Allosteric serine hydroxymethyltransferase from monkey liver: temperature induced conformational transitions

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Abstract. The homogeneous serine hydroxymethyltransferase from monkey liver was optimally activate at 60°C and the Arrhenius plot for the enzyme was nonlinear with a break at 15°C. The monkey liver enzyme showed high thermal stability of 62°C, as monitored by circular dichroism at 222 nm, absorbance at 280 nm and enzyme activity. The enzyme exhibited a sharp co-operative thermal transition in the range of 50°-70° (T_m= 65°C), as monitored by circular dichroism. L-Serine protected the enzyme against both thermal inactivation and thermal disruption of the secondary structure. The homotropic interactions of tetrahydrofolate with the enzyme was abolished at high temperatures (at 70°C, the Hill coefficient value was 1.0). A plot of *h* values vs. assay temperature of tetrahydrofolate saturation experiments, showed the presence of an intermediate conformer with an *h* value of 1.7 in the temperature range of 45°-60°C. Inclusion of a heat denaturation step in the scheme employed for the purification of serine hydroxymethyltransferase resulted in the loss of co-operative interactions with tetrahydrofolate. The temperature effects on the serine hydroxyl-methyltransferase, reported for the first time, lead to a better understanding of the heat induced alterations in conformation and activity for this oligomeric protein.

Keywords. Serine hydroxymethyltransferase; circular dichroism; thermal stability; conformational transitions.

Introduction

The kinetic, regulatory, physicochemical, immunological studies (Ramesh, 1979, 1980; Ramesh and Appaji Rao, 1978, 1980a, 1980b) and the investigation on the denaturation (Ramesh *et al.,* 1980), suggested that the oligomeric serine hydroxyl-methyltransferase from monkey liver exhibited conformational fluctuations consequent to the binding of effectors, ligands and change in the solution environment. It was quite surprising to note that the temperature-dependence of the reaction

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Abbreviations used: c.d., circular dichroism; u.v., ultraviolet; $[\theta]_{m.r.w.,}$ mean residue ellipticity; H_4folate, (+)-L-tetrahydrofolate; dimedone, 5,5-dimethylcyclohexane-l, 3-dione.

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catalyzed by serine hydroxymethyltransferase from any source had not been reported, although heat inactivation of contaminating proteins in the presence of L-serine has been used as a major step in the purification of this enzyme from several sources (Nakano *et al.,* 1980; Fujioka, 1969; Rowe and Lewis, 1973; Jones and Priest, 1976). An earlier report from this laboratory showed that this enzyme from monkey liver isolated without the heat-denaturation step, exhibited both homotropic and heterotropic co-operative interactions (Ramesh and Appaji Rao 1980b). It was therefore of interest to examine the nature of the temperature-induced effects on the protein. These temperature-induced transitions, monitored using spectroscopic and kinetic methods, are described in this paper.

Materials and methods

Materials

DL-[3—^{14}C] Serine (specific activity 48.5 mCi/mmol) was obtained from New England Nuclear (Boston, Massachussets, USA). H$_4$folate prepared by the method of Hatefi *et al.* (1960), was generously given by Dr. John H. Mangum (Brigham Young University, Provo, Utah, USA) . Pyridoxal 5'-phosphate, EDTA, and 2-mercaptoethanol were products of Sigma Chemical Co. (St. Louis, Missouri, 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 radiochemical method (Taylor and Weissbach, 1965) using DL-[3—^{14}C] serine as the substrate; and 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 catalyzed the formation of 1 µmol of formaldehyde/min at 37°C, pH 7.4. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Circular dichroic measurements were performed on a Jasco-20 scanning spectro-polarimeter. The circular dichrometer was calibrated using an aqueous solution of d-10-camphor sulphonic acid (Cassim and Young, 1969). All buffers, reagents and enzyme solutions were passed through Millipore filters (HA 0.45 µm) before recording the c.d. spectra. The spectropolarimeter was continuously purged with pre-purified 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 64 s and a wavelength expansion of 5 nm/cm.

