Role of Arg-401 of cytosolic serine hydroxymethyltransferase in subunit assembly and interaction with the substrate carboxy group

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In an attempt to identify the arginine residue involved in binding of the carboxylate group of serine to mammalian serine hydroxymethyltransferase, a highly conserved Arg-401 was mutated to Ala by site-directed mutagenesis. The mutant enzyme had a characteristic visible absorbance at 425 nm indicative of the presence of bound pyridoxal 5′-phosphate as an internal aldimine with a lysine residue. However, it had only 0.003 % of the catalytic activity of the wild-type enzyme. It was also unable to perform reactions with glycine, β-phenylserine or D-alanine, suggesting that the binding of these substrates to the mutant enzyme was affected. This was also evident from the interaction of amino-oxyacetic acid, which was very slow (8.4×10^{-4} s^{-1} at 50 μM) for the R401A mutant enzyme compared with the wild-type enzyme (44.6 s^{-1} at 50 μM). In contrast, methoxyamine (which lacks the carboxy group) reacted with the mutant enzyme (1.72 s^{-1} at 250 μM) more rapidly than the wild-type enzyme (0.2 s^{-1} at 250 μM). Further, both wild-type and the mutant enzymes were capable of forming unique quinonoid intermediates absorbing at 440 and 464 nm on interaction with thiosemicarbazide, which also does not have a carboxy group. These results implicate Arg-401 in the binding of the substrate carboxy group. In addition, gel-filtration profiles of the apoenzyme and the reconstituted holoenzyme of R401A and the wild-type enzyme showed that the mutant enzyme remained in a tetrameric form even when the cofactor had been removed. However, the wild-type enzyme underwent partial dissociation to a dimer, suggesting that the oligomeric structure was rendered more stable by the mutation of Arg-401. The increased stability of the mutant enzyme was also reflected in the higher apparent melting temperature (T_m) (61 °C) than that of the wild-type enzyme (56 °C). The addition of serine or serinamide did not change the apparent T_m of R401A mutant enzyme. These results suggest that the mutant enzyme might be in a permanently 'open' form and the increased apparent T_m could be due to enhanced subunit interactions.

INTRODUCTION

Serine hydroxymethyltransferase (SHMT; EC 2.1.2.1) catalyses the reversible conversion of serine and 5,6,7,8-tetrahydrofolate (H_4-folate) to glycine and 5,10-methylene-H_4-folate. This enzyme has an important role in one-carbon metabolism, providing the cell with approx. 70 % of the one-carbon units required for the synthesis of thymidine, purines, choline and methionine, for example [1]. It has been shown that the incorporation of the β-carbon of serine into DNA via the SHMT reaction increases when cells are stimulated to proliferate during the S-phase of the cell cycle [2,3]. Further, SHMT activity is elevated in a variety of tumour tissues [4,5]. It has therefore attracted widespread attention as a model pyridoxal 5′-phosphate (PLP) protein and a target for cancer chemotherapy [6].

It has been suggested that the PLP-dependent enzymes have the same spatial fold as aspartate aminotransferase (AAT), even though these enzymes have less than 15 % sequence identity [7,8]. Pascarella et al. [9] proposed that SHMT also has the same general spatial fold as AAT, and structural alignment analysis indicates that Arg-363 of *Escherichia coli* SHMT is equivalent to Arg-383 of AAT. Crystal structure and site-directed mutagenesis studies of AAT reveal that the Arg-383 has a role in binding the substrate carboxy group and in the conversion of the enzyme from 'open' to the 'closed' form [10,11]. Site-directed mutagenesis studies on *E. coli* SHMT indicate that this Arg-363 residue has a similar functional role in binding the substrate carboxy group [12].

