England Nuclear (Boston, Massachussets, USA). H_4 folate was generously given by Dr. J. H. Mangum (Brigham Young University, Provo, Utah, USA). GdmCl (ultra pure grade) and urea (recrystallized) were obtained from Schwarz/Mann Research Laboratories, Orangeburg, New York, USA). Trifluoroethanol was purchased from Eastman Kodak Co., Rochester, New York, USA. 2-Chloroethanol was a product of British Drug House Chemicals Ltd., Poole, England. Millipore filters (type HA 0.45 μm, Millipore Corporation, Massachussets, USA) were kindly given by Dr. A. Ashok Kumar (University of Cincinnati Medical Centre, Cincinnatti, Ohio, USA).

Methods

Serine hydroxymethyltransferase from monkey liver was purified as described earlier (Ramesh, 1979; Ramesh and Appaji Rao, 1980b). The enzyme was assayed by a radio-chemical method (Taylor and Weissbach, 1965) using DL-[3$-^{14}$C] serine as the substrate; the amount of [^{14}C] formaldehyde formed in 15 min was estimated (Ramesh and Appaji Rao, 1978). One unit of enzyme activity was defined as the amount that catalysed the formation of 1 μmol of formaldehyde/min at 37° C at pH 7.4. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

GdmCl was purified by the method described by Nozaki (1972) and urea by repeated crystallization from absolute ethanol. Trifluoroethanol and 2-chloro-ethanol were distilled before use.

C.d. measurements were performed at 25°C on a Jasco-20 scanning spectro-polarimeter. The circular dichrometer was calibrated using an aqueous solution of d-10-camphor sulphonic acid (Cassim and Yang, 1969). All buffers, reagents and protein solutions were passed through Millipore filters before recording the

c.d. spectra. The spectropolarimeter was continuously purged with prepurified nitrogen before and during the experiments. All the c.d. spectra were recorded with the slit programmed for 1 nm-spectral band width, a chart speed of 4 nm/min, a time constant of 64s and a wavelength expansion of 5 nm/cm. All c.d. spectral data are plotted as mean residue ellipticity which was calculated using the equation,

$$[\theta]_{m.r.w} = (\theta \times m.r.w.)/(10 \times l \times c)$$

where θ is the observed ellipticity in degrees, m.r.w. is the mean residue weight which is assumed to be 115, l is the optical path in cm and c is the concentration of the enzyme in g/ml.

Fluorescence measurements were made using a Perkin-Elmer model 203 fluorescence spectrophotometer equipped with a 150-W Xenon lamp source. The instrument was standardized using quinine sulphate solution. The emission spectra reported are uncorrected. All readings were made at room temperature, 26±1°C. Quartz cuvettes (1 cm path length) containing 3 ml of enzyme solution were used in the experiments. The enzyme was extensively dialysed against 50 mM-potassium phosphate buffer, pH 7.4 and filtered through a Millipore filter before fluorescence spectral measurements were made. The absorbance of the enzyme at 280 nm was always maintained below 0.10. Appropriate corrections were applied where the total volume change was significant. Fluorescence spectral measurements were made against appropriate controls.

Results

Circular dichroism of monkey liver serine hydroxymethyltransferase

In the near-u.v. c.d. spectral region, the monkey liver enzyme showed negative ellipticity maxima at 280 nm and 270 nm and a shoulder at 276 nm (figure la). The spectrum was characterized by a fine structure in the region of absorbance of aromatic amino acids. Both tyrosine and tryptophan residues contribute to the c.d. band at 276 nm (Beychok, 1966) and the 280 nm band may be due to tryptophan transitions (Beychok, 1967).