Temperature effects were studied in jacketed cells through which an ethylene glycol-water mixture was circulated from a thermostatically controlled water circular bath. The temperature of the sample was increased in a stepwise manner at a constant rate. Temperature was measured in the cell holder by making use of the mercury contact. A quartz cell of 1 nm-pathlength was used in melting-profile

experiments. All c.d. spectral data were plotted as mean residue ellipticity 0, and was calculated using the formula:

$$[0]_{m.r.w.} = (\theta \times m.r.w.)/(10 \times l \times c),$$

and expressed in units of degree $cm^2.dmol^{-1}$; where 0 is the observed ellipticity in degrees, m.r.w. is the mean residue weight (assumed to be 115), l is the optical pathlength in cm and c is the concentration of the enzyme in g/ml.

Results

Effect of temperature on the velocity of the reaction catalyzed by monkey liver serine hydroxymethyltransferase

Arrhenius plot of log v (velocity at saturating concentration of substrates) vs. the reciprocal of absolute temperature (*T*) and a plot of *v* vs. the temperature (°C) are shown in figure 1. The inset plot shows 60°C as the optimal temperature for the
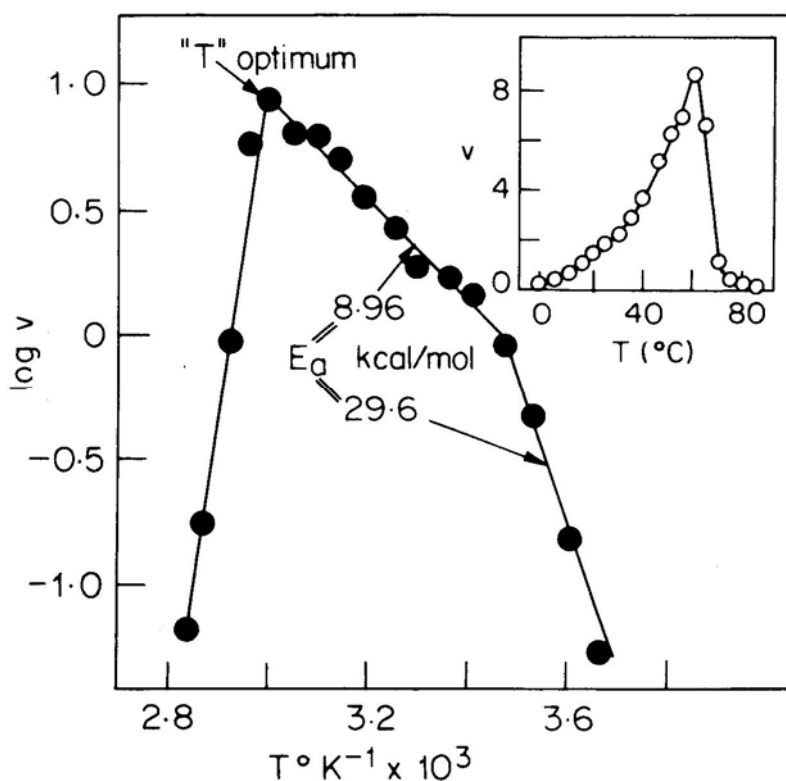

Figure 1. Arrhenius plot for the monkey liver serine hydroxymethyltransferase. The activity of the enzyme (1.1 µg in 50 mM potassium phosphate buffer, pH 7.4) was assayed at different fixed temperature (0-80°C) in the presence of saturating concentrations of L-serine (3.6 mM) and H₄folate (1.8 mM). The H₄folate concentration was increased to 2.1 mM, when the enzyme activity was measured above 50°C. This was to ensure that saturating concentrations of the substrates were present at all temperatures. The inset shows the plot of initial velocity vs. temperature of the assay in degrees centigrade.

reaction catalyzed by serine hydroxymethyltransferase. The Arrhenius plot showed a linear increase between 0°C to 60°C with a break at 15°C. Activation energies (E_a) calculated from the slopes in figure 1, were 29.6 K cal mol^{-1} (0-15°C) and 9 K cal mol^{-1} (15-60°C). The velocity decreased rapidly at higher temperatures (> 62°C) due to inactivation of the enzyme.

