Function of the Active-site Lysine in *Escherichia coli* Serine Hydroxymethyltransferase*

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Serine hydroxymethyltransferase has a conserved lysine residue (Lys-229) that forms the internal aldimine with pyridoxal 5'-phosphate. In other pyridoxal 5'-phosphate enzymes investigated so far, this conserved lysine residue also plays a catalytic role as a base that removes the α-proton from the amino acid substrate. Three mutant forms of *Escherichia coli* serine hydroxymethyltransferase (K229Q, K229R, and K229H) were constructed, expressed, and purified. The absorbance spectra, rapid reaction kinetics, and thermal denaturation of the mutant analogs were studied. Only the K229Q mutant serine hydroxymethyltransferase resembled the wild-type enzyme. The results indicate that Lys-229 plays a critical role in expelling the product by converting the external aldimine to an internal aldimine. In the absence of Lys-229, ammonia can also catalyze the same function at a much slower rate. However, Lys-229 apparently is not the base that removes the α-proton from the amino acid substrate. The K229Q mutant enzyme could catalyze one turnover of either serine to glycine or glycine to serine at rates approaching those of the wild-type enzyme. After one turnover, the mutant enzyme could not expel the product and bind new substrate. The K229Q mutant enzyme can also transaminate p-alanine, which, like the hydroxymethyltransferase activity, also requires removing the α-proton from the substrate. The absorbance spectra of the K229R and K229H serine hydroxymethyltransferases showed that their pyridoxal 5'-phosphate could not readily form an external aldimine with substrates, suggesting that Lys-229 in the wild-type enzyme may never bear a positive charge, further evidence that it is not the base that removes the α-proton.

Serine hydroxymethyltransferase (SHMT) catalyzes the interconversion of serine and glycine with tetrahydrofolate and H4PteGlu serving as the one-carbon carrier. The enzyme contains covalently bound pyridoxal-P, which forms an internal aldimine with the α-amino group of Lys-229 in the *Escherichia coli* enzyme (Schirch et al., 1985). SHMTs from rabbit liver cytosol and mitochondria, Neurospora crassa, *E. coli*, and Bradyrhizobium japonicum have a conserved α-amino acid sequence containing this lysyl residue (McChung et al., 1992; Byrne et al., 1992). Previous studies have shown that 2 other residues in this conserved sequence, His-228 and Thr-226, play critical roles in determining reaction and substrate specificity (Ker et al., 1992; Angelaccio et al., 1992). The goal of this study was to determine if Lys-229 also plays a critical catalytic role in addition to its role in forming an internal aldimine with pyridoxal-P.

All pyridoxal-P enzymes appear to have the coenzyme bound to the α-amino group of a lysyl residue as an internal aldimine. This Lys has been changed to another α-amino acid by site-directed mutagenesis in several pyridoxal-P enzymes (Bhatia et al., 1993; Lu et al., 1993; Zia et al., 1993; Ilag and Jahn, 1992; Yoshimura et al., 1992; Grimm et al., 1992; Toney and Kirsch, 1991, 1992; Nishimura et al., 1991; Planas and Kirsch, 1991; Smith et al., 1989). In most cases, the mutant enzymes contained low observable catalytic activity. One recurring theme in these studies was that substitution of lysine with arginine results in an enzyme with either low levels of activity (<3%) or the ability to interconvert enzyme intermediates. The most extensive study with a lysine-to-arginine mutant was with aspartate aminotransferase, where it was shown that arginine stabilized the key quinonoid intermediate (Toney and Kirsch, 1991). A second recurring theme in these studies is that when alanine is substituted for the active-site lysine, small amines aid in restoring some catalytic activity. For each of the pyridoxal-P enzymes, the low level of activity was interpreted as the active-site Lys amino group serving as the base to remove the α-proton of the substrate in forming a quinonoid intermediate. This view is supported by the three-dimensional structure of aspartate aminotransferase (Arnone et al., 1985; Jansonius and Vincent, 1987). Recently, studies with α-amino acid transaminase suggested that a second lysine residue could partially substitute for the active-site lysine in the catalytic mechanism (Yoshimura et al., 1992).

