Multifunctional Role of His$^{159}$ in the Catalytic Reaction of Serine Palmitoyltransferase*

Received for publication, November 24, 2008, and in revised form, February 27, 2009 Published, JBC Papers in Press, April 5, 2009, DOI 10.1074/jbc.M808916200

Yuka Shiraiwa, Hiroko Ikushiro, and Hideyuki Hayashi

From the Department of Biochemistry, Osaka Medical College, Takatsuki 569-8686, Japan

Serine palmitoyltransferase (SPT) belongs to the fold type I family of the pyridoxal 5′-phosphate (PLP)-dependent enzyme and forms 3-ketodihydrophosphoginosine (KDS) from l-serine and palmitoyl-CoA. Like other α-oxamine synthase subfamily enzymes, SPT is different from most of the fold type I enzymes in that its re face of the PLP-Lys aldimine is occupied by a His residue (His$^{159}$) instead of an aromatic amino acid residue. His$^{159}$ was changed into alanine or aromatic amino acid residues to examine its role during catalysis. All mutant SPTs formed the PLP-l-serine aldimine with dissociation constants several 10-fold higher than that of the wild type SPT and catalyzed the abortive transamination of l-serine. However, the reaction rate is diminished 505-nm absorption band of the quinonoid species during catalysis. Global analysis of the time-resolved spectra suggested the presence of the two quinonoid intermediates, the first formed from the PLP-l-serine aldimine and the second from the PLP-KDS aldimine. Accumulation of these quinonoid intermediates indicated that His$^{159}$ not only the anchoring site for l-serine but regulates the α-deprotonation of l-serine by fixing the conformation of the PLP-l-serine aldimine to prevent unwanted side reactions. Only H159A SPT retained activity and showed a prominent 505-nm absorption band of the quinonoid species during catalysis. Global analysis of the time-resolved spectra suggested the presence of the two quinonoid intermediates, the first formed from the PLP-l-serine aldimine and the second from the PLP-KDS aldimine. Accumulation of these quinonoid intermediates indicated that His$^{159}$ promotes both the Claisen-type condensation as an acid catalyst and the protonation at Cα of the second quinonoid to form the PLP-KDS aldimine. These results, combined with the previous model building study (Ikushiro, H., Fujii, S., Shiraiwa, Y., and Hayashi, H. (2008) J. Biol. Chem. 283, 7542–7553), lead us to propose a novel mechanism, in which His$^{159}$ plays multiple roles by exploiting the stereochemistry of Dunathan’s conjecture.

Coenzymes act as catalysts in biological systems, and many enzymes require coenzymes as the important catalytic group. In most cases, coenzymes can carry out the catalysis in the absence of the enzyme protein. However, the reaction rate is much lower than the rate in the system containing the enzyme protein. Furthermore, the reaction specificity is reduced in the nonenzymatic system; coenzymes without the enzyme protein tend to undergo side reactions. A remarkable example is the coenzyme pyridoxal 5′-phosphate (PLP). PLP is a versatile catalyst catalyzing transamination, decarboxylation, elimination, aldol cleavage, Claisen-type condensation, etc. of amino acids. Therefore, a pyridoxal enzyme is required to have a structure that enables elaborated chemical mechanism by which only a specific reaction proceeds at each catalytic step.

Serine palmitoyltransferase (SPT) catalyzes the condensation reaction of l-serine and palmitoyl-CoA to produce 3-ketodihydrophosphoginosine (KDS) (1). This is the first step in the sphingolipid biosynthesis. SPT belongs to the PLP-dependent α-oxamine synthase subfamily containing 5-aminolevulinate synthase, 8-amino-7-oxononanoate synthase, and 2-amino-3-ketobutyrate CoA ligase (2–6). All of them have been successfully crystallized, and their three-dimensional structures have been determined (7–12). These enzymes belong to the fold type I family of the PLP-dependent enzymes according to their folding pattern (5, 6). The commonly known fold type I PLP-dependent enzymes have an aromatic amino acid residue locating at the re face of the PLP-Lys internal aldimine and stacking with the pyridine ring of PLP. On the other hand, all members of the PLP-dependent α-oxamine synthase subfamily known to date have a His residue in this position. Therefore, the His residue is expected to play unique roles in the reaction mechanism of the PLP-dependent α-oxamine synthase subfamily enzymes.