Thermal stability

The thermal stability was monitored by measuring enzyme activity, the absorbance at 280 nm and the c.d. spectrum in the region, 200-250 nm. In these experiments, the enzyme was heated at different temperatures and for different time intervals and quickly cooled to 0°C. The enzyme activity and the spectral properties were measured at 37°C and 25°C, respectively.

It is evident from figure 2 that the enzyme was stable at 50°C for 20 min. At 65°C, there was a progressive loss of enzyme activity with increasing periods of heating. However, at 70°C, the enzyme was rapidly inactivated, with complete loss of activity occurring in 6 min. Similar denaturation profiles were obtained when the enzyme concentration was increased from 100 μg to 1 mg/400 μl buffer; suggesting that the rate of thermal inactivation was not dependent on the concentration of the enzyme.
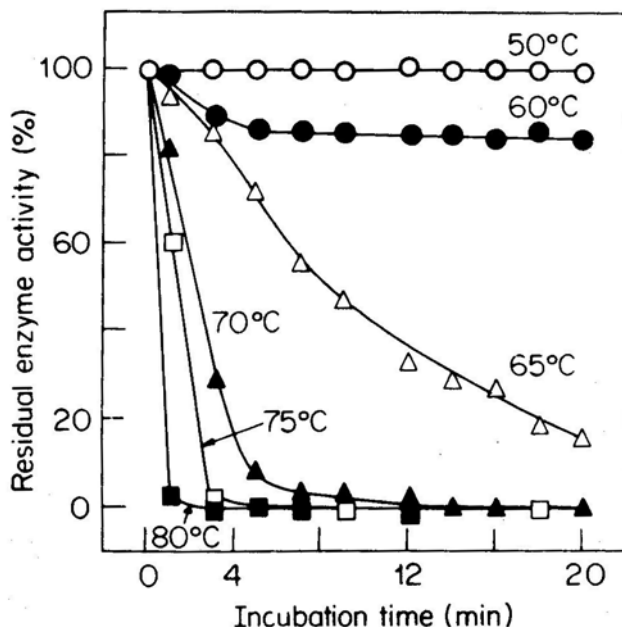

Figure 2. Thermal stability: Effect of pre-incubation temperature on the enzyme activity. The enzyme (100 μg/400 μl of 50 mM-potassium phosphate buffer, pH 7.4) was heated at different temperatures (50°-80°C) and aliquots were withdrawn at the time intervals indicated in the figure. The aliquots were cooled in ice. All the samples were warmed to 37°C by incubating for 5 min in a water-bath and the enzyme assayed at 37°C. The activity of a control sample maintained at 37°C was normalized to 100. The results of the experiment are expressed as per cent of the activity of unheated controls.

Figure 3 shows the family of far-u.v. c.d. spectral curves observed on increasing the temperature from 25° to 70°C. The prominent features of the helix related-c.d. spectrum remained unperturbed upto 50°C. Above 50°C, the amplitudes of the c.d. spectral bands decreased and at 67°C, the spectrum observed was consistent with that of a largely unfolded polypeptide chain.
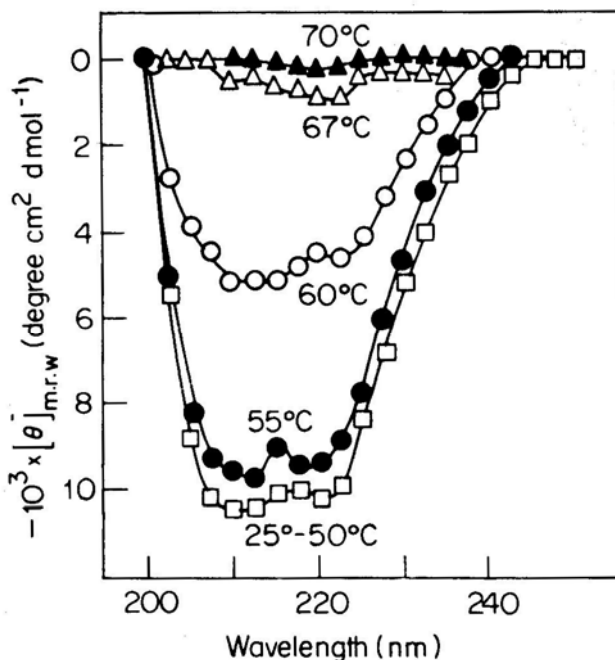