In the far-u.v. c.d. spectral region (200-250 nm), the c.d. spectrum of the native enzyme was qualitatively that of a protein with α-helical conformation (figure 1b). The spectrum is characterized by two negative ellipticity bands centred at 220-222 nm and 207-209 nm with amplitudes of $(-9.82 \pm 0.11) \times 10^3$ and $(-9.91 \pm 0.10) \times 10^3$, deg. cm^2. dmol^{-1}, respectively. The characteristic c.d. spectrum of the enzyme was not sensitive to protein concentration (0.1 to 3 mg/ml) and to the ionic strength of the solvent (0.03 to 0.2 M). Estimation of the α-helical content from the far-u.v. c.d. spectral data was obtained both by the method of (i) Chen and Yang (1971), using the mean residue ellipticity value at 222 nm and (ii) Greenfield and Fasman (1969), using the mean residue ellipticity at 208 nm. Estimates of the α-helical content by the two procedures gave values of 20 ±0.3% by the method of Greenfield and Fasman (1969) and 25 ±0.25% when the analysis was carried out according to Chen and Yang (1971). Since the contributions of α-helix to the overall secondary structure of the protein, calculated by different methods vary
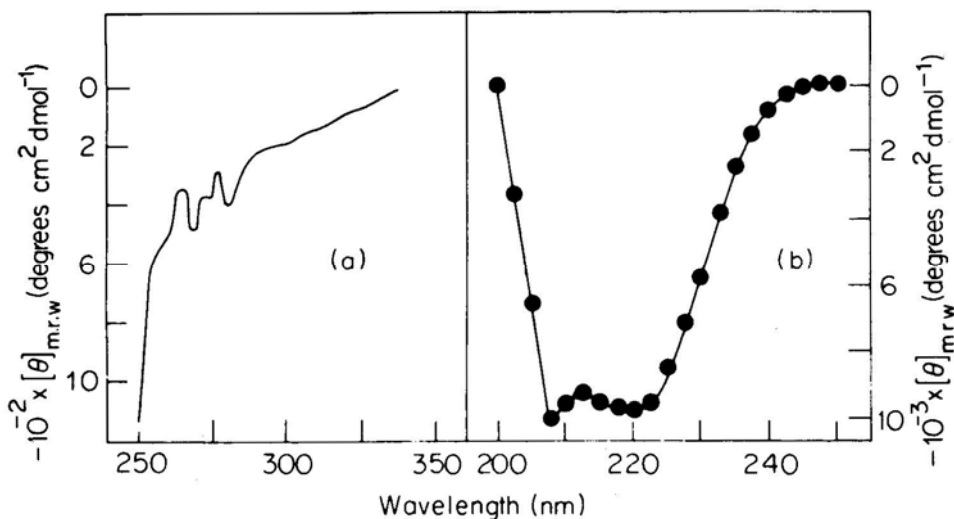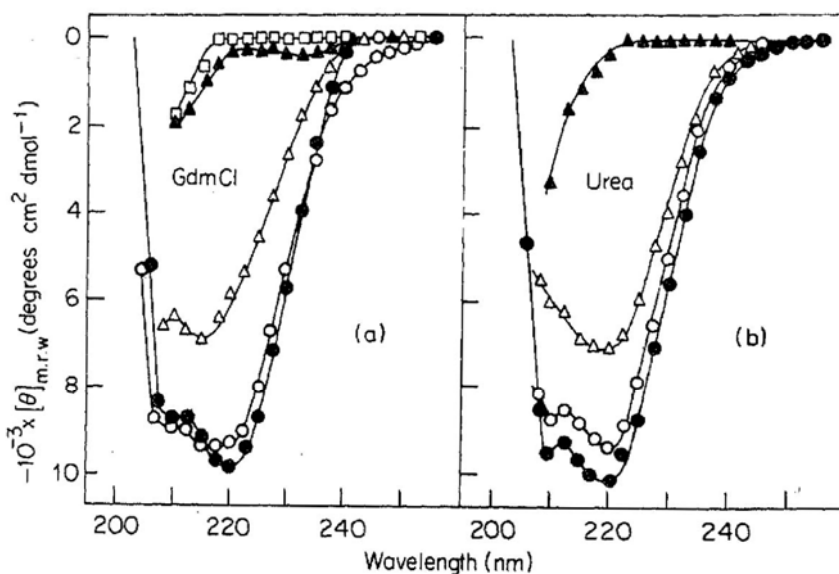
Figure 1.   Near-and far-u.v. circular dichroic spectra of monkey liver serine hydroxymethyl-transferase. a. The near-u.v. c.d. spectrum of the enzyme (2 mg/ml) in 50mM-potassium phosphate buffer, pH 7.4 containing 1 mM 2-mercaptoethanol and 1 mM EDTA, was recorded in a cell of 1 cm-pathlength containing 3 ml of the enzyme solution. b. The far-u.v. c.d. spectrum of the enzyme (3.28 mg/ml) in 50 mM potassium phosphate bufer, pH 7.4/1 mM 2-mercapto-ethanol/1 mM EDTA was recorded by using a demountable quartz cell of 0.1 mm light path. (figure lb).