The mammalian cytosolic SHMTs are tetramers of identical subunits of 53 kDa with 4 mol of PLP per mol of enzyme [13,14], unlike the *E. coli* SHMT, which is a dimer [15]. The sheep liver cytosolic enzyme has been characterized in our laboratory with respect to its primary structure [16], catalysis [17,18] and the interaction of substrate and substrate analogues at the active site [19–23]. This enzyme has been cloned and overexpressed in *E. coli* [24] with the aim of identifying the amino acid residues involved in subunit and active-site interactions. Alignment of the amino acid sequence of SHMTs showed that Arg-401 in the mammalian enzyme was equivalent to Arg-363 in the *E. coli* enzyme. Here we report the construction, expression and characterization of the R401A mutant of sheep liver cytosolic recombinant SHMT (rSHMT). The results indicate that the carboxy group of the substrate interacts with Arg-401; in the absence of this positively charged residue, the subunit interactions are strengthened.

EXPERIMENTAL

Materials

[α-^{32}P]dATP (3000 Ci/mmol), L-[3-^{14}C]serine (55 mCi/mmol), restriction endonucleases, Sequenase® version 2.0 DNA sequencing kit and DNA-modifying enzymes were obtained from

Abbreviations used: AAA, amino-oxyacetic acid; AAT, aspartate aminotransferase; H_4-folate, 5,6,7,8-tetrahydrofolate; MA, methoxyamine; PLP, pyridoxal 5′-phosphate; rSHMT, sheep liver cytosolic recombinant SHMT; SHMT, serine hydroxymethyltransferase; TSC, thiosemicarbazide.

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Amersham International. CM-Sephadex and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals. Glycine, L-serine, L-serinamide, D-alanine, amino-oxyacetic acid (AAA), methoxyamine (MA), thiosemicarbazide (TSC), hydroxylamine, L-cysteine hydrochloride, β-phenylserine, 2-mercaptoethanol, folic acid, PLP and EDTA were obtained from Sigma Chemical Company. H_4-Folate was prepared by the method of Hatefi et al. [25]. All other chemicals were of A.R. grade. The mutant oligonucleotide was purchased from Bangalore Genei Private Limited (Bangalore, India).

Bacterial strains and growth conditions

E. coli strain DH5α (BRL) was the recipient for all the plasmids used in subcloning. The BL21(DE3) pLysS strain [26] was used for the bacterial expression of pETSH and R401A mutant. Luria–Bertani medium or 'terrific' broth with 50 μg/ml of ampicillin was used for growing *E. coli* cells containing the plasmids [27].

DNA manipulations

Plasmids were prepared by the alkaline lysis procedure as described by Sambrook et al. [27]. Restriction endonuclease digestions, Klenow filling and ligations were performed in accordance with the manufacturers' instructions. The preparation of competent cells and transformation was performed by the method of Alexander [28]. The DNA fragments were eluted from the agarose gel by the low-melting agarose gel method [27].

Site-directed mutagenesis

The R401A mutant was constructed by a PCR-based megaprimer method, as described previously [29]. The mutant was constructed from pUCSH (containing the SHMT cDNA fragment lacking 227 bp at the 5′ end in pUC 19) as a template. The mutant oligonucleotide (25-mer) 5′-CCC AGT GGC TTG GCC CTG GGG ACC C-3′ was used for the construction of the R401A mutant. The full-length PCR product obtained after two rounds of PCR was subcloned into pUC 19 at *Kpn*I and *Bam*HI sites. The clones obtained after the mutagenesis procedure were identified by sequencing the gene at the mutated region. The pUC 19 plasmid containing the mutated SHMT cDNA was purified and digested with the *Pma*CI and *Bam*HI restriction enzymes flanking the Arg → Ala mutation. The 738 bp *Pma*CI–*Bam*HI-mutated DNA fragment was gel-purified and swapped at the same sites of the pETSH vector [24]. The entire 738 bp *Pma*CI–*Bam*HI DNA fragment of the R401A mutant clone was sequenced with a Sequenase® version 2.0 DNA sequencing kit to rule out the presence of other non-specific mutations.