Figure 3. Thermal stability: Effect of pre-incubation temperature on the far u.v. circular dichroic spectra of the enzyme. The enzyme (270 µg/ml) was heated at different temperature (25°-70°C) for 10 min and immediately chilled in ice. C.d. spectra were recorded at 25°C using a demountable quartz cell of 0.5 mm-light path. Above 65°C, the enzyme solution became slightly turbid and these samples were centrifuged before recording the c.d. spectra.

Figure 4 summarizes the relationship obtained between (i) $^-[\theta]_{222}$, a sensitive parameter of polypeptide backbone conformation, (ii) A_{280} a parameter related to tertiary structure of the protein, and (iii) enzyme activity, a parameter related to structural integrity of the active site, and the preincubation temperature. As can be seen from the figure, there was no decrease of either the activity or the $[\theta]_{222}$ or A_{280} values upto 52°C. Above this temperature, there was a sharp and parallel decrease in these parameters indicative of a major conformational change. The mid-points of the unfolding transition calculated from these curves are around 62°C.

Thermal melting profile

The melting-profile of the enzyme was determined by continuously monitoring the c.d. signal at 222 nm in the temperatue range of 10°-85°C. Figure 5 shows the temperature dependence of the mean residue ellipticity, $[\theta]$ at 222 nm, with a sharp
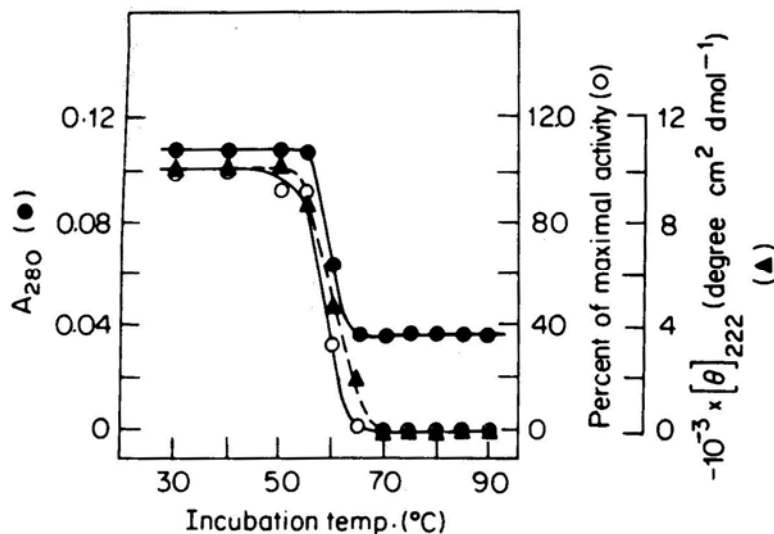
Figure 4. Effect of preincubation temperature on the structure (A_{280} and $-[\theta]_{222}$) and enzyme activity. The enzyme (270 μg/ml) was heated at different temperatures (30°-90°C) for 10 min and immediately chilled in ice. The sample was used to measure the $-[\theta]_{222}$ value (Δ), A_{280}(O) at 25°C and enzyme activity at 37°C (O). Activity of the unheated enzyme was normalized to 100.
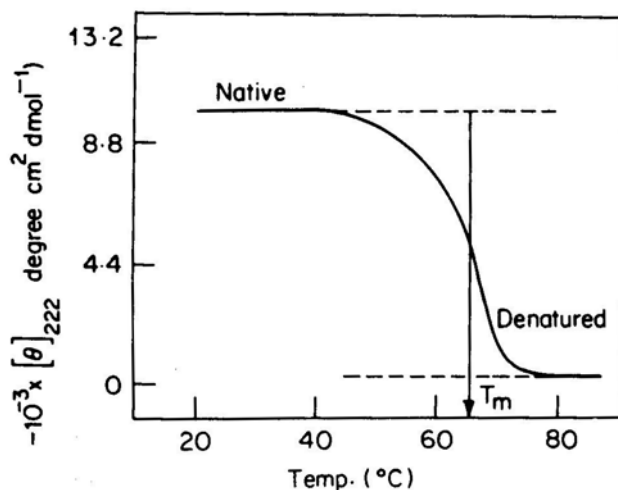