Using site-directed mutagenesis, Lys-229 in *E. coli* SHMT was changed to a Gln, a His, or an Arg residue (K229Q, K229H, and K229R). The spectral and catalytic properties of these three mutant enzymes were studied. Evidence suggests that Lys-229 in SHMT is not the base that removes a proton from the α-carbon of glycine in its conversion to serine.

EXPERIMENTAL PROCEDURES

Materials—All amino acids, coenzymes, and buffers were purchased from Sigma. 1-[U-¹⁴C]Serine (170 mCi/mmol) was purchased from Du
Past New England Nuclear, and [2-14Clglycine (58 mCi/mmol) was purified as described previously (Schirch et al., 1985; Villar et al., 1985), with the exception that 0.1 mM pyridoxal-P was included at each stage of the purification of the mutant enzymes, except on the DEAE column. Mutant oligonucleotides for the construction of E. coli K229Q, K229H, and m29R forms of SHMT and C1-tetrahydrofolate synthase were purified and used for the mutant SHMTs was removed by adding 0.4 mM NaCNBH3 from a 75 mM stock solution in methanol, followed by a 20-min incubation at 30 °C. The SHMT-catalyzed retroaldol cleavage of serine with H,PteGlu to glycine and 5,10-CH2-H4PteGlu was prepared immediately prior to use by adding 0.1 mM H,PteGlu to 5,10-CH2-H4PteGlu (Schirch, 1978). The cleavage of Ser bound as an external aldimine, shifted to 388 nm, which is free pyridoxal-P bound to the mutant forms of SHMT. Samples (50 fM) of K229Q SHMT were added to a Sephadex G-25 column in a 1-ml plastic syringe. An additional 50 fM of buffer was added to the column. The pyridine was placed in a test tube and centrifuged at 2000 rpm for 2 min. The enzyme (~90% recovery) was isolated in the test tube in 270 µl. One-hundred µl of the sample were diluted to 800 µl in 20 mM KP, pH 7.3. The concentration of enzyme active sites was determined from the pyridoxal-P content, as described above. To the remaining 270 µl of K229Q SHMT were added 25 µl of 9 N perchloric acid to precipitate the protein and to free any bound amino acids. The precipitate was removed by centrifugation and washed with two 100-µl aliquots of water. The combined supernatants were added 70 µl of 4.5 N KOH to precipitate excess perchlorate. Again, the precipitate was removed by centrifugation and washed twice with 50-µl aliquots of water. The combined supernatants were dried and redissolved in 500 µl of water. Small aliquots of this sample were then analyzed for amino acids. All aqueous solutions described in this procedure used high pressure liquid chromatography-grade water.

**Enzyme Assays—** All assays were performed either on a Hewlett-Packard 8425A spectrophotometer or a Kinetic Instruments stopped-flow spectrophotometer as previously described (Stover et al., 1992). Contaminating activity by low levels of the wild-type SHMT was eliminated by using a preparative CE separation. The concentration of enzyme active sites was determined from the pyridoxal-P content, as described above. To the remaining 270 µl of K229Q SHMT were added 25 µl of 9 N perchloric acid to precipitate the protein and to free any bound amino acids. The precipitate was removed by centrifugation and washed with two 100-µl aliquots of water. The combined supernatants were added 70 µl of 4.5 N KOH to precipitate excess perchlorate. Again, the precipitate was removed by centrifugation and washed twice with 50-µl aliquots of water. The combined supernatants were dried and redissolved in 500 µl of water. Small aliquots of this sample were then analyzed for amino acids. All aqueous solutions described in this procedure used high pressure liquid chromatography-grade water.

**Differential Scanning Calorimetry—** Thermograms for the denaturation of K229Q SHMT were obtained with an MC-3 scanning calorimeter from Microcal Inc. (Amherst, MA). Each mutant SHMT and apoSHMT were dialyzed for 24 h against 20 mM KP, 20 mM l-serine, pH 7.3, and 10 mM 2-mercaptoethanol prior to analysis. The scanning rate was 30 °C/h. Enthalpy of denaturation (ΔHf) values were determined as described previously (Schirch et al., 1991).

**Circular Dichroism—** Spectra were recorded from 260 to 200 nm using a Jasco Model 500C with a 0.1-cm cell containing 0.2 mg/ml protein in 20 mM KP, pH 7.3, at 25 °C.