Scheme 1 shows the chemical reaction mechanism of SPT (1, 13). At the active site of SPT, PLP forms an aldimine with the ε-amino group of Lys$^{265}$ (internal aldimine, I). The internal aldimine undergoes transaldimination with the first substrate l-serine to yield the PLP-l-serine aldimine (external aldimine, II). After binding of the second substrate palmitoyl-CoA, α-deprotonation occurs to form the first quinonoid intermediate (III). The carbanionic Cα of III attacks palmitoyl-CoA (Claisen-type condensation) to generate a condensation product (IV), which, by decarboxylation, yields the second quinonoid intermediate (V). Protonation at Cα of V gives the external aldimine of PLP-KDS (VI). Finally, release of KDS regenerates the internal aldimine (I). For this reaction mechanism, we proposed by model building studies that His$^{159}$ of SPT is the anchoring site for both l-serine and palmitoyl-CoA and possibly involved in the catalytic steps (13). However, no experimental analyses have been made to confirm this proposal or to gain further insight into the function of the residue. To determine the catalytic role of His$^{159}$, especially its role in the reaction specificity of PLP-dependent α-oxamine synthase subfamily enzymes.

---

* This work was supported in part by Grants-in-Aid for Scientific Research 21570120 (to H. H.) and 21570149 (to H. I.) from the Japan Society for the Promotion of Science and a grant from the Osaka Medical Research Foundation for Incurable Diseases (to H. I.).
1 To whom correspondence may be addressed: 2-7 Daigakumachi, Takatsuki 569-8686, Japan. Fax: 81-72-684-6516; E-mail: ikushiro@art.osaka-med.ac.jp.
2 To whom correspondence may be addressed: 2-7 Daigakumachi, Takatsuki 569-8686, Japan. Fax: 81-72-684-6516; E-mail: hayashi@art.osaka-med.ac.jp.
3 The abbreviations used are: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; HPLC, high performance liquid chromatography; KDS, 3-ketodihydrophosphoginosine; SPT, serine palmitoyltransferase; WT, wild type.
enzymes, we constructed mutant *Sphingomonas paucimobilis* SPTs, in which His\(^{159}\) was replaced by Ala and aromatic amino acid residues, and analyzed the reaction of these mutant enzymes. The results showed that His\(^{159}\) has at least two additional distinct functions: one as a residue that controls the reaction pathway by adjusting the conformation of the PLP-L-serine external aldimine and the other as an acid catalyst that promotes the reactions of the Claisen-type condensation and the following steps.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Palmitoyl-CoA was obtained from Funakoshi (Tokyo, Japan). *Escherichia coli* BL21 (DE3) pLysS and plasmid pET21b were from Novagen (Madison, WI). S-(2-Oxoheptadecyl)-CoA was synthesized as previously described (13). 3-Ketodihydrosphingosine hydrochloride was obtained from Matreya LLC (Pleasant Gap, PA). All other chemicals were of the highest grade commercially available.

**Expression and Purification of SPT**—The mutation of His\(^{159}\) to Ala (H159A), Phe (H159F), Tyr (H159Y), and Trp (H159W) was introduced by a two-step PCR as previously described (13). H159A was purified in the same way as the wild type enzyme (14). Other mutant proteins were purified accordingly except for the addition of 200 mM L-serine at the active site of SPT is accelerated by phenylalanine residues in SPT (15).

**Kinetic Analysis**—Stopped flow spectrophotometry was performed using an Applied Photophysics (Leatherhead, UK) four-syringe SX.18MV spectrophotometer equipped for both conventional and sequential stopped flow measurements. The reactions were carried out in 50 mM HEPES-NaOH, 150 mM KCl, and 0.1 mM EDTA, pH 7.5, at 298 K. For the sequential stopped flow measurements, two solutions (enzyme and L-serine) were mixed and allowed to stand for an appropriate time in the aging loop. After a programmable delay of 1 s, when most of the PLP-L-serine external aldimine had been formed, the contents of the aging loop were mixed with the third solution, palmitoyl-CoA. The dead time was 2.3 ms under a gas pressure of 600 kPa. Time-resolved spectra were collected using the SX.18MV system equipped with a photodiode array accessory and the XScan (version 1.0) controlling software. The absorption changes were analyzed using the software Pro-KII (Applied Photophysics).

