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Arginine Residues Involved in Binding of Tetrahydrofolate to Sheep Liver Serine Hydroxymethyltransferase*

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The arginine residue(s) necessary for tetrahydrofolate binding to sheep liver serine hydroxymethyltransferase were located by phenylglyoxal modification. The incorporation of [7-^{14}C]phenylglyoxal indicated that 2 arginine residues were modified per subunit of the enzyme and the modification of these residues was prevented by tetrahydrofolate. In order to locate the sites of phenylglyoxal modification, the enzyme was reacted in the presence and absence of tetrahydrofolate using unlabeled and radioactive phenylglyoxal, respectively. The labeled phenylglyoxal-treated enzyme was digested with trypsin, and the radiolabeled peptides were purified by high-performance liquid chromatography on reversed-phase columns. Sequencing the tryptic peptides indicated that Arg-269 and Arg-462 were the sites of phenylglyoxal modification. Neither a spectrally discernible 495-nm intermediate (characteristic of the native enzyme when substrates are added) nor its enhancement by the addition of tetrahydrofolate, was observed with the phenylglyoxal-modified enzyme. There was no enhancement of the rate of the exchange of the α-proton of glycine upon addition of tetrahydrofolate to the modified enzyme as was observed with the native enzyme. These results demonstrate the requirement of specific arginine residues for the interaction of tetrahydrofolate with sheep liver serine hydroxymethyltransferase.

Serine hydroxymethyltransferase (SHMT,[1] EC 2.1.2.1), a pyridoxal 5′-phosphate (PLP) protein functions in the generation of one-carbon units for a variety of end products. The enzyme catalyzes the conversion of serine and tetrahydrofolate (H_4-folate) to form glycine and 5,10-methylene H_4-folate. In addition, a number of aldolytic cleavage reactions are catalyzed by the enzyme, but H_4-folate is required only when formaldehyde is formed (1, 2). Although the catalytic mechanism of the enzyme has been studied in some detail, the amino acids that form the active site have not been clearly defined. Chemical modification studies with the sheep, human liver, and mung bean enzymes indicated that arginine, histidine, and cysteine residues were essential for enzyme activity (3–5). These studies did not pinpoint the specific residues in the primary structure which were essential for activity.

In this paper, we report the identification of 2 Arg residues, Arg-269 and Arg-462, in the primary structure of the sheep liver enzyme which are involved in binding H_4-folate and in enzyme catalysis.

EXPERIMENTAL PROCEDURES

Materials—[7-^{14}C]Phenylglyoxal (23.1 mCi/mmol) was purchased from Amersham International (Bucks, England). [2-^{3}H]Glycine (53.3 Ci/mmol) was obtained from Du Pont-New England Nuclear Research Products. Nonradioactive phenylglyoxal (PG), *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-trypsin, trifluoroacetic acid were from Sigma. Phenyl isothiocyanate sequencing grade was from Pierce Chemical Co. Polyamide sheets were obtained from Schleicher & Schuell. High-performance liquid chromatography (HPLC) grade acetonitrile was obtained from Spectrochem (Bombay, India). H_4-Folate was prepared by the method of Hatefi *et al.* (6). HPLC columns: Aquapore RP-300 (4.6 × 250 mm, 7 μm) was from Pyeunicam, Cambridge, UK, Spherisorb ODS 2 (4 × 250 mm, 5 μm) was from Pharmacia, LKB (Uppsala, Sweden). All other reagents were of the highest grade available from commercial sources.

Incorporation of 7-[^{14}C]PG—Sheep liver SHMT (5 μM) was incubated with 4 mM PG (23.1 mCi/mmol) in 50 mM potassium phosphate buffer, pH 7.4. The reaction was carried out in the presence or absence of 2 mM H_4-folate at 37 °C for 1 h. After incubation, a 1-μl aliquot was spotted on a glass fiber filter paper disc, dried, and counted to determine the input radioactivity. The remaining aliquot was precipitated with 10% trichloroacetic acid. The precipitate was washed extensively with 10% trichloroacetic acid followed by ethanol and dried. The precipitate was dissolved in 50 μl of formic acid and the radioactivity measured.