**RESULTS**

**Spectral Properties of K229Q, K229R, and K229H Mutant SHMTs—** The spectra of wild-type SHMT exhibits a single absorption maximum at 422 nm in addition to the protein band at 278 nm. The 422 nm band is due to the protonated internal aldimine of pyridoxal-P bound to Lys-229. The spectra of the three mutant enzymes, as isolated from a final hydroxylapatite column, are shown in Fig. 1. For K229Q SHMT, the spectrum is virtually identical to the spectrum of the wild-type enzyme, with absorption maxima at 278 and 422 nm and a 278:422 ratio of 0.3.

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![Absorption spectra of K229Q (---), K229R (--), and K229H (---) SHMTs. Proteins were 2.4 mg/ml in 20 mM KP, pH 7.2. Each sample was pretreated with NaCNBH3 as described under "Experimental Procedures."](image-url)

**Fig. 1.** Absorption spectra of K229Q (---), K229R (--), and K229H (---) SHMTs. Proteins were 2.4 mg/ml in 20 mM KP, pH 7.2. Each sample was pretreated with NaCNBH3 as described under "Experimental Procedures."
of 7.6 (Schirch et al., 1985). Since K229Q SHMT has no active-site lysine residue to form an internal aldime, the absorption peak at 422 nm suggests that the enzyme is isolated as an external aldime with a bound amino acid at the active site. The K229H enzyme exhibits absorption maxima at 278 and 352 nm. However, it also displayed increased absorption at 300 nm compared to the wild-type enzyme, probably due to bound pyridoxal-P. Dialysis of K229H SHMT with 0.2% SDS overnight did not result in significant loss of the absorption band at 352 nm. This suggests that the pyridoxal-P is bound as a covalent complex. The spectrum of K229R SHMT exhibits absorption maxima at 330 and 390 nm. The 390 nm band is characteristic of the free aldehyde form of pyridoxal-P and suggests that in this mutant enzyme, the coenzyme is not bound as an external aldime as observed with K229Q SHMT.

Saturation of wild-type SHMT with Gly results in three spectral absorption maxima at 345, 425, and 495 nm, which have been shown to be complexes on the reaction path (Schirch et al., 1985). Addition of either 50 nm glycine or serine to K229Q SHMT did not change the spectrum of the purified enzyme. Addition of either serine or glycine to K229H SHMT also did not result in any change in its spectral properties. However, addition of either glycine or serine to K229R SHMT resulted in a shift of the absorption maxima from 390 nm to a spectrum with maxima at 406 and 332 nm. The 406 nm band was ~25% lower than the 390 nm band, and the 332 nm band was ~15% higher than the 330 nm band of the enzyme in the absence of amino acids as shown in Fig. 1. This suggests that this mutant enzyme can form complexes with these amino acids. Rapid removal of the excess amino acids by gel filtration in spin columns resulted in a return to the spectrum, shown in Fig. 1, within 30 s. Prolonged incubation with serine, however, rendered the enzyme inert to further changes, suggesting that some covalent modification had occurred.

Previous studies with SHMT have shown that addition of H₄PteGlu₄, (or its 5-methyl and 5-formyl derivatives) to the SHMT-Gly complex shifts the equilibrium to the quinonoid complex absorbing near 500 nm, resulting in a 2-order magnitude increase in absorbance (Schirch et al., 1985). The addition of glycine and H₄PteGlu to the three mutant enzymes did not produce this large increase in absorbance near 500 nm. Only K229Q SHMT showed any evidence of quinonoid formation. Even with this mutant enzyme, the absorbance after saturation with glycine and H₄PteGlu was at least 2 orders of magnitude lower than with wild-type SHMT.

Another characteristic spectral property of wild-type SHMT is the shift of the 422 nm absorbing band to 325 nm after addition of NaCNBH₃. This is the result of the rapid reduction of the internal aldime to a secondary amine (Schirch et al., 1985). Complete reduction occurs in a few minutes. Addition of excess NaCNBH₃ to the three mutant enzymes resulted in no significant change in spectral properties during a 1-h incubation. Prolonged incubation of K229Q SHMT suggested that a slow reduction was occurring by evidence of the partial appearance of the 422 nm band and the concomitant appearance of a band at 325 nm. However, after several hours, this reaction had resulted in only 10–15% change in these spectral bands.