Figure 5. The thermal melting profile. A quartz cell of 1 mm path length and a protein concentration of 0.5mg/ml were used to monitor the c. d. signals at 222nm. It was ascertained that no significant changes in the concentration of the enzyme occurred at high temperatures due to evaporation, by tightly closing the cell with a cap and parafilm. The temperature of the enzyme solution in the cell was gradually increased in a step-wise manner from 20° to 85°C. The circular dichroic signals were recorded after equilibrating the cell for at least 10min, after each desired temperature was attained.

co-operative thermal transition in the range of 50°-70°C. A melting temperature (T_m, defined as the temperature at which the melting-profile crosses the mid-point between the two values extrapolated from the native and the denatured region of the macromolecule) of 65°C was observed. When the enzyme was heated at temperatures above 50°C, cooled and $[\theta]_{222}$ values determined, the values obtained were identical to those observed at the corresponding elevated temperatures.

Effect of substrates on the thermal stability of the enzyme

The susceptibility of the enzyme to thermal inactivation in the absence and in the presence of added substrates is shown in figure 6. In the absence of any substrate,
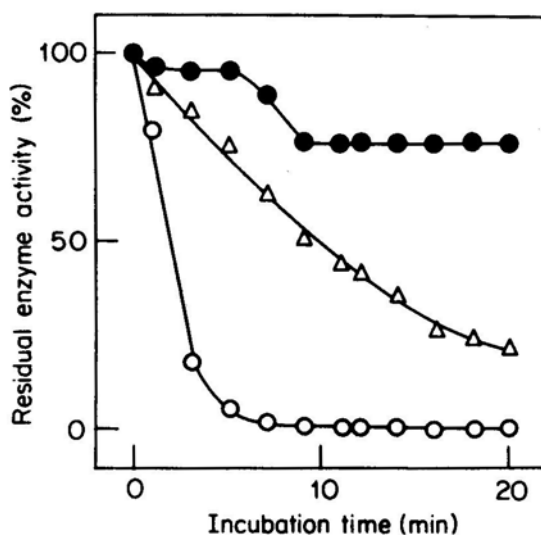

Figure 6. Heat-inactivation patterns of the enzyme in the presence of L-serine and H₄folate
The enzyme (100 μg/400 μ/ of 50 mM potassium phosphate buffer, pH 7.4) in the presence of 18 mM L-serine (●), 9 mM H₄folate (Δ) and enzyme alone (O) was heated at 70°C. Aliquots were removed at definite time intervals and the thermal inactivation of the enzyme was terminated by chilling them in ice. The enzyme activity was assayed at 37°C. The enzyme activity of the sample which was not subjected to heat treatment was normalized to 100.

the enzyme lost its activity within 6-7 min on heating at 70°C. However, in the presence of L-serine (18 mM), there was only slight inactivation (<2%) within a 5 min period and at 10 min, 20% inactivation of the enzyme occurred. After this time, there was no further loss of activity upto 20 min. Addition of H₄folate to the enzyme prior to heating at 70°C, did not result in significant protection of the enzyme from inactivation. NADH and NADPH (allosteric activators, upto 10 mM) exerted no significant influence on the thermal inactivation of the enzyme at 70°C.