Isolation of the PG-modified Peptides from Sheep Liver SHMT—SHMT (5 μM) was incubated at 37 °C for 45 min with 4 mM PG in the presence of 2 mM H_4-folate. The modified enzyme was dialyzed against 50 mM potassium phosphate buffer (500 ml × 4) for 6 h to remove H_4-folate. The enzyme was modified again with 2 mM [7-^{14}C] PG for 1 h at 37 °C in the absence of H_4-folate. The enzyme was precipitated with 10% trichloroacetic acid and treated with performic acid (7). The conditions for digestion and separation of peptides are given (see the legends to Figs. 1*A*, 1*B*, and 2).

Sequencing of the Peptides—Peptide 14 was sequenced by the manual 4-*N*,*N*′-dimethyl aminoazobenzene isothiocyanate/phenyl isothiocyanate double-coupling method (8, 9). Peptide 11 was sequenced using a gas-phase automated protein sequenator from Shimadzu, PSQ-1.

Spectral Studies—All spectra were recorded on a Shimadzu UV-240 double-beam spectrophotometer.

RESULTS

Stoichiometry of the Modification of Sheep Liver SHMT by [7-^{14}C]PG—The radioactivity incorporated into the enzyme in the presence of H_4-folate (3.01×10^4 cpm) was approximately 10 times less than that in its absence (2.2×10^5 cpm). This observation suggested that the Arg residue(s) were protected by H_4-folate against modification. The stoichiometry of the reaction was calculated by deducting the incorporation

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[1] The abbreviations used are: SHMT, serine hydroxymethyltransferase; PG, phenylglyoxal; H_4-folate, 5,6,7,8-tetrahydrofolate; PLP, pyridoxal 5′-phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; HPLC, high-performance liquid chromatography; DABTH, 4-*N*,*N*′-dimethylaminoazobenzene thiohydantoin.

of PG in the presence of H_4-folate from that in its absence. Approximately 4 mol of [7-^{14}C]PG were bound per mol of subunit (M_r 50,000) of SHMT when the enzyme was completely inactivated. As in most instances, two molecules of PG react with 1 Arg residue (10); this result suggested that the modification of 2 Arg residues were essential for complete loss of activity.

Separation of Tryptic Peptides Obtained from Control and PG-modified SHMT—The profiles of peptides from the control and PG-modified enzymes are shown in Fig. 1. The estimation of radioactivity in each fraction allowed the identification of two radiolabeled peaks (numbered 11, 14) in the PG-modified enzyme profile but absent in the control unmodified enzyme. This is because Arg modified by PG in proteins is known to be resistant to tryptic cleavage (10). Further resolution of the two peptides was achieved by rechromatography of these peptides on spherisorb 2 ODS column. The HPLC profiles of peaks 11 and 14 upon rechromatography are given in Fig. 2.

Sequencing of Peptides—The thin-layer chromatography plates of the 4-*N*,*N*′-dimethylaminoazobenzene thiohydantoin derivatives (DABTH) of the peptide sequenced manually are given in Fig. 3. The sequence obtained was A-G-M-I-F-Y-R-K, in which DABTH-modified Arg showed a mobility different from other DABTH derivatives. The carryover from the previous cycle to the next cycle of manual sequencing was