The thermal stability of wild-type SHMT has been extensively studied using differential scanning calorimetry (Stover et al., 1992). These studies have shown that forming the serine external aldime increases both the Tₘ and ΔHₘ of the enzyme. This is correlated with the enzyme going from an open conformation to a closed conformation on forming the external aldime (Stover et al., 1992). We have also used differential scanning calorimetry to determine the values for Tₘ and ΔHₘ of K229Q SHMT-Ser to determine the effect of the mutation on the thermal stability of the enzyme. The results showed that the Tₘ was 75.9 ± 0.2 °C and the ΔHₘ was 320 ± 40 kcal/mol. Both of these parameters are only slightly lower than the observed values of 78 °C and 370 kcal/mol observed with the serine complex of wild-type SHMT. This suggests that the K229Q Ser complex is in the closed form and that the active-site lysyl does not play a significant role in stabilizing the enzyme.

Both K229H and K229R SHMTs were also investigated by differential scanning calorimetry in the presence of serine. The thermal denaturation of both of these mutant proteins showed very broad thermal transitions with multiple thermal peaks. With respect to Tₘ, the mutant proteins were intermediate between the wild-type Tₘ of 58.3 ± 0.1 °C for apoSHMT and 68 ± 1 °C for holoshMT without serine. The values of ΔHₘ for the K229H and K229R mutants were within experimental error of the 200 kcal/mol for apoSHMT. These results suggest that solutions of these two mutant proteins consist of multiple forms with respect to tertiary structure. To analyze if there were differences in secondary structure, circular dichroism spectra were obtained for the mutant proteins and compared to the wild-type enzyme. These results showed that in the 200–240 nm regions, there were no differences in band shape or intensity of optical activity between the wild-type and the K229Q and K229H mutant proteins. K229H SHMT had the same band shape, but did show an ~8% decrease in optical activity.

Catalytic Properties of K229Q, K229R, and K229H SHMTs—The ability of the three mutant enzymes to catalyze the conversion of serine and H₄PteGlu to glycine and 5,10-CH₂-H₄PteGlu was tested. For each mutant enzyme, a small amount of catalytic activity was found in different preparations, suggesting that the Vₘₐₙ values of the mutant enzymes were anywhere from 0.05 to 2% of the activity of the wild-type enzyme. However, this activity rapidly disappeared when the mutant enzymes were treated with NaCNBH₃. The rate of activity loss was the same as that observed with wild-type SHMT. The low level of catalytic activity was used to determine the Kₘ values for L-serine and allo-threonine. These values were found to be the same as those for wild-type SHMT. These two observations suggest that the small amount of activity of the three mutant enzymes was the result of contamination with a nonmutant form of SHMT. This was further supported by the observation that different preparations resulted in different amounts of catalytic activity. Extended studies on the origin of this small amount of activity resulted in the conclusion that there may be reversion of the mutation at the active site to form the wild-type gene. This was most often found with the K229Q and K229H mutants. We could never obtain a preparation that was completely devoid of a small amount of catalytic activity without treating the enzyme with NaCNBH₃. All subsequent studies were therefore performed with purified enzymes that had been pretreated with NaCNBH₃, which reduced catalytic activity to <0.01%, but did not cause observable changes in the spectral properties of the enzyme. The only exception to this is that K229H SHMT did show some absorption at 422 nm, which disappeared with the NaCNBH₃ treatment. The results of these kinetic studies suggest that none of the three mutant enzymes has steady-state catalytic activity.

All forms of SHMT that have been studied slowly catalyze the transamination of D-alanine and, to a lesser extent, L-alanine to pyruvate and pyridoxamine-P (Shostak and Schirch, 1988). The reaction results in the disappearance of the absorption band of bound pyridoxal-P at 422 nm with the concomitant appearance of the absorption band of pyridoxamine-P at 325 nm. The pyridoxamine-P has a low affinity for the enzyme and can be removed by either dialysis or gel filtration. These slow transamination reactions are important because they occur by the enzyme proceeding through a normal catalytic pathway of
removing the α-proton of the amino acid, but then replacing the proton on the 4'-carbon of the coenzyme. For wild-type SHMT, the transamination reactions are first order and can be used to

validation of both two-bound pyridoxal-P is reactive. Addition of 200 mM ω-alanine, pH 7.6, to K229R and K229H SHMTs showed no evidence of transamination. These are the conditions used to determine the rate of transamination with wild-type SHMT, which has a \( K_{m} \) for ω-alanine of 30 mM.