Expression and purification of R401A SHMT

The pETSH and R401A mutant constructs were transformed into BL21(DE3) pLysS strain. A single colony was inoculated in 50 ml of Luria–Bertani medium containing 50 μg/ml ampicillin and grown overnight at 37 °C; 4 % of these cells grown overnight were inoculated into 1 litre of 'terrific' broth medium containing 50 μg/ml ampicillin. After 3.5 h of growth at 37 °C, the cells were induced for 4 h with 0.3 mM isopropyl β-D-thiogalactoside. The cell pellet was harvested, resuspended in 150 ml of extraction buffer (50 mM potassium phosphate buffer, pH 7.4, containing 5 mM 2-mercaptoethanol, 1 mM EDTA and 100 μM PLP) and sonicated until it was optically clear. The supernatant was subjected to 0–65 %-satd. $(NH_4)_2SO_4$ precipitation; the pellet obtained was resuspended in 20 ml of 20 mM potassium phosphate buffer, pH 6.4, containing 1 mM 2-mercaptoethanol, 1 mM EDTA and 50 μM PLP, then dialysed for 24 h against the same buffer (1 litre with two changes). The dialysed sample was loaded on a CM-Sephadex column (2 cm × 20 cm) equilibrated with 20 mM potassium phosphate buffer, pH 6.4, containing 1 mM 2-mercaptoethanol, 1 mM EDTA and 50 μM PLP. The column was washed with the same buffer (1 litre) and the bound enzyme was eluted with 100 ml of 200 mM potassium phosphate buffer, pH 7.4, containing 1 mM 2-mercaptoethanol, 1 mM EDTA and 50 μM PLP. The eluted sample was subjected to 0–65 %-satd. $(NH_4)_2SO_4$ precipitation. The pellet obtained was resuspended in 4 ml of 200 mM potassium phosphate buffer, pH 7.4, containing 1 mM 2-mercaptoethanol, 1 mM EDTA and 50 μM PLP and subjected to Sephacryl S-200 column chromatography as described previously [24]. Fractions containing the enzyme were pooled and precipitated at 65 %-satd. $(NH_4)_2SO_4$ and the pellet was resuspended in 50 mM potassium phosphate, 1 μM 2-mercaptoethanol and 1 mM EDTA (buffer A) and dialysed against 1 litre of the same buffer (with two changes) for 24 h; this enzyme preparation was used in these studies.

Enzyme assay and protein estimation

The aldol cleavage of serine with H_4-folate to glycine and 5,10-methylene-H_4-folate catalysed by SHMT was monitored with L-[3-^{14}C]serine and H_4-folate as substrates [30,31].

Purified rSHMT (1 ml; 10 A_{280} units/ml) enzyme was passed through a Centricon filter with 10 ml of doubly distilled water. After Centricon filtration, the absorbance was measured at 280 nm, then the sample was freeze-dried and weighed. The concentration at 1 A_{280} unit was found to be 1.2 mg with a molar absorption coefficient of 176 333 $M^{-1} \cdot cm^{-1}$. A similar value was obtained when the protein was estimated by the method of Gill and von Hippel [32]. This value was used for the estimation of the mutant enzyme because A_{280} nm did not change compared with the wild-type enzyme.

Estimation of PLP content

The purified rSHMT and R401A SHMT were dialysed extensively against buffer A. These dialysed enzyme samples (1.2 mg/ml) were incubated separately with 0.1 M NaOH for 5 min and the absorbance was measured at 388 nm. The PLP content was determined, taking the molar absorption coefficient of PLP in 0.1 M NaOH as 6600 $M^{-1} \cdot cm^{-1}$ at 388 nm [33].

Spectroscopy

The visible spectra were recorded at 25 °C in buffer A with a Shimadzu UV–visible (UV-160A) spectrophotometer. CD measurements were made with a JASCO-J-500A automated recording spectropolarimeter. The CD spectra were recorded at 22 ± 2 °C in buffer A, with the same buffer as blank. The protein concentration corresponding to 0.12 mg/ml was used for far-UV CD studies. Rapid reaction kinetic studies were performed with a Biologic Stopped Flow Module with Biokine software for monitoring the interaction of AAA (50 μM) and MA (250 μM), with rSHMT (10 μM) and the R401A SHMT (10 μM) mutant at 425 nm. The pseudo-first-order rate constants were calculated by a curve-fitting analysis of Sigma Plot software by fitting the data to a first-order rate equation.