significantly (Lee *et al.,* 1978; Luer & Wong, 1978), we have used $^{-}[\theta]_{222}$ values to describe the conformational changes in the enzyme, as it is a sensitive probe of the polypeptide backbone conformation (Leavis *et al.,* 1978).

Effect of urea and GdmCl on the circular dichroic and fluorescence spectra of monkey liver serine hydroxymethytransferase

The far-u.v. c.d. spectra of the enzyme in the presence of different fixed concentrations of GdmCl are shown in figure 2a. At 0.4 M GdmCl, there was a shift of the negative 221 nm maximum to 217 nm, and at 1.6 M GdmCl, the negative band shifted further to 216 nm. At 6.4 M GdmCl, the c.d. spectrum of the enzyme assumed a pattern similar to that of a randomly coiled polypeptide chain (Stevens *et al.,* 1968) with no characteristic c. d. bands at 221 nm and 208 nm.

   The effect of increasing concentrations of urea on the c.d. spectrum of the enzyme in the far-u.v. region is depicted in figure 2b. As is evident from the figure, the presence of 0.8 M urea did not alter the position of the negative ellipticity bands (209 nm and 221 nm), although there was a slight decrease in the peak amplitudes. However, the presence of higher concentration of urea (>1.0M) resulted in a shift of the 221 nm negative ellipticity band to 216 nm. In the presence of 8 M urea, the far-u.v. region c.d. spectrum indicated extensive denaturation.

Figure 2. The far-u.v. circular dicbroic spectra of monkey liver serine hydroxymethyltrans-ferase as a function of GdmCl and urea. The enzyme 50 µl (3.50 mg/ml) was mixed with 200µl of urea or GdmCl (concentrations ranging between 0.5 to 10 M) to yield the denaturant concen-tration indicated below. The c.d. spectra were recorded lh after mixing the enzyme with the denaturants using 0.5 mm pathlength demountable quartz cell. a. GdmCl, OM (●); 0.4 M (O); 1.6 M (Δ); 4.8 M (▲) and 6.4 M (□). b . Urea, ,0 M (●);0.8 (O); 1.6 M (Δ) and 8 M (▲).

The enzyme exhibited fluorescence emission maxima at 335 nm on excitation at 285 nm and at 340 nm on excitation at 295 nm. The addition of 6 M GdmCl and 8 M urea to the enzyme resulted in a red-shift of the 340 nm-fluorescence emission peak to 365 nm and 350 nm, respectively. This could be attributed to the exposure of tryptophan residues to the solvent or their shift into a more hydrophobic environment and may in turn reflect an altered three dimensional conformation of the enzyme induced by these denaturants (data not shown).

Effect of urea and GdmCl on the enzyme activity and mean residue ellipticity at 222 nm

GdmCl and urea, both inactivated the serine hydroxymethyltransferase and the enzyme inactivation was dependent on the concentration of the denaturant. The enzyme underwent a conformational transition as indicated by the decrease of $^-[\theta]_{222}$ values, when the GdmCl concentration was increased from 0 to 5 M, with the mid point of transition at 1.9 M GdmCl. On the other hand, when urea was used as the denaturant, the mid point of transition from native to the denatured state occurred at 3.6 M urea (data not shown). In the case of GdmCl, the loss of enzyme activity was rapid and 50% activity was lost at only 0.4 M GdmCl. In contrast, 2.4 M urea was needed to effect a 50% loss of enzyme activity. The loss of

activity and secondary structure of the enzyme was completed within 15 min of exposure to the denaturants. Removal of the denaturants from the fully denatured enzyme solution, by dialysis against potassium phosphate buffer (50 mM/pH 7.4) did not result in the recovery of either the enzyme activity or the secondary structure.

