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Identification of active-site residues of sheep liver serine hydroxymethyltransferase

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Chemical modification of amino acid residues with phenylglyoxal, *N*-ethylmaleimide and diethyl pyrocarbonate indicated that at least one residue each of arginine, cysteine and histidine were essential for the activity of sheep liver serine hydroxymethyltransferase. The second-order rate constants for inactivation were calculated to be $0.016\,mM^{-1}\cdot min^{-1}$ for phenylglyoxal, $0.52\,mM^{-1}\cdot min^{-1}$ for *N*-ethylmaleimide and $0.06\,mM^{-1}\cdot min^{-1}$ for diethyl pyrocarbonate. Different rates of modification of these residues in the presence and in the absence of substrates and the cofactor pyridoxal 5'-phosphate as well as the spectra of the modified protein suggested that these residues might occur at the active site of the enzyme.

Although the catalytic mechanism of serine hydroxymethyltransferase (5,10-methylenetetrahydrofolate:glycine hydroxymethyltransferase, EC 2.1.2.1) has been studied in considerable detail (Schirch, 1982), there is little direct evidence for the presence of specific amino acid residues at the active site of this enzyme. The identification of these residues is of importance as the enzyme from rabbit liver has been crystallized (Schirch, 1981) and the elucidation of its three-dimensional structure has now become possible. The present paper describes the identification of arginine, cysteine and histidine at the active site of sheep liver serine hydroxymethyltransferase.

Materials and methods

Chemicals

All biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Tetrahydrofolate (tetrahydropteroylglutamate) was prepared by the method of Hatefi *et al.* (1960). L-[3-^{14}C]Serine (specific radioactivity 58.5 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Enzyme

Homogeneous sheep liver serine hydroxymethyltransferase was prepared and assayed as described previously (Manohar *et al.*, 1982). One unit of enzyme activity is defined as the amount that catalysed the formation of 1 μmol of formaldehyde/min. Specific activity is expressed as units/g of protein. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Modification by phenylglyoxal, N-ethylmaleimide and diethyl pyrocarbonate

The enzyme (0.5 mg/ml) in 50 mM-potassium phosphate buffer, pH 7.1, containing 1 mM-EDTA was incubated at 37°C with phenylglyoxal (0–4 mM), and after various intervals 10 μl samples were withdrawn into tubes that contained, in addition to the usual components of the assay mixture, L-arginine (10 mM). The enzyme was assayed as described previously (Manohar *et al.*, 1982) with L-[3-^{14}C]serine as substrate, and residual enzyme activity was expressed as percentage of the activity in the absence of phenylglyoxal. Arginine had no effect on the rate of the enzyme reaction. The apoenzyme modification by phenylglyoxal was carried out similarly, except that after reaction with phenylglyoxal it was reconstituted with an excess (2.5 mM) of pyridoxal 5'-phosphate in the assay mixtures employed for determining residual enzyme activity.

Inactivation with *N*-ethylmaleimide (0–0.55 mM) was carried out as described for phenylglyoxal except that 2-mercaptoethanol (10 mM) was present in the assay for residual enzyme activity instead of arginine.

Inactivation by diethyl pyrocarbonate in 50 mM-potassium phosphate buffer, pH 6.8, was carried out essentially as described for phenylglyoxal, with

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L-histidine (10mM) instead of arginine added to inactivate excess reagent.

Reversal of diethyl pyrocarbonate modification by hydroxylamine

The holoenzyme or the apoenzyme (0.5mg/ml) in 50mM-potassium phosphate buffer, pH6.8, containing 1mM-EDTA was incubated with diethyl pyrocarbonate (1mM) for 20min at 37°C. L-Histidine (50mM) followed by 1ml of hydroxylamine hydrochloride (adjusted to pH 7.0 with KOH) was added to the inactivation mixture. After 20h at 22°C the mixture was dialysed for 6h at 4°C against four changes of 50mM-potassium phosphate buffer, pH7.1, containing 1mM-2-mercaptoethanol and 1mM-EDTA. The enzyme was assayed after incubation for 15min in the presence of a large excess (2.5mM) of pyridoxal 5'-phosphate. In a control experiment the enzyme was not treated with diethyl pyrocarbonate but was incubated with hydroxylamine and processed in the same manner.