Size-exclusion chromatography

To analyse the oligomeric structure of the mutant holoenzyme and apoenzyme, a Superose-12 HR 10/30 analytical gel-filtration

column was used on a Pharmacia FPLC system. The column was calibrated with standard molecular mass markers, i.e. apoferritin (440 kDa), sheep cytosolic SHMT (213 kDa), yeast alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa). The buffer used for this study was buffer A containing 0.1 M KCl and 0.05% sodium azide. The absorbance was recorded at 280 nm. The holoenzyme and apoenzyme (100 μg) were analysed immediately after dialysis as described above. The apoenzymes (100 μg) were reconstituted by incubation with 100 μM of PLP for 10 min and then analysed on the gel-filtration column. The percentages of tetramer and dimer were calculated from the corresponding areas on the chromatogram.

Apoenzyme preparation

The rSHMT and R401A SHMT (2 mg/ml) in buffer A were incubated with 50 μM hydroxylamine at 25 ± 2 °C for 30 min. The sample was subsequently dialysed for 24 h against buffer A (1 litre with two changes) to remove the PLP-oximes.

Thermal stability

Thermal denaturation of rSHMT and R401A SHMT was performed in a Gilford Response II spectrophotometer from Ciba Corning Diagnostics (Oberlin, OH, U.S.A.) as described by Bhaskar et al. [34], with the following modifications. The protein samples (300 μl, 0.3 mg/ml) in buffer A were heated from 30 to 80 °C at the rate of 1 °C/min. The absorbance change in each case was monitored at 287 nm and data were averaged from two experiments. The first derivative of the thermal denaturation profiles was used to evaluate the apparent transition temperatures of both proteins. Similar conditions were used to monitor the thermal denaturation profiles of rSHMT and R401A SHMT in the presence of serine, glycine, serinamide or alanine at 100 mM concentration, with the buffer blank in the reference cuvette with the same ligand concentration.

RESULTS

Properties of R401A SHMT

The visible absorption spectra of rSHMT and R401A SHMT indicated the presence of an internal aldimine in the mutant enzyme, as in rSHMT. The absorbance values at 425 nm were 0.115 and 0.08 per mg of the mutant and rSHMT respectively. The increase in absorbance could be caused by either an increased PLP content or a change in the environment of the active site. The PLP content of R401A and rSHMT were therefore estimated as described in the Experimental section. R401A SHMT and rSHMT contained PLP at 4.1 mol/mol and 3.5 mol/mol respectively, indicating that a small amount of apoenzyme might have formed during the isolation of the rSHMT. The secondary structure of R401A SHMT, as monitored by far-UV CD (195–250 nm), did not show any alteration in the structure as a consequence of the mutation. The subunit and native molecular masses of the mutant enzyme as monitored by SDS/PAGE and gel filtration analysis confirmed that it was a homotetramer, as is the wild-type enzyme.

Interaction of substrate analogues

The R401A SHMT was unable to catalyse the hydroxymethyl transfer from serine. The specific activity of the mutant was only 1.6×10^{-4} units/mg compared with 4.8 units/mg for rSHMT. Similarly, it was unable to catalyse the reactions with β-phenylserine, D-alanine or glycine. Cysteine, a substrate analogue, reacts with the internal aldimine of the enzyme to yield a thiazolidine complex [35]. It can be seen from Figure 1 that the reaction of cysteine with rSHMT was complete within 30 s, as indicated by the disappearance of 425 nm absorbance and the concomitant appearance of a 330 nm peak. In contrast, the absorbance changes (at 425 and 330 nm) with the R401A SHMT were very slow and even after 4 h the reaction was not complete, suggesting that the absence of Arg-401 had affected the interaction of cysteine with the enzyme.