To understand the relationship between the observed structural transition and the catalytic function, circular dichroism was used in order to confirm the presence of conformational transition in the enzyme which exhibited substrate-induced

thermal stability (Ramesh and Appaji Rao 1980b). As can be seen in figure 7, the c.d. spectrum of the enzyme heated at 70° C (10 min) in the presence of L-serine (18 mM) showed significant c d. ellipticitybands at 221and 208 nm, as compared to the c.d. spectrum of the enzyme obtained on heating at 70°C without the addition of L-serine. The latter spectrum indicated the absence of even the traces of secondary structure in the enzyme. For comparison, the spectrum of the enzyme, which was not subjected to heating at 70°C is shown in figure 7.
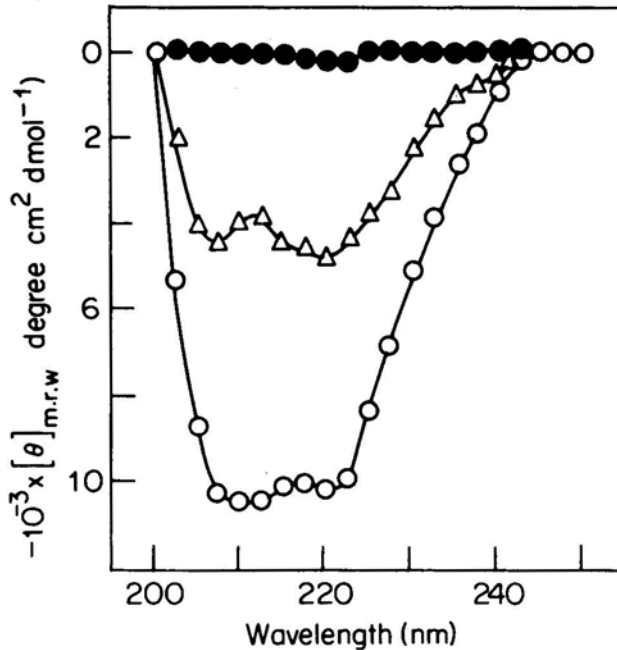

Figure 7. Protective effect of L-serine against temperature-induced loss of circular dichroic properties of the enzyme. The enzyme (270 μg/ml) was heated for 10 min at 70°C in the absence (●) and in the presence (Δ) of 18 mM L-serine. The sample was immediately chilled in ice and the c.d. spectra recorded at 25°C using a 0.5 mm light path demountable quartz cell. For comparison, the spectrum of the enzyme which was not heated at 70°C is shown (O).

Effect of temperature on the positive homotropic interaction of H_4folate (i.e.,Hill coefficient, h) with the enzyme

Figure 8 depicts the *h* values plotted vs. incubation temperature. It is evident from the figure that the *h* value of 2.5 was not altered upto 38°C and above this temperature there was a rapid decrease in the *h* value upto 45°C (*h* = 1.7). The *h* value of 1.7 remained constant upto 60°C and above this temperature there was a further decline in the *h* value; at 70°C, the Hill coefficient reached a value of 1.0. The H_4folate saturation curve at 70°C was hyperbolic and the Lineweaver-Burk plot was linear.