Absorption spectra

Absorption spectra were recorded with a Cary 219 double-beam spectrophotometer. Difference absorption spectra were recorded with a Shimadzu UV-190 spectrophotometer. All enzyme solutions were previously dialysed extensively against the appropriate buffers.

Results

Inactivation of the enzyme by phenylglyoxal

From the inactivation curves obtained at different concentrations of phenylglyoxal (0–4mM), a first-order plot was constructed (Fig. 1). The pseudo-first-order rate constant ($k_{app.}$) values at different concentrations of phenylglyoxal obtained from this plot were: 0.018 min^{-1} at 1mM; 0.032min^{-1} at 2mM; 0.048min^{-1} at 3mM; 0.067min^{-1} at 4mM. A plot of $\log k_{app.}$ versus log[phenylglyoxal] (Fig. 1 inset) gave a slope value of 1.1, indicating that reaction with a single molecule of phenylglyoxal was probably sufficient to inactivate the enzyme. From these data a second-order rate constant of 0.016$mM^{-1} \cdot min^{-1}$ for phenylglyoxal was calculated. The theoretical basis for the calculation of the rate constants as well as the number of mol of reagent reacting/mol are given in Levy *et al.* (1963).

The rate of inactivation of the holoenzyme was slightly faster than that of the apoenzyme at the same concentration (4mM) of phenylglyoxal ($k_{app.}$ = 0.027min^{-1} and 0.067min^{-1} for apoenzyme and holoenzyme respectively), showing that pyridoxal 5'-phosphate did not appear to protect the enzyme against inactivation by phenylglyoxal. Both L-serine and tetrahydrofolate protected the holoenzyme against inactivation by phenylglyoxal (Table 1). In the case of the apoenzyme, however, although tetrahydrofolate almost completely protected the enzyme against inactivation, L-serine had no effect (Table 1).

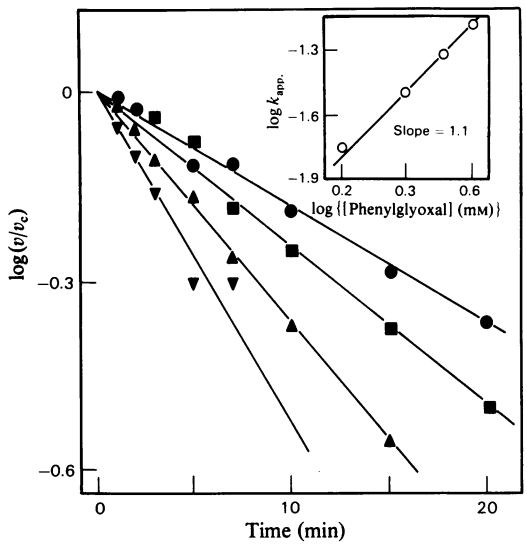

Fig. 1. *First-order plots of the inactivation of serine hydroxymethyltransferase by phenylglyoxal*
Inactivation of the holoenzyme (0.5mg/ml) was carried out at different concentrations (●, 1mM; ■, 2mM; ▲, 3mM; ▼, 4mM) of phenylglyoxal as described in the Materials and methods section. v is the enzyme activity remaining at any given time, and v_c is the activity of the uninhibited enzyme. Inset: second-order plot of the pseudo-first-order rates ($k_{app.}$) of inactivation at different concentrations (1–4mM) of phenylglyoxal.

Spectral changes in the holoenzyme on interaction with phenylglyoxal

The intensity of the absorbance peak of the enzyme-bound pyridoxal 5'-phosphate progressively increased and reached a maximum value after 30min; the changes were also characterized by a broadening of the absorbance peak and its shift to lower wavelengths (390–395nm) (Fig. 2).

Inactivation of the enzyme by diethyl pyrocarbonate

The rates of inactivation of the holoenzyme at different concentrations (0.25–1mM) of diethyl pyrocarbonate were determined as described in the Materials and methods section, and $k_{app.}$ values at 0.25mM-, 0.50mM-, 0.75mM- and 1.0mM-diethyl pyrocarbonate were 0.013, 0.035, 0.048 and 0.059min^{-1} respectively. A slope of 1.0 was obtained in a plot (not shown) of $\log k_{app.}$ versus log[diethyl pyrocarbonate], suggesting that a single

Table 1. *Effects of substrates and cofactor on the rate of inactivation of serine hydroxymethyltransferase by phenylglyoxal, diethyl pyrocarbonate and N-ethylmaleimide*
For experimental details see the text.