Addition of 200 mM ω-alanine to K229Q SHMT, pH 7.6, at 37 °C resulted in the biphasic disappearance of the absorption band at 422 nm and the appearance of a new peak at 325 nm. The complete spectral shift suggested that transamination had occurred. Gel filtration resulted in enzyme with no absorbing bands above 300 nm, indicating the formation of apoSHMT. Addition of pyridoxal-P to K229Q apoSHMT resulted in a slow spectral change to a form of enzyme exhibiting absorption maxima at 390 and 335 nm. Addition of either glycine or serine to this solution resulted in K229Q SHMT with a single band absorbing at either 418 or 424 nm, respectively, and exhibiting properties of the purified K229Q enzyme. Analysis of the biphasic rate of the disappearance of absorption at 422 nm during reaction with ω-alanine suggested that the reaction could be described as two first-order reactions with rate constants of \( x \times 10^{-4} \) and \( 5.5 \times 10^{-3} \) min\(^{-1}\). The faster rate is \( \approx 40\% \) of the rate of transamination observed with wild-type SHMT (21.6 \( \times \) 10\(^{-3} \) min\(^{-1}\)) (Shostak and Schirch, 1988).

For transamination to occur with ω-alanine and K229Q SHMT, any amino acid bound as an external aldime would have to be displaced to form the K229Q-ω-Ala complex. The displacement of a bound substrate amino acid in pyridoxal-P enzymes lacking an active-site lysine has been shown to be very slow (Toney and Kirsch, 1992). To determine if the slower rate of transamination of ω-alanine by K229Q SHMT was determined by the rate of a bound amino acid being displaced rather than the rate of transamination of ω-alanine, the purified enzyme was analyzed for released amino acids. Only serine and glycine were found, suggesting that the purified enzyme contained \( \approx 0.7 \) eq of bound serine and \( 0.3 \) eq of glycine (Table I). The ability of ω-alanine to convert the holoenzyme to apoenzyme was used to make K229Q SHMT with either only L-serine or glycine bound at the active site (see Experimental Procedures). The use of these two forms of the enzyme were reinvestigated for their ability to catalyze the transamination of ω-alanine. In each case, the spectral changes could be described by a single first-order reaction with rate constants of 2.9 \( \times \) 10\(^{-3} \) min\(^{-1}\) for the K229Q-Ser complex and 8.9 \( \times \) 10\(^{-3} \) min\(^{-1}\) for the K229Q-Gly complex. This difference in rate between the enzyme-serine and enzyme-glycine complexes accounts for the biphasic rate observed with the purified enzyme since it is a mixture of the serine and glycine complexes.

The ability of K229Q SHMT to transaminate L-alanine was also tested. The wild-type enzyme catalyzes the transamination of this amino acid at a rate that is about one-fourth the rate of ω-alanine (Shostak and Schirch, 1988). However, with K229Q-Gly, spectral changes occurring in the presence of 200 mM L-alanine suggested that a significantly slower transamination was occurring. After 7 h, the absorbance at 422 nm had decreased by only 16%, with a slightly larger increase in absorbance at 325 nm. Normally, the decrease in absorbance at 422 nm is nearly equivalent to the increase in absorbance at 325 nm since these two forms of the coenzyme have similar molar absorptivity coefficients. Longer incubations could not be followed because of slow precipitation of the enzyme. However, assuming that these spectral changes are the result of the transamination of L-alanine, the rate constant would be \( 0.4 \times 10^{-3} \) min\(^{-1}\), which is 15-fold slower than the rate of transamination of ω-alanine found for wild-type SHMT.