**Determination of PLP and PMP in H159A SPT**—The high performance liquid chromatography (HPLC) analysis was performed according to Ref. 16 with minor modifications. H159A SPT was incubated in the HEPES buffer in the presence or absence of 200 mM L-serine at room temperature overnight, and then perchloric acid was added to a final concentration of 5% (w/v). The samples were centrifuged at 16,000 \(\times\) g for 15 min, and the supernatant was analyzed by HPLC using a prepacked C\(_{18}\) reversed phase column (Cosmosil 5C\(_{18}\)-AR-II, 4.6 \(\times\) 150 mm; Nacalai Tesque, Kyoto, Japan). The mobile phase was 50 mM potassium phosphate, pH 3.2, acetonitrile (99/1, v/v), and the flow rate was 1 ml/min. PLP and PMP were detected by fluorescence at 395 nm after excitation at 290 nm.

**Synthesis of \([\alpha\text{-}^2\text{H}]\text{L-serine}\)**—The synthetic strategy for the preparation of \([\alpha\text{-}^2\text{H}]\text{-serine}\) is based on the fact that deprotonation at C\(_{\alpha}\) of the L-serine at the active site of SPT is accelerated by the presence of S-(2-oxoheptadecyl)-CoA, an analogue of palmitoyl-CoA (13). A mixture containing 100 mM L-serine, 100 \(\mu\)M S-(2-oxoheptadecyl)-CoA, and 40 \(\mu\)M SPT was incubated overnight at room temperature in 50 mM potassium phosphate buffer (D\(_{2}\)O, pH 7.5). The \(\alpha\)-proton peak in the \(^1\)H NMR spectrum disappeared after 24 h (data not shown). At this point, SPT was removed from the reaction mixture by ultrafiltration using VivascipTM (Sartorius Stedim Biotech, Aubagne, France). The filtrate solution was adjusted to pH 3 with HCl and applied to a Dowex-50 column. \([\alpha\text{-}^2\text{H}]\text{-serine}\) was eluted with 1 M \(\text{NH}_4\text{OH}\) and collected. It was then dried and recrystallized. The yield was 72.8%.
where $\varepsilon_{\text{PLP}}$ and $\varepsilon_{\text{holo}}$ denote the absorptivity at 430 nm of the free PLP and the holoenzyme, respectively, and $K_d$ is the dissociation constant for the enzyme and PLP. A theoretical line based on Equation 1 was fitted to the experimental values (Fig. 1B), and the parameters were determined to be $\varepsilon_{\text{PLP}} = 1320 \pm 3 \text{ M}^{-1} \text{ cm}^{-1}$, $\varepsilon_{\text{holo}} = 2840 \pm 70 \text{ M}^{-1} \text{ cm}^{-1}$, and $K_d = 18.9 \pm 3.0 \text{ mM}$.

When H159A SPT saturated with PLP was subjected to gel filtration to remove the excess PLP, the enzyme essentially showed the same spectra as WT SPT (Fig. 1A, dashed line) with two absorption bands at 332 and 412 nm, each corresponding to the enolimine and ketoenamine forms of the internal aldimine. Contrary to H159A SPT, the purified H159F, H159Y, and H159W SPTs were not complete apoenzymes, and the apoenzymes of these mutant SPTs were obtained by treatment with phenylhydrazine. In the spectra of these mutant enzymes saturated with PLP, the intensity of the 412-nm absorption band was greater than that in H159A SPT (data not shown). This increase in intensity was considered to occur because the ketoenamine form was stabilized in H159F, H159Y, and H159W SPTs. Titration with PLP was carried out in the same way as for H159A SPT. The apoenzyme of WT SPT was also treated with phenylhydrazine and titrated with PLP. The apparent dissociation constant ($K_d$) is summarized in Table 1. The value of $K_d$ for the mutant SPTs was lower than that of WT. This indicates that His$^{159}$ does not play significant roles in the binding of PLP to the enzyme.