Substrate/cofactor	$k_{app.}$ (min^{-1}) With phenylglyoxal (4mM)	With diethyl pyrocarbonate (1mM)	With *N*-ethylmaleimide (0.05mM)
None	0.027	0.33	0.18
Pyridoxal 5′-phosphate	0.067	0.059	0.09
Tetrahydrofolate	0.006	0.014	0.03
L-Serine	0.027	0.33	0.18
Pyridoxal 5′-phosphate + tetrahydrofolate	0.009	0.014	0.011
Pyridoxal 5′-phosphate + L-serine	0.013	0.023	0.032

Table 2. *Reversal of diethyl pyrocarbonate modification of serine hydroxymethyltransferase by treatment with hydroxylamine*
For experimental details see the text. Apoenzyme was reconstituted with excess (2.5mM) of pyridoxal 5′-phosphate before being assayed for enzyme activity.

Treatment	Specific activity (units/mg of protein) Holoenzyme	Apoenzyme
Untreated	6.3	5.8
Enzyme + diethyl pyrocarbonate	0.54	0
Enzyme + NH_2OH	5.6	5.4
Enzyme + diethyl pyrocarbonate + NH_2OH	3.61	3.18

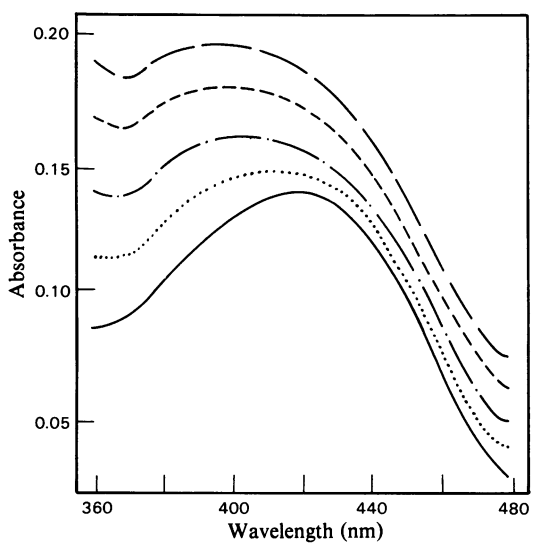

Fig. 2. *Changes in the visible-region absorbance spectrum of serine hydroxymethyltransferase holoenzyme on interaction with phenylglyoxal*
The enzyme (1.5mg/ml) in 50mM-potassium buffer, pH7.1, containing 1mM-EDTA was mixed with phenylglyoxal (5mM), and the visible-region absorbance spectrum was recorded with a Cary 219 spectrophotometer after various intervals (——, 0min; ·····, 1min; —·—, 7min; ----, 20min; ——, 30min) at 22°C.

molecule of diethyl pyrocarbonate was adequate for inactivating the holoenzyme.

The apoenzyme was inactivated at a faster rate (0.33min^{-1}) than was the holoenzyme (0.09min^{-1}) at 1mM-diethyl pyrocarbonate, showing that pyridoxal 5′-phosphate protected the enzyme against inactivation. Both L-serine and tetrahydrofolate protected the holoenzyme against the inactivation, whereas with the apoenzyme tetrahydrofolate could protect against inactivation by the reagent but L-serine had no effect (Table 1).

It has been reported that the 1:1 diethyl pyrocarbonate–histidine complex can be cleaved by reaction with a nucleophilic reagent such as hydroxylamine (Miles, 1977). Table 2 shows that with hydroxylamine about 65% of the control activity was recovered for the holoenzyme and about 60% for the apoenzyme.