### Table I

| Enzyme form | Serine | Glycine |
|-------------|--------|---------|
| Purified K229Q SHMT | 0.7 | 0.3 |
| K229Q SHMT·Ser | 1.0 | 0 |
| +H\(_2\)PteGlu | 0.16 | 1.2 |
| K229Q SHMT·Gly | 0 | 1.0 |
| +CH\(_3\)H\(_2\)PteGlu | 0.65 | 0.17 |

![Fig. 2. A, off-rates for glycine (●) and L-serine (○) with their respective complexes with K229Q SHMT. The enzyme complexed with \(^{14}\)C[Gly or \(^{14}\)C]Ser in 50 mM KP, 10 mM 2-mercaptoethanol, 0.1 mM pyridoxal-P, and a 10 mM concentration of the respective radiolabeled amino acid, pH 7.6, was rapidly desalted into 50 mM KP, 10 mM 2-mercaptoethanol, and a 20 mM concentration of the respective nonradioabeled amino acid. During incubation at 37 °C, aliquots were removed at timed intervals, and the rate of radiolabeled amino acid to enzyme was determined as described under "Experimental Procedures." B, the off-rate for serine was determined as described above with either 100 mM Na\(_2\)SO\(_4\), (●) or (NH\(_4\))\(_2\)SO\(_4\), (○) included in the incubation buffer.](image-url)
had been converted to serine. This may be due to the equilib-

rium constant for this reaction, which favors the formation of
glycine over serine by a factor of 10 (Schirch et al., 1977). To
determine if it was an equilibrium problem, we coupled the
reaction to the conversion of the product H_4PteGlu to 10-CHO-
H_4PteGlu by adding formate, MgATP, and the enzyme 10-
formyltetrahydrofolate synthetase, which is one activity of the
trifunctional enzyme C_1-tetrahydrofolate synthase (Strong et
al., 1987). By removing the product H_4PteGlu, we were able to
convert nearly all of the bound glycine to serine (Table I).
Previous studies have shown that this assay works only if a poly-
glutamate form of the folate is used (Strong and Schirch, 1989).
We also found that to be true in this study. Using H_4PteGlu
instead of H_4PteGlu resulted in only ~20% of the glycine being
converted to serine.

The rate at which K229Q-Ser was converted to K229Q-Gly and
5,10-CH_2-H_4PteGlu was determined by adding an excess of
the enzyme methylenetetrahydrofolate dehydrogenase-cyclo-
ydrolase and NADP^+, which converts the CH_2-H_4PteGlu to
10-CHO-H_4PteGlu and NADPH (Strong et al., 1987). The rate
of the reaction was monitored at 340 nm. The results showed
that 1.0 eq of NADP^+/eq of K229Q-Ser was formed in this
reaction (Fig. 3). A trace of the absorbance change at 340 nm
at limiting dehydrogenase-cyclohydrolase concentration is shown
in Fig. 3A. The amplitude of the absorbance change was
proportional to the amount of K229Q-Ser used in the reaction.

To determine the rate of conversion of K229Q-Ser to K229Q-Gly,
ingcreasing amounts of the dehydrogenase-cyclohydrolase
zyme were added until no further increase in rate was ob-
served. The results are shown in Table II and suggest that the
rate of conversion of K229Q-Ser to K229Q-Gly is 0.21 s^{-1},
which is ~2% of the value of k_cat for wild-type SHMT in the
conversion of serine to glycine (Stover et al., 1992).

The rate at which K229Q-Gly and 5,10-CH_2-H_4PteGlu_3 are
converted to K229Q-Ser was investigated by coupling the conversion
of the product H_4PteGlu back to 5,10-CH_2-H_4PteGlu_3 by
adding of formate, MgATP, NADPH, and the trifunc-
tional enzyme C_1-tetrahydrofolate synthase. This is the same
system used to determine the conversion of bound glycine to
bound serine in the amino acid analysis experiments described
above. The first reaction in the coupled assay is the conversion
of H_4PteGlu to 10-CHO-H_4PteGlu by the synthetase activity
of the trifunctional enzyme. The k_cat for this reaction is an
order of magnitude smaller than the rate of conversion of glycine
to serine by wild-type SHMT (Strong and Schirch, 1989). Fig.
3B shows a stopped-flow spectrophotometer trace at 340 nm
after flowing K229Q-SHMT-Gly against 5,10-CH_2-H_4PteGlu_3
and the other components of the coupled enzyme system.
In this experiment, the C_1-tetrahydrofolate synthase was rate-
determining. The rate of decrease in absorbance at 340 nm was
first-order. The magnitude of the absorbance change at 340 nm
was proportional to the concentration of K229Q SHMT-Gly. A
linear rate of decrease in absorbance followed the first-order
burst and was also observed in the absence of K229Q-Gly. The
results reported in Fig. 3B are consistent with the absorbance

![Graph](https://via.placeholder.com/150)