Activity of Mutant SPTs—When [14C]L-serine was used as the substrate, the radiolabeled product KDS was detected only in H159A among the mutant SPTs (Fig. 2). Therefore, steady state kinetic analysis of H159A SPT was performed. When PLP was not added to the reaction mixture, the rate of product formation by H159A SPT rapidly declined with time. On the other hand, the time course was almost linear up to 20 min in the presence of 0.2 mM PLP, provided that the L-serine concentration was below 20 mM (data not shown). At higher concentrations of L-serine, it was necessary to increase the concentration of PLP to obtain the linear time course up to 20 min. These nonlinear behaviors are due to the abortive transamination catalyzed by H159A (see the next section), which becomes pronounced with increasing substrate concentration. The rate for the production of KDS was plotted versus the substrate concentrations (supplemental Fig. S1), and the theoretical lines based on the steady state ordered Bi-Bi mechanism were fitted to the experimental values. The kinetic parameters were obtained under two different conditions: condition A (0.2 mM PLP, 2–20 mM L-serine, and 0.01–2 mM palmitoyl-CoA), $K_m$ (L-serine) = 128 ± 51 mM, $K_m$ (palmitoyl-CoA) = 1.6 ± 0.7 mM, and $k_{cat} = 0.200 \pm 0.070 \text{ s}^{-1}$; and condition B (1 mM PLP, 2–200 mM L-serine, and 0.01–5 mM palmitoyl-CoA), $K_m$ (L-serine) =
Multifunctional Histidine Residue

58.1 ± 5.5 mM, $K_m$ (palmitoyl-CoA) = 0.72 ± 0.08 mM, and $k_{cat} = 0.112 ± 0.004$ s$^{-1}$. The corresponding values of WT SPT are $K_m$ (l-serine) = 6.2 ± 0.6 mM, $K_m$ (palmitoyl-CoA) = 1.0 ± 0.1 mM, and $k_{cat} = 0.69 ± 0.03$ s$^{-1}$, which were obtained under the condition of 10 μM PLP, 2–20 mM l-serine, and 0.01–2 mM palmitoyl-CoA (13). Unfortunately, neither of the two conditions is ideal for obtaining the kinetic parameters of H159A SPT; condition A uses the l-serine concentrations lower than the $K_m$ value, and condition B uses a high concentration of PLP, which tends to undergo nonspecific modification of Lys residues of enzyme proteins and may alter their properties (18). However, we can say that the $K_m$ for l-serine is apparently increased (20-fold under condition A and 10-fold under condition B) by the H159A mutation. The H159A mutation did not significantly decrease the $k_{cat}$ value; H159A SPT retained 29% (condition A) or 16% (condition B) of the activity of WT SPT. The $K_m$ for palmitoyl-CoA was also essentially unaffected by the H159A mutation.

Abortive Transamination from the External Aldimine in H159A SPT—The addition of l-serine to H159A SPT caused an increase in the absorption band at 416 nm and a concomitant decrease in the absorption band at 332 nm (Fig. 3, line 1). As in the case of WT SPT, this is considered to reflect the formation of the external aldimine, in which the ketoenamine tautomer became dominant as compared with the internal aldimine. However, the spectrum of the external aldimine was not stable, and the intensity of the 416-nm absorption band gradually decreased, and a new absorption band appeared at 326 nm (Fig. 3). After the spectral transition was over, the enzyme solution was deproteinized and subjected to HPLC analysis. A stoichiometric amount of PMP was found to be formed from the PLP of the holoenzyme incubated with l-serine (supplemental Fig. S2). These results indicate that the external aldimine in H159A SPT undergoes an abortive transamination. The decrease in the absorption at 416 nm was fitted to an exponential curve, and the rate constant for the abortive transamination was calculated to be (4.43 ± 0.02) × 10$^{-4}$ s$^{-1}$. H159F, H159Y, and H159W SPT reacted with l-serine in a manner similar to H159A SPT, and the rate constants for the abortive transamination were obtained (Table 1).