Effect of trifluoroethanol and 2-chloroethanol on the far-u.v. region c. d. spectra and activity of the monkey liver serine hydroxymethytransferase

The effect of 50 to 90 vol.-% trifluoroethanol and 10-35 vol.-% 2-chloroethanol on the conformation of the enzyme was tested. It was found that 50-90 vol.-% trifluoroethanol altered the conformation significantly (figure 3a) as indicated by
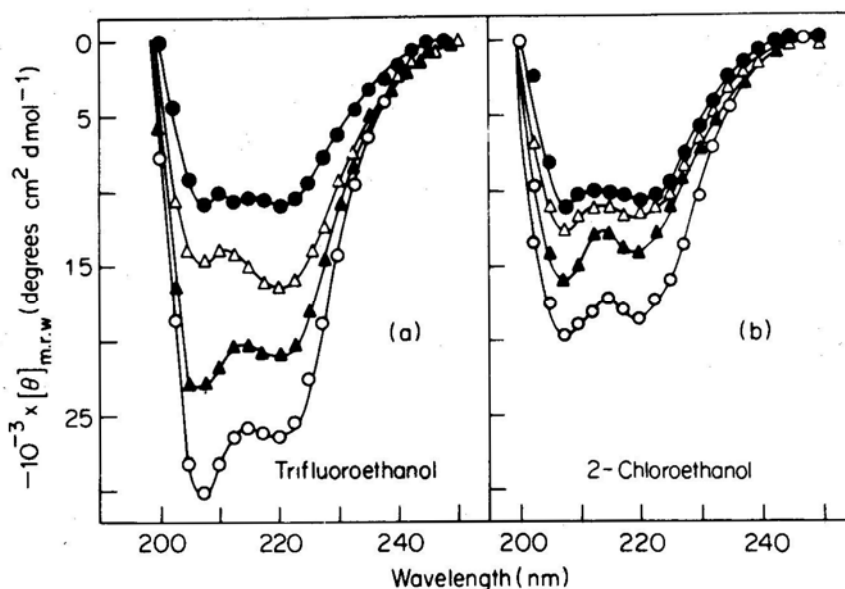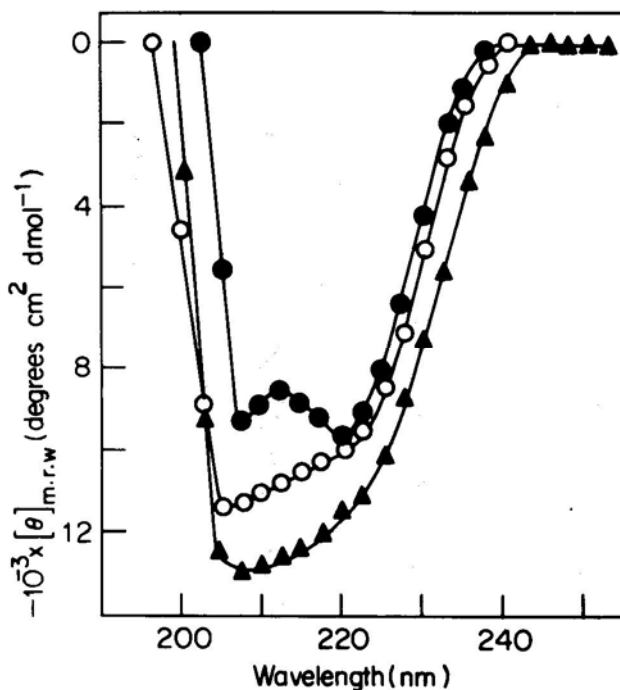