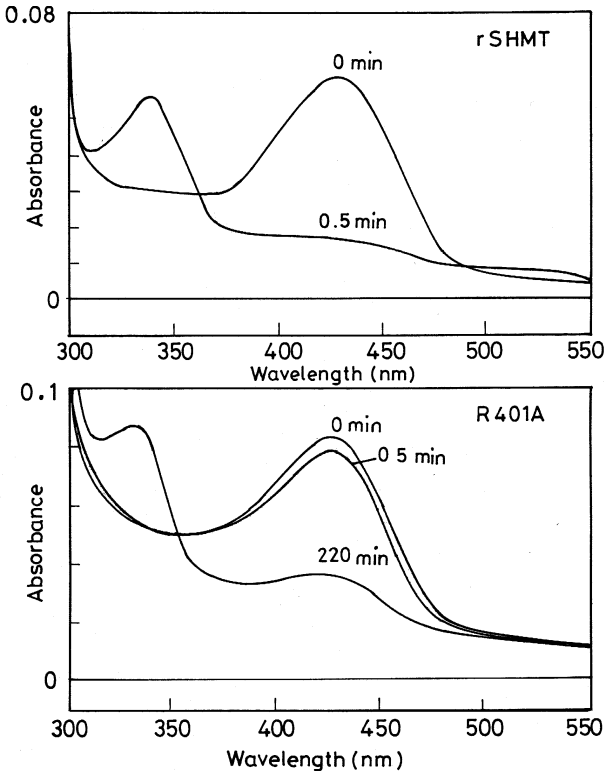

Figure 1 Interaction of cysteine with rSHMT and R401A

The spectra of rSHMT (0.6 mg/ml) were recorded 0 and 0.5 min after the addition of cysteine (10 mM) in buffer A. For R401A SHMT (0.6 mg/ml) the spectra were recorded 0, 0.5 and 220 min after the addition of cysteine (10 mM) in buffer A. The reference cuvette contained all components except enzyme.

We had earlier shown that amino-oxy compounds such as *O*-amino-D-serine and AAA reacted more efficiently with the enzyme than MA, which lacks the carboxy group; the presence of a carboxy group had a profound influence on the rate of reaction [20–22]. These compounds were used to probe the role of the Arg-401 residue in the interaction of the carboxy group of ligands. It can be seen from Figure 2 that AAA reacts rapidly with rSHMT to yield the oxime with the formation of an intermediate absorbing at 388 nm with an isosbestic point at 376 nm. In contrast, AAA reacted very slowly with R401A SHMT and yielded the oxime without the formation of an intermediate absorbing at 388 nm. MA, which lacks the carboxy group, reacts with rSHMT and the mutant enzyme (Figure 3) in a similar manner to yield the oxime with an intermediate absorbing at 388 nm and an isosbestic point at 364 nm.

The rate constants for the interaction of AAA with rSHMT and of MA with rSHMT and the mutant enzyme were determined by rapid reaction kinetics because the rate of disappearance at

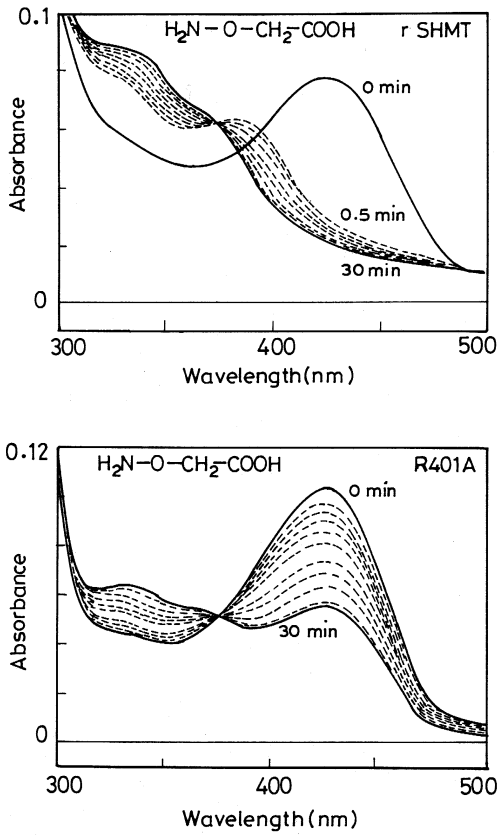

Figure 2 Interaction of AAA with rSHMT and R401A SHMT

The spectra of rSHMT (1 mg/ml) were recorded 0, 0.5 and 30 min after the addition of AAA (50 μM) in buffer A. For R401A SHMT (1 mg/ml), the spectra were recorded 0, 0.5 and 30 min after the addition of AAA (50 μM) in buffer A. The reference cuvette contained all components except enzyme.