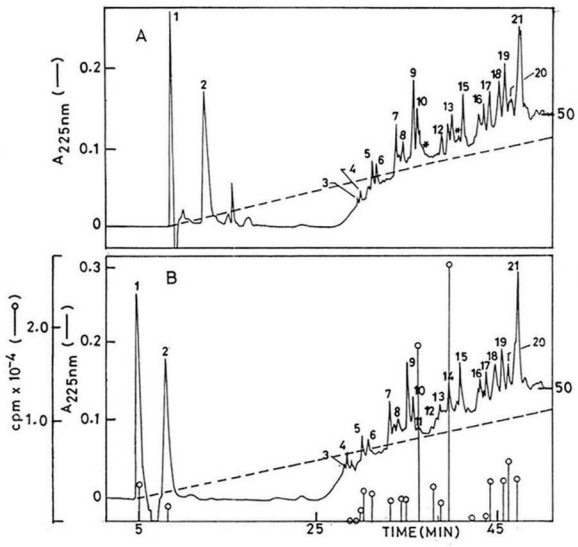

FIG. 1. *A*, the elution profile of the tryptic peptides from unmodified sheep liver SHMT. Unmodified sheep liver SHMT (400 μg) was hydrolyzed with TPCK-treated trypsin at a ratio of 1:100 (protease:protein) in 0.1 M NH_4HCO_3 for 4 h at 37 °C. The digest was lyophilized, dissolved in 0.1% trifluoroacetic acid in water (solvent A), and subjected to HPLC using Shimadzu LC-6A. The tryptic peptides were initially separated on an Aquapore RP-300 column, and the absorbance of the eluate monitored at 225 nm. The peptides were eluted with increasing concentrations of 0.1% trifluoroacetic acid in acetonitrile (solvent B) as follows: 0–5 min, 0% solvent B; 5–60 min, 55% solvent B. The flow rate was 0.5 ml/min. *B*, the elution profile of the tryptic peptides from the PG-modified SHMT. Sheep liver SHMT (400 μg) was modified with 4 mM [7-^{14}C]PG as described under "Experimental Procedures." The tryptic peptides were separated as described in *A*. An aliquot (100 μl) of each fraction was dissolved in aqueous scintillation fluid, and the radioactivity was measured in LKB Rackbeta scintillation counter. The *bars* indicate the radioactivity incorporated in each fraction. In order to obtain peptide 14 (*B*) in sufficient amounts for manual sequencing, sheep liver SHMT (10 mg in 10 ml) was modified with unlabeled PG and digested with TPCK-treated trypsin as described above. Before fractionating the digest, a small aliquot (5000 cpm) of the labeled peptide (peak 14, *B*) was added as a tracer. As peak 11 (*B*) was sequenced using the PSQ-1 automated sequenator. The amounts required were small, and hence, large-scale digestion of the enzyme was not carried out.

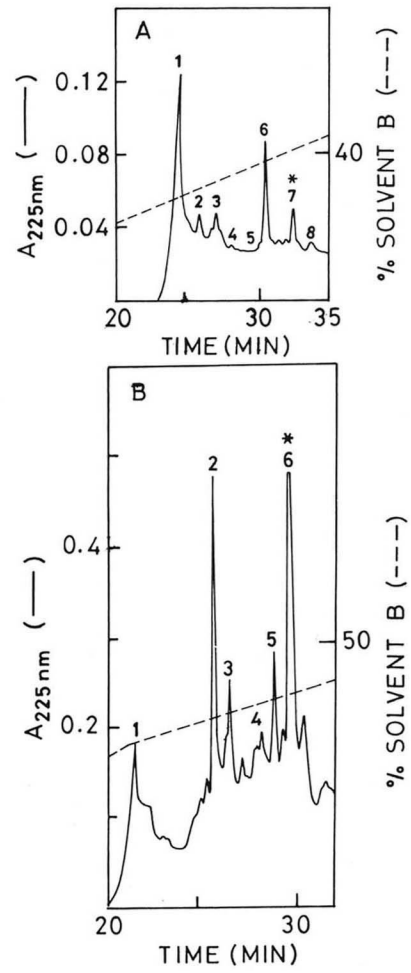

FIG. 2. **Purification of the radioactive peptides.** The tryptic peptides 11 and 14 from Fig. 1*B* were rechromatographed (*A* and *B*) on Spherisorb ODS 2 with 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B) on a Shimadzu LC-6A system. The gradients used are indicated. The *asterisk* marks the peptides carrying radioactivity. The fraction containing radioactivity was collected for sequencing.