Figure 3.   Effect of trifluoroethanol and 2-chloroethanol on the far-u.v. circular dichroic spectra of monkey liver serine hydroxymethyltransferase. The enzyme (0.2 mg/ml) in 10 mM potassium phosphate buffer, pH 7.4/1 mM 2 mcercaptoethanol/1 mM EDTA, was mixed with either trifluoroethanol or 2-chloroethanol to obtain a final concentration indicated in figure. The c.d. spectra were recorded after lh of equilibration using a demountable quartz cell of 0.5 mm-light path. Trifluoroethanol below 50 vol.-% precipitated the protein and 2-chloroethanol above 35 vol.% failed to increase the $-[\theta]_{m.r.w}$ values. a. Trifluoroethanol, 0% ($\bullet$); 50 vol.-% ($\triangle$) 60 vol.-% ($\blacktriangle$) and 90 vol.-% (O). b. 2-Chloroethanol, 0% ($\bullet$); 10 vol.-% 20 vol.-% ($\blacktriangle$) and 35 vol.-% (O).

an increase in the mean residue ellipticity value of the native enzyme form -10120 at 222 nm to -15870 at 50 vol.-% trifluoroethanol and to -25530 at 90 vol.-% trifluoroethanol; the corresponding values at 208 nm were -10810, -14720 and -30130 deg. cm^2. dmol^{-1}, respectively. Interestingly, the negative ellipticity at 208 nm in the presence of 90 vol.-% trifluoroethanol was about 13% greater than at 222 nm.

2-Chloroethanol was more effective than trifluoroethanol in increasing the mean residue ellipticity values (figure 3b). Thus, 90 vol.-% trifluoroethanol was required to increase the $-[\theta]_{222}$ values from -10120 to -25530 deg. cm^2.dmol^{-1}, whereas, only 35 vol.-% 2-chloroethanol was required to increase the $-[\theta]_{222}$ values from -10120 to -17420 deg. cm^2. dmol^{-1}. The enzyme activity was completely lost on the addition of very low concentration (1%) of these alcohols. Extensive dialysis of the enzyme-alcohol mixtures did not result in the restoration of the native enzyme structure, as indicated by $-[\theta]_{222}$ values and enzyme activity, although a small decrease (10%) in the $-[\theta]_{222}$ value was observed with trifluoroethanol.

Influence of SDS on the circular diehroism and fluorescence spectrum of monkey liver serine hydroxymethyltransferase

The addition of 0.4%-SDS to the enzyme altered the native conformation to a more ordered structure. Inspection of figure 4 indicates the absence of an explicit 222 nm negative ellipticity band and the prevalence of a plateau at 208-222 nm. The enzyme was completely inactive in the presence of low concentrations of SDS (0.01%) and this process was irreversible, as monitored by activity and conformation.
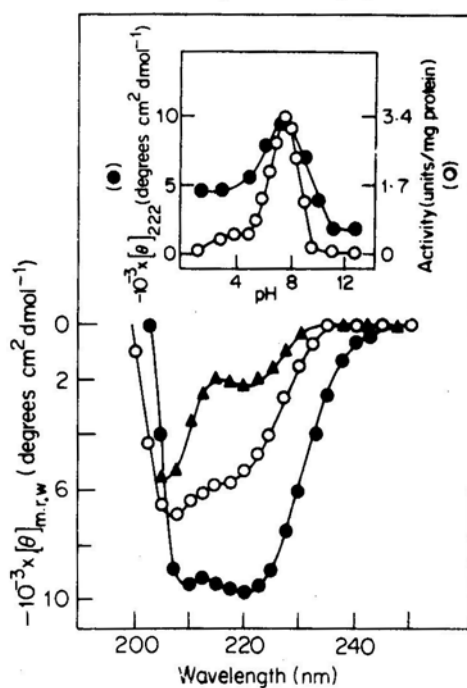

Figure 4. Faru.v. circular dichroic spectra o f monkey liver serine hydroxymethyltransferasese in the presence of SDS. The enzyme in 50mM potassium phosphate buffer, pH 7.4 was exhaustively dialysed against 50 mM sodium phosphate buffer, pH 7.4, before mixing with SDS, in order to avoid the precipitation of SDS. The enzyme (0.18 mg/ml) was mixed with SDS and the c.d. spectra were recorded after lh of equilibration at 25°C using a 0.5 mm-path length demountable quartz cell. 0% SDS (●); 0 0.2% (O) and 0.4% (▲).