Absorption spectra of the product of the enzyme reaction with diethyl pyrocarbonate

The characteristic absorption peak at 240nm in the protein difference spectrum (Fig. 3) as well as the increase in its intensity with time of reaction indicated that diethyl pyrocarbonate was probably modifying a histidine residue. It may also be noted that the difference spectrum in Fig. 3 did not show a minimum in the region of 280nm that is

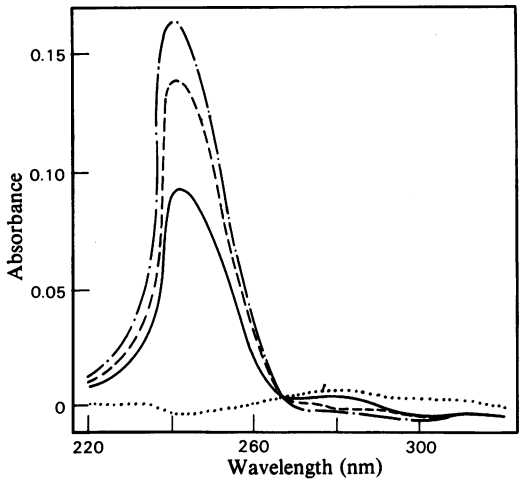

Fig. 3. *Protein difference spectra of the interaction of serine hydroxymethyltransferase apoenzyme with diethyl pyrocarbonate*

Samples (1ml) of the apoenzyme (1.5mg/ml) in 50mM-potassium phosphate buffer, pH6.8, containing 1mM-EDTA and 1mM-2-mercaptoethanol were placed in sample and reference cuvettes to record the base-line spectrum (·····). Diethyl pyrocarbonate dissolved in ethanol (10μl) was added to the sample cuvette to a final concentration of 1mM, and 10μl of ethanol was added to the reference cuvette. The difference spectrum was recorded after three different periods (——, 1min; – – – –, 3min; —·—, 6min and 9min) at 22°C.

characteristic of the modification of tyrosine by diethyl pyrocarbonate (Miles, 1977).

Diethyl pyrocarbonate also marginally perturbed the visible-region absorption spectrum of the bound pyridoxal 5′-phosphate in the holoenzyme. It was observed that the change in the visible-region absorbance spectrum was marked by a small decrease in absorbance at 415nm and the occurrence of points of inflexion at 395 and 450nm.

Time course of inactivation of the enzyme with N-ethylmaleimide

The rates of inactivation of the holoenzyme at different concentrations (0.05–0.55mM) of *N*-ethylmaleimide were determined as described in the Materials and methods section. The apparent rate constants at 0.05mM-, 0.15mM-, 0.35mM- and 0.55mM-*N*-ethylmaleimide were 0.09, 0.14, 0.22 and 0.33min^{-1} respectively. From a plot (not shown) of $\log k_{app.}$ against log[*N*-ethylmaleimide] a slope of 0.8 was obtained, indicating that the reaction of a single molecule of *N*-ethylmaleimide was sufficient to inactivate the holoenzyme.

The rate of inactivation of the apoenzyme (0.18min^{-1}) was faster than that of the holoenzyme (0.09min^{-1}) at 0.05mM-*N*-ethylmaleimide, showing that pyridoxal 5′-phosphate protected the enzyme against inactivation by *N*-ethylmaleimide. The substrates, L-serine (3.6mM) and tetrahydrofolate (2.7mM), protected the holoenzyme against inactivation by *N*-ethylmaleimide (Table 1). The apoenzyme, on the other hand, was also protected against the inactivation by tetrahydrofolate but L-serine was without effect (Table 1).

Discussion

Earlier studies with serine hydroxymethyltransferase isolated from rabbit liver had indicated that (i) a residue with a pK_a greater than 12 was involved in the binding of carboxy groups of substrates (Schirch & Diller, 1971), (ii) thiol groups were necessary for activity and that there was differential reactivity in these groups (Schirch *et al.*, 1973), and (iii) a group with a pK_a of 6.8 was essential for catalysis (Schirch & Diller, 1971). It could be postulated that an arginine residue may be responsible for observation (i), a thiol group at the active site for observation (ii), and a histidine may represent the ionizing group with a pK_a of 6.8 (observation iii).

The involvement of an arginine residue in the active site of sheep liver serine hydroxymethyltransferase was indicated by the data presented in Figs. 1 and 2. The ability of tetrahydrofolate to protect both the apoenzyme and the holoenzyme suggested that arginine might be interacting with this substrate. The amino acid composition (Bossa *et al.*, 1976) revealed the occurrence of 24 arginine residues in the rabbit liver enzyme molecule.