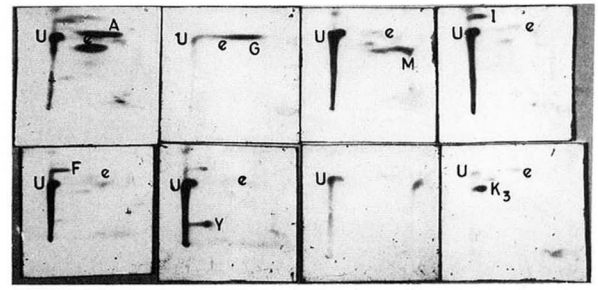

FIG. 3. **Thin-layer chromatography of DABTH-amino acid derivatives from the radioactive peptide 14-6 (Fig. 2).** The DABTH-amino acids were identified by thin-layer chromatography on polyamide sheets (3 × 3 cm) using acetic acid:water (1:2) in the first direction and toluene:*n*-hexane:acetic acid (2:1:0.9) in the second dimension. The spots were located by exposure to HCl vapors. The DABTH-amino acid derivatives are denoted by the *single-letter code*; *U*, thiourea derivative; *e*, DABTC-diethylamine reference marker. DABTH-homoserine (obtained from methionine during performic acid oxidation) overlapped with DABTH-glutamine in two-dimensional chromatography.

TABLE I
Yields of phenylthiohydantoin-amino acids from peptide 11-7

Cycle	Amino acid	Yield
		pmol
1	A	296
2	V	232
3	R	
4	A	198
5	L	172
6	R	53

minimal. The presence of Arg was confirmed by automated sequencing of a 4-kDa cyanogen bromide fragment obtained during the course of the primary structure determination of the sheep liver enzyme.[2] Automated sequencing of peptide 11-7 revealed the sequence A-V-*X*-A-L-R. The Arg at the third position (*X* represents any amino acid) was conspicuously missing from the third cycle of sequencing (Table I). The presence of Arg in the third cycle was confirmed by the sequence of a chymotryptic fragment obtained during primary structure determination. Both the sequences determined contained an Arg residue not cleaved by trypsin, an additional evidence indicating that the residue was indeed modified.

Spectral Studies—Earlier studies (1, 11, 12) with cytosolic SHMT suggested the following mechanism for the enzyme reaction: (*a*) the formation of a geminal diamine followed by; (*b*) the generation of a substrate coenzyme Schiff base; and (*c*) abstraction of the α-proton of the amino acid to form a resonance stabilized quinonoid complex. In the absence of H_4-folate, the concentration of the quinonoid complex was small, while the geminal diamine and Schiff base were present in higher amounts. The addition of H_4-folate shifted the distribution of the complexes with the quinonoid intermediate being the dominant species. The spectrum of the native enzyme (*curve 1*) and the three distinct absorption peaks obtained on the addition of glycine (200 mM) to the holoenzyme (*curve 2*) are shown in Fig. 4. The addition of H_4-folate to the mixture of enzyme and glycine greatly increased the concentration of the quinonoid intermediate absorbing at 495 nm (*curve 3*). Fig. 4 (*inset*) shows the spectrum of the PG-modified enzyme (*curve 4*). There was a broadening of the characteristic 428-nm PLP-Schiff base absorbance and a shift to 390 nm. Upon addition of saturating amounts of glycine to the PG-modified enzyme only two species were observed: the geminal diamine at 343 nm and the glycine Schiff base at 425 nm (*curve 5*). The 495-nm species was not discernible in the spectrum. Upon addition of H_4-folate (*curve 6*), increase in the absorbance of the 495-nm species was not observed. All the spectral studies were carried out at pH 7.4 and 8.3 but data at pH 7.4 only are given.

Spectral Studies with Methoxyamine and Thiosemicarbazide—Both methoxyamine (13) and thiosemicarbazide were shown to inhibit sheep liver SHMT by interacting with PLP at the active site of the enzyme. Fig. 5 (*curve 1*) represents the spectrum of the native enzyme. The spectrum obtained on the addition of methoxyamine (100 μM) to the native enzyme is shown in Fig. 5 (*curve 2*). Methoxyamine formed a characteristic 388-nm species in a fast phase of interaction with the enzyme. The PG-modified enzyme (*curve 3*) also interacted with methoxyamine to form the 388-nm intermediate as shown in Fig. 5 (*curve 4*).[3] Thiosemicarbazide interacted with PLP at the active site of sheep liver SHMT to form an enzyme-bound intermediate with absorption maxima at 464 and 440 nm (Fig. 5, *curve 5*). The PG-modified enzyme

[2] R. Usha and N. Appaji Rao, unpublished data.
[3] J. K. Acharya and N. Appaji Rao, unpublished data.