**Fig. 3.** A, a single turnover of K229Q SHMT-Ser to K229Q
SHMT-Gly. In a stopped-flow spectrophotometer, 6.6 μM K229Q
SHMT-Ser was flowed against 0.1 mM H_4PteGlu, 0.6 mM NADPH,
and 9.4 μM 5,10-CH_2-H_4PteGlu dehydrogenase, all in 20 mM
KPi, 10 mM 2-mercaptoethanol, pH 7.2, at 30 °C. B, a single
turnover of K229Q SHMT-Gly to K229Q SHMT-Ser. A solution of 10.6
μM K229Q-Gly was flowed against 40 μM 5,10-CH_2-H_4PteGlu_3, 2 mM
MgATP, 0.3 mM NADPH, 20 mM ammonium formate, and 0.072 μM
C_1-tetrahydrofolate synthase at 30 °C. The buffer for both solutions
was 50 mM KPi, 10 mM Gly, 50 μM pyridoxal-P, and 10 mM 2-mercaptoethanol.
In both assays, the C_1-tetrahydrofolate synthase was rate-limiting.

| Table II |
|-----------------|--------|--------|----------|-
| **Rate of single turnover of K229Q SHMT-Ser to K229Q SHMT-Gly** |
| Reaction | K229Q complex | C_1-TFH synthase | k |-
| Ser → Gly | 1.3 | 1.6 | 0.21 ± 0.05 |-
| Gly → Ser | 3.3 | 13.3 | 0.20 ± 0.03 |-

* C_1-tetrahydrofolate.
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change at 340 nm being the result of 0.9 eq of K229Q SHMT·Gly being converted to K229Q SHMT·Ser. To determine the rate of this reaction, increasing amounts of C1-tetrahydrofolate synthase were added to show that the rate was the result of the conversion of glycine to serine and not the conversion of the product H4PteGlu to 10-CHO-H4PteGlu by the synthase reaction. As shown in Table II, the rate of decrease in absorbance at 340 nm was dependent on the amount of C1-tetrahydrofolate synthase that was added. We were unable to add enough of this enzyme to make the SHMT-catalyzed reaction rate-determining. The greatest value of the first-order rate constant determined suggests that K229Q-Gly is converted to K229Q-Ser with a rate that may be as large as the value of $k_{cat}$ for wild-type SHMT (Stover et al., 1992).

**DISCUSSION**

In aspartate aminotransferase, considerable evidence indicates that the active-site Lys plays a dual role in the mechanism of the reaction (Toney and Kirsch, 1991). First, it is the base that accepts and donates the proton in the 1,3-protonetric shift in the interconversion of the aldimine and ketimine intermediates. Second, it also is required to expel the product amino acid from the external aldimine intermediate by forming an internal aldimine (Toney and Kirsch, 1992). Several other pyridoxal-P enzymes have now been studied by site-directed mutants with respect to the function of the active-site Lys (Bhatia et al., 1993; Lu et al., 1993; Ilag and Jahn, 1992; Yoshimura et al., 1992; Grimm et al., 1992; Toney and Kirsch, 1991, 1992; Nishimura et al., 1991; Planas and Kirsch, 1991; Smith et al., 1989). Some of these mutant enzymes can slowly interconvert the aldimine and ketimine intermediates. However, in most cases, it appears that the active-site Lys residue is also the base that accepts the α-proton from the amino acid substrate. In each of these enzymes, expulsion of the product amino acid external aldimine is very slow, as found with aspartate aminotransferase.

Scheme I shows the reactions that appear to be involved with K229Q SHMT. The purified enzyme is a mixture of external aldmines of the two amino acid substrates glycine and serine (structures I and III). A key intermediate in the interconversion of these two complexes is the quinonoid complex shown as structure II. The interconversion of structures II and III involves a proton transfer between a base on the enzyme and the α-carbon of glycine. The failure to observe a strong absorbance of the ternary complex K229Q-Gly-H4PteGlu at 500 nm, as observed with wild-type SHMT, suggests that Lys-229 is the base involved in this proton abstraction. However, there are several experiments that indicate that this conclusion is wrong. First, K229Q SHMT catalyzes the transamination of D-alanine at 40% of the rate of wild-type SHMT (when starting with the glycine complex). The transamination of D-alanine involves forming the same quinonoid complex (structure II) with subsequent placement of the proton at the 4'-carbon of pyridoxal-P (structure IV). With the wild-type enzyme, the transamination of D-alanine is accompanied by a significant absorbance band at 502 nm, which shows that structure II accumulates during the reaction. With K229Q SHMT, there is no observable absorbance at 502 nm, suggesting that the equilibrium between structures II and III favors structure III in this mutant.