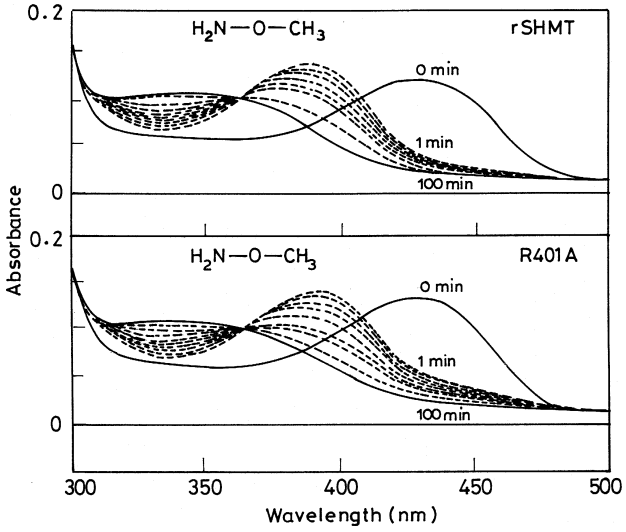

Figure 3 Interaction of MA with rSHMT and R401A SHMT

The spectra of rSHMT and R401A SHMT (1.2 mg/ml each) were recorded 0, 1 and 100 min after addition of MA (250 μM) in buffer A separately.

425 nm was very rapid and could not be measured by conventional spectroscopy. Figure 4(a) shows that when AAA (50 μM) was mixed with rSHMT (10 μM), the absorbance at 425 nm decreased rapidly and the reaction was almost complete in 75 ms. A pseudo-first-order rate constant of 44.6 s^{-1} was calculated. In contrast, the reaction of AAA with the R401A SHMT was very slow and the disappearance of the absorbance at 425 nm was monitored by conventional spectroscopy. A pseudo-first-order rate constant of 8.15×10^{-4} s^{-1} was calculated at 50 μM AAA. The disappearance of the 425 nm absorbance on addition of MA (250 μM), monitored with a stopped-flow spectrophotometer, showed that the reaction was complete in 7.5 and 2.5 s for rSHMT and the mutant enzyme respectively (Figure 4b). The pseudo-first-order rate constants for rSHMT and R401A SHMT were 0.2 and 1.72 s^{-1} respectively.

Interaction of TSC with R401A SHMT

In the absence of Arg-401, the binding of the carboxy group of the substrate cannot take place and consequently the formation of the quinonoid intermediate (λ_{max} 495 nm) cannot be monitored. It has been shown previously that TSC generates a novel quinonoid intermediate (λ_{max} 464 nm) in its interaction with SHMT [23]. It could be postulated that as TSC does not have a carboxy group, the R401A SHMT should also generate this intermediate if the other steps in the reaction are unaffected. rSHMT and the mutant enzyme were capable of forming the intermediates absorbing at 440 and 464 nm (results not shown). The intermediates slowly decomposed to yield the PLP-thiosemicarbazone with an isosbestic point at 412 nm. It is interesting to note that whereas the rate of formation of the intermediate is similar, its decomposition is faster with the mutant as indicated by rate constants of 0.036 min^{-1}, 0.085 min^{-1} for rSHMT and the mutant enzyme respectively.