Interaction of SDS with the enzyme resulted both in increased fluorescence intensity as well as a red-shift of the emission maximum to 360 nm.

pH-Induced alterations in the secondary structure and activity

The far-u.v. c.d. spectra of the enzyme at pH 1.4, 7.4 and 12.6 are shown in figure 5. The c.d. spectrum of the enzyme at pH 1.4 differed considerably from that at pH 7.4. There was an overall decrease in the c.d. band intensities at 222 nm and 208 nm. The 222 nm band shifted to a lower wavelength and these changes were consistent with a gradual loosening of the secondary structure. Even more drastic changes in the circular dichroic characteristics were observed at alkaline pH. At pH 12.6, the spectrum of the enzyme assumed the character of a denatured, but still constrained protein (Fasnian. *et al.,* 1970), with a negative maximum at 205 nm and a broad shoulder at 220 nm.

The changes in the negative ellipticity at 222 nm and the activity as a function of pH are shown as an inset to figure 5. The magnitude of the 222 nm negative ellipticcity band increased significantly from pH 1.4 to 7.5 and was followed by a more gradual decrease above pH 7.6. The pH-activity profile also followed a similar

Figure 5.   Alteration in the far-u.v. circular dichroic spectrum and the enzymic activity of serine hydroxymethyltransferase as a function of pH.

The enzyme (0.3 mg/ml) in 20 mM potassium phosphate buffer, pH 7.4, was adjusted with either 0.2 M HCl or 0.2 M NaOH to the desired pH value and the c.d. spectra recorded after 30 min equilibration at 25°C, using a 0.5 mm-light path demountable quartz cell. pH 12.6 (▲); pH 1.4 (O) and pH 7.4 (●). The inset shows the changes in mean residue ellipticity at 222 nm (●) and enzyme activity (O) as a function of pH. The activity of the enzyme was assayed by the procedure described in Methods.

pattern, the enzyme exhibiting maximal activity at pH 7.45. It was interesting to note that the enzyme had lost most of its activity between pH 1.5 and 4.0, although the negative ellipticity values at 222 nm testified the prevalence of residual structural constrains in the enzyme. The acid-transition was reversible above pH 5.0, but was irreversible at lower pH values. The loss in the secondary structure and activity of the enzyme above pH 9.8 could not be regained by readjusting the pH to neutral values.

Discussion

The elucidation of the nature of the forces that stabilize the native conformation of proteins and the steps in the pathways of protein unfolding and refolding, have been a major focus of protein research in the last few decades. A very versatile approach for the understanding of the conformation of proteins in solution is the study of protein denaturation. The results of the investigation described in this paper represent a preliminary attempt to demonstrate the usefulness of using intrinsic fluorescence and circular dichroism as probes to examine the conformational changes induced by interactions with various denaturants and enzyme activity.

The near u.v. c.d. spectrum of the monkey liver serine hydroxymethyltransferase (figure 1 a) appears unusual in the absence of positive c.d. signals. A similar type of spectrum has been reported in the case of *E. coli* ribosomal protein L7/L12 (Luer and Wong, 1979). The absorption spectrum of the enzyme (figure 5, Ramesh & Appaji Rao, 1980b) shows a peak at 420 nm, due to the presence of protein bound pyridoxal 5'-phosphate. The presence of bound pyridoxal 5'-phosphate could be one of the reasons for this rather unusual c.d. spectrum.