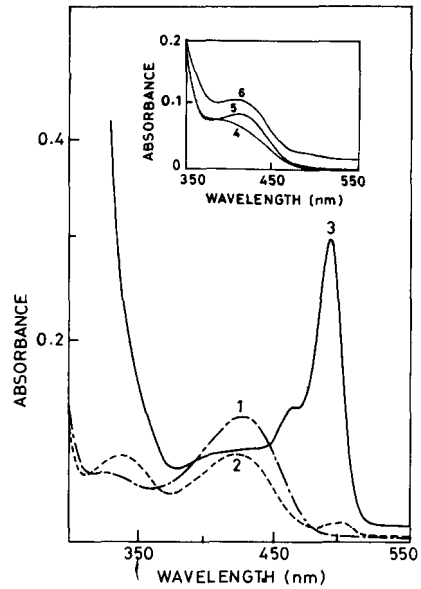

FIG. 4. **Interaction of unmodified enzyme with glycine and H_4-folate.** The enzyme (5 μM) in 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM β-mercaptoethanol was centrifuged, and the spectrum of the clear supernatant was recorded (*curve 1*). *Curve 2* represents the spectrum of the enzyme upon addition of 200 mM glycine. *Curve 3* is the spectrum obtained upon adding 120 μM H_4-folate to the enzyme-glycine mixture (*curve 2*). *Inset*, the interaction of modified enzyme with glycine and H_4-folate. The enzyme (2.5 μM) in 50 mM potassium phosphate buffer, pH 7.4, was modified with 4 mM PG at room temperature for 1 h. The enzyme was centrifuged and the spectrum of the clear supernatant recorded (*curve 4*). The reference cuvette contained 4 mM PG in buffer. To the sample cuvette containing the modified enzyme, 200 mM glycine was added and the spectrum recorded (*curve 5*). *Curve 6* shows the spectrum upon addition of H_4-folate (120 μM) to the modified enzyme-glycine mixture (*curve 5*).

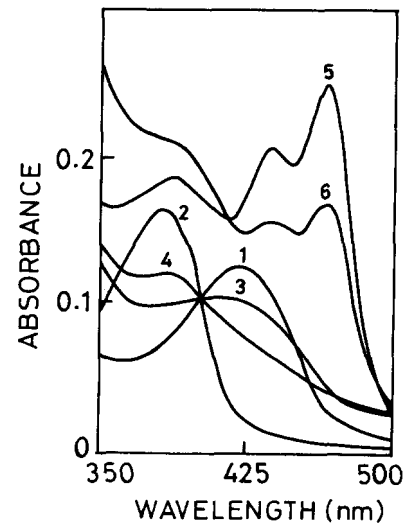

FIG. 5. **The interaction of the unmodified and the PG-modified enzymes with methoxyamine and thiosemicarbazide.** *Curve 1* represents the spectrum of the native enzyme (5 μM) in 50 mM potassium phosphate buffer. *Curve 2* represents the spectrum upon the addition of methoxyamine 100 μM to the native enzyme. *Curve 3* represents the spectrum of the PG-modified enzyme. *Curve 4* shows the spectrum of the PG-modified enzyme upon addition of 100 μM methoxyamine. *Curve 5* is the spectrum of the native enzyme with thiosemicarbazide (200 μM).[3] *Curve 6* is the spectrum of the PG-modified enzyme with thiosemicarbazide (2.2 mM).

also interacted with thiosemicarbazide (2.2 mM) to form the intermediate (Fig. 5, *curve 6*).

Tritium Exchange Studies—A key step in the mechanism of reaction of SHMT with glycine as the substrate was the removal of the *Pro* 2 S proton by a base on the enzyme during the conversion of the Schiff base to the quinonoid intermediate. The rate of exchange of the α-proton of glycine with the solvent protons and its increase on addition of H_4-folate could be correlated with the concentration of the quinonoid complex (12). Table II shows the results of the tritium exchange studies with the control and PG-modified enzymes. There was a small amount of exchange (462 cpm, Table II) of the α-hydrogen of glycine with the solvent protons in the case of the control enzyme. The rate of this exchange reaction was enhanced 200-fold (from 462 to 94,232 cpm, Table II) on the addition of H_4-folate. The α-proton exchange was negligible (60 cpm) with the PG-modified enzyme, and there was no enhancement (100 cpm) of the exchange rate upon addition of H_4-folate. The exchange experiments were in agreement with the fact that the PG-modified enzyme did not show a spectrally discernible quinonoid intermediate, and there was no increase in its amount on the addition of H_4-folate.