Stability of R401A SHMT

We have shown earlier that PLP has a role in maintaining the oligomeric structure of SHMT ([36,37], and B. Venkatesha, J. B. Udgaonkar, N. Appaji Rao and H. S. Savithri (unpublished work). Because R401A SHMT has a higher absorbance at 425 nm, it might also be more stable than rSHMT. To test this possibility, the apoenzymes of R401A SHMT and rSHMT were prepared by reaction with hydroxylamine rather than the more conventional method of reaction with D-alanine or L-cysteine as the mutant enzyme was unable to interact with these compounds efficiently. The oligomeric status of the holoenzyme, as shown in Table 1, was essentially tetrameric. The freshly prepared apoenzyme of rSHMT eluted as a mixture of tetramer (60%) and dimer (40%), whereas the apoenzyme of R401A SHMT was mostly in the tetrameric form (more than 90%). Both apoenzymes could be reconstituted to holoenzymes as monitored by the regaining of 425 nm absorbance and visible CD. The oligomeric structure of the reconstituted rSHMT was still a mixture of tetramer and dimer (75% and 25% respectively). In contrast, the reconstituted R401A SHMT was eluted as a single symmetrical peak of tetramer with barely detectable amounts of dimer. The apoenzyme of rSHMT stored at 4 °C for 48 h (after dialysis) was present predominantly as a dimer (more than 75%) (Table 1). On addition of PLP, only 5% of the dimer could be converted to tetramer. Unlike rSHMT, the R401A SHMT apoenzyme was present predominantly as a tetramer even after 48 h and it could be reconstituted to a holotetramer almost completely (Table 1). These results indicate that R401A SHMT apoenzyme was more stable than rSHMT.

The stability of the enzyme can also be monitored by deter-

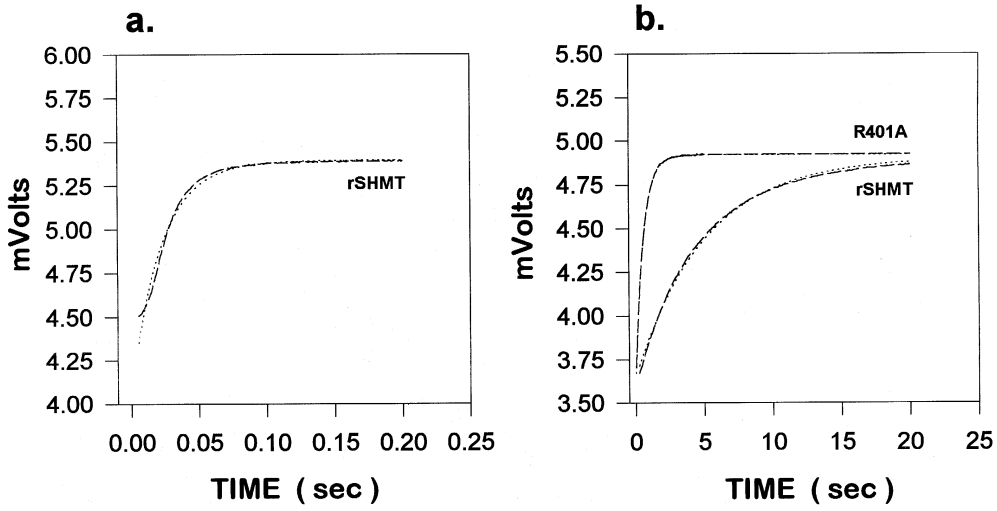

Figure 4 Rapid-reaction kinetics of interaction AAA and MA

rSHMT (10 μM) and AAA (50 μM) were mixed in a Biologic Stopped Flow Module and the decrease in absorbance at 425 nm was monitored. The increase in potential (mV) as a function of time (s) is shown in (**a**). Similarly the interaction of rSHMT (10 μM) and R401A SHMT (10 μM) with MA (250 μM) is shown in (**b**). The experimental and curve-fitted curves are shown as broken and dotted lines respectively.