The far-u.v. c.d. spectrum of the enzyme (figure 1b) was typical of a protein which contained an unique conformation with a well organized secondary structure. The c.d. spectrum of the enzyme in 6.4 M GdmCl and 8M urea (figure 2) was characteristic of a protein which was unfolded into an extremely disordered conformation (Nozaki *et al.,* 1974). There was a good correlation between the loss of native structure (as monitored by $-[\theta]_{222}$ values) and loss of enzyme activity, when urea was used as the denaturant; whereas, in the case of GdmCl, the enzyme activity was lost much earlier compared to the loss of secondary structure. These results suggested that GdmCl might initiate conformational changes close to or at the active site; whereas, urea might initiate denaturetion at a site distal to the substrate binding site of the enzyme. It was also noted that higher concentrations of urea were required to effect complete unfolding as compared to GdmCl and this might be due to the differences in the structure of the two denaturants. It was interesting to observe that the final c.d. spectra of the enzyme in 6.4 M GdmCl or 8 M urea were nearly similar. This would suggest that the pathways of protein unfolding induced by GdmCl and urea might be different, although the resulting denatured products were similar (Hibbard and Tulinsky, 1978). The fact that the GdmCl-or urea-denatured enzyme, failed to regain either the catalytic activity or the native structure, would imply that the process of denaturation was essentially irreversible. This is not surprising, since, in oligomeric proteins, the reassociation of individual subunits to form the native quaternary structure plays an important role in the overall renaturation process.

At a high concentration of trifluoroethanol or 2-chloroethanol, the enzyme showed a transition from the native structure to a conformation with a considerable proportion of α-helix (figure 3) as indicated by enhanced mean residue ellipticity values at 208 nm and 222 nm. The effects of ethanol and similar water-miscible solvents on protein conformation have been discussed by several investigators (Tanford, 1968; Timasheff, 1970; Jirgensons, 1978; Toniolo *et al.,* 1978; Tamura and Jirgensons, 1980). Jirgensons (1978) reported that non-helical proteins (e.g., *ß*-lactoglobulins and caseins) were reorganized into new conformations of higher helical content than in the native state. Our results of the helix promoting effects of trifluoro ethanol and 2-chloroethanol on the structure of the oligomeric serine hydroxymethyltransferase appears to be an unique example of a large mol.wt. protein undergoing these transitions. The fact that the activity of the enzyme was lost in the presence of the haloalcohols, suggested that the active site geometry and its integrity might have been lost during the interactions.

Similar to alcohols, the interaction of SDS with serine hydroxymethyltransferase resulted in the enzyme adopting a new conformation with greater helical content than in the native state (figure 4). Jirgensons (1976) proposed that SDS-induced conformational transitions occurred in two stages: (i) interaction in the first stage resulted in the unfolding of the ordered globular proteins and (ii) the unfolded polypeptide chain refolded into another conformation of higher order, which differed from the native conformation. The loss of enzymic activity in the presence of SDS might be due to the altered structure of the enzyme in the presence of the denaturant. Although a number of models have been put forward to explain the varied hydrodynamic behaviour of protein-SDS complexes, which contain approximately 1.4g SDS/g of protein (Reynolds and Tanford, 1970; Shirahama *et al.,* 1974; Wright *et al.,* 1975; Mattice *et al.,* 1976) none of these models provide a molecular explanation for their formation.

Significant alterations in the c.d. spectra of the enzyme were induced by pH alterations (figure 5) suggesting an unfolding of the native conformation. A permanent loss of the secondary structure of the enzyme occurred below pH 4.0, since the spectrum obtained at pH 4.0 remained irreversibly distorted upon restoration to neutral pH values. At extremes of pH, the c.d. spectrum of the enzyme became increasingly dominated by a disordered structure and resembled the spectrum of a randomly coiled protein and these processes were irreversible. The enzyme was not dissociated into subunits over the entire pH range as monitored by isoelectrofocusing and Polyacrylamide gel electrophoresis (Ramesh, 1980). The interpretation of the c.d. spectra of the enzyme at alkaline pH values was complicated due to the increased reactivity of −SH groups at high pH values and peptide bond cleavage (Lapanje, 1978). The good correlation observed between negative ellipticity values at 222 nm and enzymic activity at different pH values (inset to figure 5), suggested that, for the enzyme to be optimally active, its structure should exist in a precise conformation. Thus at pH 7.4, the enzyme had maximal activity as well as $-[\theta]_{222}$ value.

In conclusion, the solution coformation of the monkey liver serine hydroxyl-methyltransferase reported for the first time, possesses a well defined secondary

structure, which when perturbed by denaturants or pH alterations, produces changes both in the structure and the activity of the enzyme. Our results establish that this enzyme offers a useful handle for understanding the mechanism of protein denaturation.

Acknowledgements

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