The enzyme purified using heat denaturation step (70°C, 5 min) failed to exhibit positive homotropic co-operative interactions with H_4folate. This is indicated by a hyperbolic saturation pattern (figure 9) with a Hill coefficient (*h*) value of 1.1 (inset to figure 9).
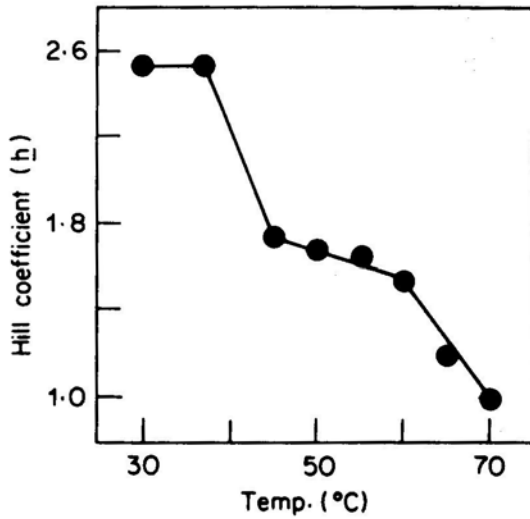
Figure 8. Effect of temperature on the Hill coefficient of H4folate saturation of the enzyme The enzyme (1 μg) was preincubated with different concentrations of H_4folate (0.036-2.2 mM) for 5 min and then for 15 min with 3.6mM L-[$3^{-14}C$] serine at different fixed temperatures (30°-70°C). After terminating the reaction by the addition of dimedone, the amount of [^{14}C] formaldehyde formed was estimated (Ramesh and Appaji Rao, 1978). The Hill coefficient(h) was calculated from a Hill plot of the H_4folate saturation, curve at each temperature (Segel, 1975).
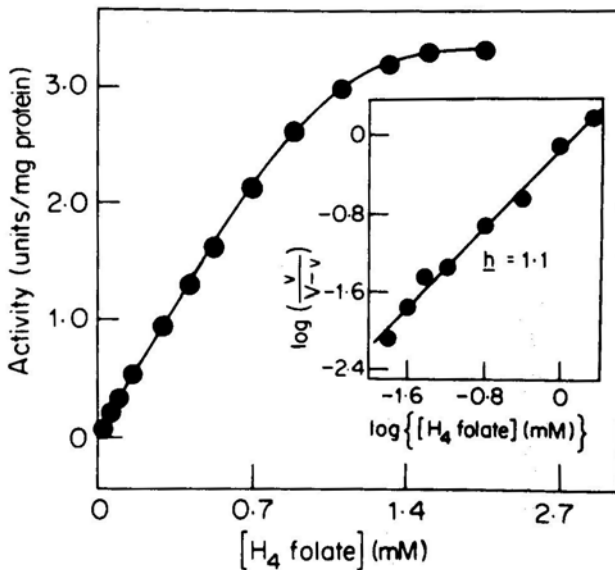

Figure 9. H_4folate saturation pattern of the enzyme purified using heat denaturation step. The enzyme from monkey liver was purified by heating the crude extract for 5 min at 70°C, followed by $(NH_4)_2SO_4$ fractionation, CM-Sephadex chromatography, ultrogel-ACA34 gel filtration and Blue Sepharose CL-6B affinity chromatography (Ramesh & Appaji Rao, 1980b). This enzyme preparation was used for the H_4folate saturation experiment (for details see Ramesh and Appaji Rao, 1978). The inset shows the Hill plot of the data obtained.

Discussion

Thermally induced conformational transitions have been accepted as explanations for the temperature dependence of a number of properties of macromolecules (Massey *et al.,* 1966; Berman and Boyer, 1972; Kelley and Von Hippel, 1976; Zav'yalov *et al.,* 1977; Sturtevant and Matco, 1978; Fujita *et al.,* 1979; Matheson and Scheraga, 1979a, b; Ralston and Dunbar, 1979; Vita *et al.,* 1979 Ugarovo *et al.,* 1979; Luer and Wong, 1980) and have helped in the understanding of the forces that stabilize the higher order structure of proteins and of protein-ligand interactions. The Arrhenius plot for the monkey liver serine hydroxymethyltrans-ferase showed a biphasic curve, with a break at 15°C (figure 1) suggesting tempera-ture-induced conformational change in the molecule. Non-linear Arrhenius plots similar to that observed in the present study have been reported for a few proteins (Sizer, 1943; Levy *et al.,* 1959; Massey *et al.,* 1966; Saint-Blancard *et al.,* 1977; Matheson and Scheraga, 1979b; Berge *et al.,* 1980) and have been interpreted to suggest temperature induced conformational changes. Before invoking this, possibilities such as (i) phase change in the solvent, (ii) failure to maintain satura-tion of the enzyme with the substrates at elevated temperatures, (iii) artifacts of mixing, (iv) reversible enzyme inactivation, and (v) buffer effects have to be ruled out (Dixon and Webb, 1958). These have been carefully considered and ruled out before invoking conformational change in the enzyme. Thus the nonlinear Arrhenius plot provides evidence in favour of the existence of non-denaturational transitions and also illustrates the concept of limited molecular rearrangement as a possible mechanism for metabolic regulation.