DISCUSSION

The involvement of Arg residues in the catalytic function of sheep liver SHMT was indicated by the inactivation of the enzyme by PG, a specific Arg-modifying reagent (3). The kinetic evidence presented earlier indicated that at least 1 residue was necessary for catalysis and that the positively charged guanidinium side chain of Arg was probably involved in binding the carboxyl groups of the substrate amino acids and H_4-folate. However, the role of Arg in catalysis and its position in the primary structure was not established. Hence it was necessary to isolate the peptides containing the modified arginine residues, determine their sequence and place them in the primary structure.

Stoichiometric studies with [7-^{14}C]PG suggested that the Arg residue(s) that were interacting with H_4-folate were probably modified with PG and that 2 Arg residues were probably modified per subunit of the enzyme. Amino acid composition of the modified enzyme indicated that only 2 Arg and no other residues were modified by PG under the experimental conditions used.[2] The sequences of the peptides containing the modified Arg residues were A-G-M-I-F-Y-R-K and A-V-R-A-L-R. In view of the extensive homology between the sheep and rabbit liver sequences, the modified Arg residues would correspond to Arg-269 and Arg-462 in the rabbit liver enzyme sequence.

TABLE II

Proton exchange catalyzed by native and modified SHMT

To sheep liver SHMT (2.5 μM) in HEPES buffer, pH 7.4, 7.5 mM [2-^{3}H]glycine (2×10^5 cpm) was added and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 10% trichloroacetic acid. The denatured protein was removed by centrifugation, and the supernatant was loaded onto a Dowex 50-W 12 column packed in a 1-ml syringe. The column was washed with 10 ml of 0.01 M HCl, the eluate containing the exchanged ^{3}H was collected, and the radioactivity was measured. In a second experiment, an identical reaction was carried out in the presence of H_4-folate (2 mM) for 1 min. Sheep liver SHMT (2.5 μM) was mixed with 4 mM PG in 0.1 M HEPES buffer, pH 7.4, and incubated at 37 °C for 1 h. The modified enzyme was assayed for α-proton exchange form [2-^{3}H]glycine as described above.

Enzyme	Addition	cpm
Unmodified		462
Unmodified	H_4-folate	94,232
PG-modified		60
PG-modified	H_4-folate	100

H_4-Folate has a unique role in the transfer of the hydroxymethyl group from serine. In all other reactions catalyzed by SHMT such as decarboxylation of amino malonate (14) and transamination of alanine (15) H_4-folate had no effect on the reaction rate. Earlier studies with the rabbit liver enzyme showed that serine was cleaved by the enzyme in the absence of H_4-folate, but the formaldehyde dissociated very slowly from the active site (11). Therefore a role for H_4-folate in the serine reaction was postulated, namely, to catalyze the removal of formaldehyde as 5,10-methylene H_4-folate. It could be postulated that if H_4-folate interaction with the enzyme was hindered, the earlier steps in the catalysis could be unaffected, but the later events might not occur. The interaction of the putative Arg residues with H_4-folate can be characterized by monitoring the spectral change of the quinonoid intermediate and the α-proton exchange. The data presented in Fig. 4 show that there is no characteristic absorbance at 495 nm but the spectra of the geminal diamine and the Schiff base, which are the early events in catalysis are essentially unaffected. The proton exchange was enhanced by H_4-folate in the control unmodified enzyme, whereas in the PG-modified enzyme there was no such increase (Table II). These observations clearly point out that the Arg residue(s) identified in this study are probably involved in H_4-folate interaction, a later event in catalysis, while the earlier reactions are probably unaffected.