Arginine residues can serve as positively charged recognition sites for negatively charged substrates containing phosphate or carboxylate groups and anionic cofactor in enzyme active sites (Riordan *et al.*, 1977; Riordan, 1979; Wong & Wong, 1981). By using a number of amino acid substrate analogues it was demonstrated that the carboxy group of the substrate was essential for binding at the active site but that this was not dependent on the pH of the reaction (Schirch & Diller, 1971). This was explained by assuming that the exposure of this site occurred only on binding of the amino group of the substrate. The presence of an arginine residue with a pK_a value of >12 can also explain these results. In the case of serine hydroxymethyltransferase, besides the amino acid substrate, the second substrate, tetrahydrofolate, also has a free carboxy group, which is largely ionized at neutral pH, suggesting that arginine at the active site could also bind the free carboxy group of tetrahydrofolate. This is feasible, since

there occur as many as three possible anion-binding centres on a single arginine residue (Cotton *et al.*, 1973; Riordan & Scandura, 1975; Gilbert & O'Leary, 1975; Azaryan *et al.*, 1978).

The postulation of a histidine residue at the active site of this enzyme was supported by the following observations: (i) at pH6.8, at which the modification was carried out, diethyl pyrocarbonate is specific for histidine; (ii) there was a sharp increase in the absorption at 240nm due to modification by diethyl pyrocarbonate (Fig. 3); (iii) the absence of an absorbance minimum at 278nm accompanied by a sharp decrease in absorbance at wavelengths below 240nm in the protein difference spectrum due to *O*-carbethoxylation of tyrosine residues (Fig. 3) indicated that a tyrosine modification might not be occurring; (iv) after modification with diethyl pyrocarbonate the enzyme activity was partially restored by treatment with hydroxylamine (Table 2), suggesting that a thiol or a primary amino group was not being modified in this reaction.

Bossa *et al.* (1976) sequenced a nonapeptide at the pyridoxal 5'-phosphate-binding site of the rabbit liver serine hydroxymethyltransferase, and reported the occurrence of a histidine residue adjacent to the *N*-terminal side of the pyridoxal-lysine residue. The present study (Tables 1 and 2 and Fig. 3) provides direct chemical evidence that a histidine residue may be present at the active site of sheep liver serine hydroxymethyltransferase.

The involvement of a thiol group in the active site of the enzyme was indicated by the data presented in Table 1. The faster rate of inactivation of the apoenzyme as compared with the holoenzyme (Table 1) reflected a possible partial masking of the reactive thiol group by pyridoxal 5'-phosphate. In rabbit liver cytosolic serine hydroxymethyltransferase two classes of thiol groups have been detected, one reacting rapidly with dithionitrobenzaldehyde and the other slowly (Schirch *et al.*, 1973). From such studies it was suggested that there could be two thiol groups in the active site of the enzyme. The studies reported in the present paper are in partial agreement with this postulation.

On the basis of the results reported in the present paper in combination with the observations made by other workers, a probable mechanism for the reactions at the active site of sheep liver serine hydroxymethyltransferase can be postulated. The holoenzyme has pyridoxal 5'-phosphate at the active site in a Schiff-base complex with the ε-amino group of a lysine residue (Bossa *et al.*, 1976). The active site is postulated to contain, in addition, a histidine, an arginine and a thiol group. The arginine residue could provide a locus for the binding of the carboxy group of tetrahydrofolate.

In the initial phase of the reaction, L-serine displaces the ε-amino group of lysine to form a Schiff-base complex with pyridoxal 5'-phosphate. The carboxy group of the substrate amino acid could be bound at a locus on the arginine residue. At this time there probably occurs a transfer of a proton from the serine hydroxy group to the imidazole ring of histidine, concerted with the transfer of the proton from the other side of the imidazole ring to the ε-amino group of the lysine residue (Ulevitch & Kallen, 1977). The formation of the alkoxide anion facilitates $C_{(\alpha)}$–$C_{(\beta)}$ bond cleavage in L-serine, leading to the transfer of a hydroxymethyl group to tetrahydrofolate (Ulevitch & Kallen, 1977). Thus lysine is the ultimate base that accepts and stores the proton until in the final step of the reaction, in which it gives up the proton to the α-carbon of the product, glycine (Schirch, 1982).

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