Temperature induced transitions in the enzyme structure are evident from the changes in A_{280} which is indicative of alterations in the tertiary structure; $^{-}[\theta]_{222}$ values, which are correlated to secondary structure of the protein; and the loss of enzyme activity reflecting alterations in the catalytic site (figure 4). By using thermal unfolding as a sensitive probe to measure changes in the different orders of structural organization in the enzyme molecule, it was found that the loss of enzyme activity, and the decrease in A_{280} and $-[\theta]_{222}$ values followed a similar pattern suggesting that these properties are closely related. A similar T_m (62°C) value was obtained, for all the parameters plotted as a function of pre-incubation temperature. A similarity of the melting profiles and the occurrence of an identical T_m value for different conformational probes have been used as evidence for a two-state co-operative unfolding process, without observable intermediates (Lumry *et al.,* 1966).

The changes in $^{-}[\theta]_{222}$ values displayed a sigmoidal dependence on the tempera-ture (figure 5) in the range of 50°-70°C. A T_m value of 65°C was calculated from this curve. It was also seen that in the region of the major thermal transition (50°-70°C), no evidence for intermediates were found. These results are compatible with the hypothesis that two-state co-operative transitions occur during the thermal denaturation of monkey liver serine hydroxymethyltransferase. However, this can be confirmed only after performing precise differential scanning microcalorimetric studies and fast reaction temperature-jump experiments.

The binding of L-serine to the enzyme confers significant thermal stability to the enzyme, whereas H_4folate affords only meagre protection against thermal inactivation (figure 6). These results suggest that the binding of L-serine promotes a conformational change in the native enzyme to a thermostable conformer and this conclusion has been supported by circular dichroism studies (figure 7). Ligand induced stabilization against heat inactivation may also be explained by invoking protection by the substrate of heat labile groups at or near the active site on the enzyme molecule. Similar explanations for substrate protection have been given by several workers (Grisolia and Joyce, 1960; Segel *et al.,* 1968; Theorell and Tatemoto, 1971; Citri, 1973).

At high temperatures, above 65°C, the H_4folate saturation curve became hyperbolic, as indicated by a Hill coefficient value of 1.0 (figure 8). In other words, the homotropic co-operative interactions of H_4folate with the enzyme was abolished at high temperatures. At temperatures above 60°C, the critical interaction between subunits seems to have been lost. These results are further confirmed by desensitization of the enzyme using a heat step during the purification (figure 9). Although the studies on the effect of temperature on the structure of the enzyme clearly indicated the absence of intermediates in the process of denaturation (as monitored by c.d., A_{280}, and enzyme activity), the effect of temperature on the Hill coefficient for the interaction of H_4folate with the enzyme, showed the presence of an intermediate conformer with an *h* value of 1.7. The mol. wt of the native as well as the temperature desensitized enzyme were identical indicating that dissociation of the enzyme may not have occurred at elevated temperatures (data not given). These results would suggest that intermediate conformations are possible during the process of thermal denaturation. The apparent discrepancy in the results obtained by spectroscopic methods and the kinetic methods, may be due to the differences in the sensitivity of these two probes.

In conclusion, the results presented in this paper suggest that temperature induced conformational changes may have a role in the metabolic regulation of serine hydroxymethyltransferase.

Acknowledgements

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