Table 1 Oligomeric status of R401A SHMT holoenzyme, apoenzyme and reconstituted holoenzyme

		Proportion in oligomeric form (%)			
		rSHMT		R401A SHMT	
Enzyme	Form	Day 1	Day 3	Day 1	Day 3
Holoenzyme	Tetramer	100	100	100	100
	Dimer	0	0	0	0
Apoenzyme	Tetramer	60	< 25	> 90	> 80
	Dimer	40	> 75	< 10	< 20
Reconstituted holoenzyme	Tetramer	75	< 30	> 98	> 90
	Dimer	25	> 70	–	< 10

Table 2 Thermal stability of R401A SHMT in the presence of ligands

The apparent T_m values were determined from the first-derivative plots of the denaturation curves (see the text for details).

	Apparent T_m (°C)	
Ligand (100 mM)	rSHMT	R401A SHMT
None	56	61
L-Serine	65	60
L-Serinamide	55	58
Glycine	58	58
D-Alanine	56	60

mining the apparent melting temperature (T_m) values. It can be seen from Table 2 that R401A SHMT had an apparent T_m of 61 ± 1 °C, compared with 56 ± 1 °C for rSHMT. The addition of serine enhanced the apparent T_m of rSHMT from 56 ± 1 to 64 ± 1 °C, whereas this enhancement was not observed with the mutant enzyme. Other ligands such as glycine, D-alanine and serinamide also did not change the apparent T_m values.

DISCUSSION

It has been suggested from model studies that Arg residues are convenient anchors for carboxy groups of several substrates and the formation of the ion pair provides the necessary chemical pull to facilitate catalysis [38]. Mutation of these active-site Arg residues results in the inability of substrates containing carboxy groups to bind to the enzyme. An approach to the study of interactions at the active site especially of such inactive mutants is by the use of substrate analogues and inhibitors [11]. The R401A mutation of SHMT resulted in a marked decrease in the enzyme activity by 30000-fold with serine as the substrate. The rate constant for the interaction of AAA with R401A mutant enzyme was 1/50000 that of rSHMT. The decrease in the rate constant was similar to the decrease in the specific activity observed for R401A SHMT. However, the interaction of MA, which lacks the carboxy group, was 9-fold faster with the mutant enzyme than with rSHMT. TSC interacted with rSHMT and the mutant enzyme, generating similar quinonoid intermediates. The decomposition of this quinonoid intermediate to PLP-thiosemicarbazone was 3.5-fold faster in the case of mutant enzyme compared to rSHMT. These results suggest that the Arg-401 residue has a role in the interaction of the enzyme with the substrate carboxy group. The enhanced reactivity of the mutant with the ligands lacking a carboxy group could be due to the removal of the constraints imposed by the positive charge of the Arg residue.

The results presented in Table 1 clearly indicate that R401A SHMT apoenzyme was more stable as a tetramer than rSHMT. A plausible explanation for the increased stability is that the mutation of the Arg residue could have removed the charge repulsion between subunits, resulting in more stable tetramers.

The increased stability of the mutant was also reflected in the higher apparent T_m (Table 2) and more tightly bound cofactor than in rSHMT. It has previously been suggested that the increase in T_m on the addition of the substrate serine results in the conversion of the enzyme from an 'open' to 'closed' form [39]. It was shown in the *E. coli* SHMT R363A mutant that the rate of cleavage of L-serine ethyl ester and L-serinamide was lower than expected, being only about 0.1% of the wild-type enzyme with serine as a substrate [12]. These results suggested that Arg-363 might have additional roles other than forming a tight ion pair with the substrate's carboxy group. It was suggested that R363A mutant enzyme might be in an 'open' form owing to the absence of an enhanced T_m with substrate (serine or serinamide) and its ability to catalyse the transamination of D-alanine methyl ester [12]. Similarly, the addition of serine or serinamide did not result in a further increase in apparent T_m for R401A SHMT. These results suggest that the mutant enzyme did not undergo the 'open' to 'closed' conformational change on addition of these ligands and that the increased stability of R401A could be the result of enhanced subunit interactions in the 'open' form. In other words, Arg-401 has a role not only in providing the positive charge for the formation of an ion pair with the substrate's carboxy group but also in maintaining the conformational flexibility of the enzyme.

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