In order to examine this possibility, the interaction of PLP with the inhibitors was studied. We had earlier demonstrated that hydroxylamine derivatives of the substrates such as methoxyamine, *O*-amino-D-serine, and aminooxyacetate interact with PLP at the active site of SHMT and generate characteristic intermediates (13, 16, 17). The formation of the characteristic intermediates on interaction of the PG-modified and the native enzymes indicated that the interaction of PLP with the substrates (Fig. 4) or analogues was probably unaffected (Fig. 5).

In most PLP-dependent enzymes, the positively charged guanidinium side chain of Arg has been implicated in binding the phosphate ester of PLP, as in serine dehydratase (EC 4.2.1.14) PMP oxidase (EC 1.4.3.5) (18, 19). A situation analogous to that seen in PG-modified SHMT was noticed in tryptophanase (EC 4.1.99.1), a PLP-dependent enzyme which catalyzes a β-elimination reaction. Tryptophanase modified with PG did not show the 500-nm absorption band characteristic of the native enzyme when substrates are added (20). SHMT, in addition to being a PLP enzyme is a constituent of the thymidylate cycle along with dihydrofolate reductase (EC 1.5.1.3) and thymidylate synthase (EC 2.1.1.45) (21). Recent site-directed mutagenesis of Arg-70 of human dihydrofolate reductase to Lys revealed that the mutant enzyme was unable to bind methotrexate, the α-carboxyl which interacted with Arg-70 (22). In thymidylate synthase, labeling with [^{14}C]pteroylheptaglutamate indicated that the analogue was covalently attached to Lys-58 of one subunit and Lys-50 and Lys-51 of the other subunit (23).

The most facile conclusion and one that is supported by our results is that the Arg residues are involved in the binding of H_4-folate. Since H_4-folate exists as a polyglutamate (24) it is not unreasonable to assume that 2 Arg residues would play a role in binding and orientation of H_4-folate at the active site. An Arg-carboxylate interaction might be preferred over simple charge interactions since the guanidinium group would present charged hydrogen bonds to the carboxylate substrate rather than the single bond formed by lysine- or histidine-

TABLE III

Comparison of sequences around Arg-269 and Arg-462 in sheep liver and other SHMTs

Top part: positions of the amino acid residues aligned are as follows. *E. coli* enzyme, residues 232–241 (27); rabbit cytosolic enzyme, residues 259–274 (25), rabbit mitochondrial enzyme, residues 254–269 (26); and sheep liver enzyme sequence.[2] *Bottom part*: positions of the amino acid residues aligned are as follows. *E. coli* enzyme, residues 402–415 (27); rabbit cytosolic enzyme, residues 452–465 (25); rabbit mitochondrial enzyme, residues 444–457 (26); and the sheep liver enzyme sequence.[2]

The conserved residues are *boxed* by the *solid line*.

Sheep SHMT	R G C R A G M I F Y R K G V R S
Rabbit cytosolic SHMT	R G C R A G M I F Y R R G V R S
Rabbit mitochondrial SHMT	R G A R S G I I F Y R K G V R V
E. coli SHMT	A G P R G G L I L A - - - - - -
Sheep SHMT	A G A E E H Q R A V R A L R
Rabbit cytosolic SHMT	A G D E K H Q R A V R A L R
Rabbit mitochondrial SHMT	L K D P E T S Q R L A D L R
E. coli SHMT	A K E L A G W M C D S I N D

carboxylate interactions. Table III shows the sequences flanking the Arg residues in the sheep liver, the rabbit liver cytosolic and mitochondrial protein sequences (25, 26), and the deduced amino acid sequence of the *Escherichia coli* enzyme (27). Both the Arg residues identified in the sheep liver sequence are well conserved in the rabbit liver cytosolic enzyme sequence, but there is no apparent local sequence homology in the *E. coli* enzyme. Detailed investigations are in progress with the PG-modified *E. coli* enzyme.[4]

The data reported herein show that Arg residues are present at the active site of the sheep liver enzyme. We have localized these residues to short stretches of the primary sequence. The data suggest that these residues are involved in binding H_4-folate. Further studies designed to test the role of other amino acid residues in the sheep liver enzyme which are important for catalysis should help in defining the architecture of the active site of the enzyme.

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