A second experiment that suggests that Lys-229 is not the base involved in the interconversion of structures III and I is that when 5,10-CHO-H4PteGlu is added to the K229Q-Gly complex, it is converted to the K229Q-Ser complex (Table I). We could not determine a true rate for this reaction because of the low $k_{cat}$ value for the coupling enzyme. However, it appears that the rate in this direction at least approaches the $k_{cat}$ value for wild-type SHMT. The rate-determining step in the interconversion of glycine and serine by wild-type SHMT is not known, so a direct comparison of the rates of the interconversion of structures III and I is not possible. However, the rate of conversion of Gly to Ser is sufficiently fast for K229Q SHMT that Lys-229 cannot be the base involved in this reaction.

K229Q SHMT also catalyzes the conversion of the bound serine to glycine when H4PteGlu is added (Table I). The rate of this conversion is 2% of the $k_{cat}$ value for the wild-type enzyme (Table II). If Lys-229 was the base accepting the proton in the conversion of glycine to serine, then it must be the acid that donates the proton in the conversion of the quinonoid complex to the glycine external aldimine. Even when the rate is only 2% of the wild-type rate, it would be difficult to argue that Lys-229 is the acid involved as the proton donor.

The second role played by the active-site Lys residue in aspartate aminotransferase appears also to be a role in SHMT. The observation that K229Q SHMT is purified with a mixture of serine and glycine bound at the active site suggests that these amino acids cannot be expelled readily by the mutant enzyme. Indeed, the off-rates of 6.9 $\times$ 10$^{-3}$ and 27 $\times$ 10$^{-3}$ min$^{-1}$ are similar to the slow release of the amino acid substrates found with aspartate aminotransferase (Toney and Kirsch, 1991, 1992). Small amines were found to accelerate the off-rate for amino acid products with aspartate aminotransferase. Likewise, we have found that 200 mM ammonia at pH 7.6 gives an ~3-fold increase in the rate of release of serine. This may be due to the ability of ammonia to form an imine by the mechanism shown in Scheme I (structures VI and VII). An unanswered question is if the ammonia attacks the external aldimine from the same or opposite face of the 4'-carbon as the active-site lysine.

We have previously shown that in the transamination of D-alanine and L-alanine by wild-type SHMT, different bases on the enzyme were involved in the removal of the (2S)-proton of
t-alanine and the (2R)-proton of L-alanine (Shostak and Schirch, 1988). In aspartate aminotransferase, it is the R-protons of aspartate and glutamate that are removed by the active-site lysine. Since it appears that the same face of the pyridoxal-P ring is facing the solvent in SHMT as in aspartate aminotransferase, one might expect that the expelled Lys-229 would be on the side of the (2R)-proton of L-alanine (Dunathan and Voet, 1974). It would then be in a position to be a base to remove the (2R)-proton. In support of this conclusion is the observation that the base that removes the (%)-proton of L-alanine is transaminated extremely slowly by K229Q SHMT. However, because there appeared to be a very slow rate of transamination, we cannot conclude without reservation that the base that removes the (2R)-proton of L-alanine is Lys-229.

Substitution of Lys-229 with Arg results in the isolation of an enzyme with at least part of the pyridoxal-P bound as the free aldehyde (Fig. 1). The addition of serine or glycine does convert some of the bound pyridoxal-P to the external aldimine. However, the affinity for serine must be low because as soon as the serine is removed, the external aldimine is converted in a few seconds back to the aldehyde form. The substitution of Arg for Lys-258 in aspartate aminotransferase results in an enzyme that can form the quinonoid intermediate (Toney and Kirsch, 1991). The observation that K229R SHMT cannot even form a stable external aldimine suggests that the active site does not tolerate a positive charge next to the imine-positive charge. This may further substantiate that Lys-229 is not the active-site base and that it never goes through a positive charged intermediate, as required for the aspartate aminotransferase mechanism.

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