Titration with l-Serine—Because the external aldimine is not stable in the His$^{159}$ mutant enzymes, the dependence of the formation of the external aldime with l-serine was followed by stopped flow spectrophotometry. The absorption at 416 nm exponentially increased and then gradually decreased upon mixing the enzymes with l-serine (data not shown). Therefore, the spectra at which $A_{416}$ reached the maximum value (generally 1 s after mixing, depending on the l-serine concentration) were collected from each set of the time-resolved spectra. The absorbance at 416 nm was plotted versus the l-serine concentration, and the dissociation constant ($K_d$) was obtained in the same way as described in Ref. 14. The $K_d$ value of H159A SPT was 77.1 mM and was 50-fold greater than that of WT SPT. The other mutant enzymes showed $K_d$ values greater than that of WT SPT but severalfold lower than that of H159A SPT (Table 1).

Accumulation of the Quinonoid Intermediate in the Reaction of H159A SPT, l-Serine, and Palmitoyl-CoA—Among the His$^{159}$ mutant SPTs, only H159A SPT showed any enzyme activity. Therefore, a transient kinetic analysis of the reaction of H159A SPT with l-serine and palmitoyl-CoA was carried out using a sequential stopped flow system. This system was necessary because the external aldime complex of H159A SPT with l-serine must react with the palmitoyl-CoA before it undergoes abortive transamination. The enzyme solution (200 μM) was mixed with an equal volume of 400 mM l-serine and allowed to stand for 1 s in the aging loop, and then the enzyme-l-serine complex was mixed 1:1 with various con-
centrations of palmitoyl-CoA containing 200 mM L-serine. PMP formed during the 1 s was considered to be negligible as calculated from the rate constant of the abortive transamination. The time-resolved spectra showed a new absorption band at 505 nm, indicating the formation of the quinonoid intermediate. The singular value decomposition implemented to the Pro-KII software indicated that the spectral eigenvector suggests the presence of two spectroscopically distinct species. The simplest model that accounts for this is the following equation,

\[
A \rightarrow \text{PalCoA} \rightarrow B \rightarrow D \rightarrow A + KDS
\]

where \(A\) and \(B\) denote the enzyme-L-serine complex and the enzyme-L-serine-palmitoyl-CoA ternary complex, respectively, and \(A\) and \(B\) are assumed to be in rapid equilibrium. Because the experiment was carried out in the presence of a nearly saturating concentration of L-serine, and the rate constant for the formation of the external aldimine is of the order of 1000 s\(^{-1}\) (14), the free enzyme generated from \(D\) by dissociating KDS was assumed to be rapidly converted to \(A\) (the symbol \(D\) was used here for the sake of consistency with the model of Equation 3). The global analysis based on this model yielded a quinonoid-like spectrum for the intermediate \(D\) (data not shown), but its absorptivity at 505 nm reached the value of \(4.1 \times 10^8\) M\(^{-1}\) cm\(^{-1}\), which was 10⁴-fold higher than that of the typical quinonoid intermediate (13). Additionally, the rate constant for \(B \rightarrow D\) was obtained to be \(2.6 \times 10^{-5}\) s\(^{-1}\), which was far lower than the \(k_{cat}\) value of H159A SPT. Therefore, the model of Equation 2 was considered to be inadequate for describing the results of Fig. 4A. We then added an intermediate \(C\) to the above model.

\[
A + \text{PalCoA} \rightarrow B \rightarrow C \rightarrow D \rightarrow A + KDS
\]

The global analysis based on this model yielded the spectra of the intermediates and the values of the kinetic parameters, each shown in Fig. 4B and Table 2, respectively. Both \(C\) and \(D\) have absorption maximum at around 500 nm with molar absorptivity values above 30,000 M\(^{-1}\) cm\(^{-1}\). Thus, \(C\) and \(D\) are the quinonoid intermediates and are reasonably considered to correspond to III and V, respectively, in Scheme 1. The abortive transamination was not included in Equation 3. This is rationalized by the fact that \(k_{+4} = 0.46\) s\(^{-1}\) was much greater than \(4.4 \times 10^{-4}\) s\(^{-1}\) of the rate constant of the abortive transamination. Considering the \(K_d\) value for L-serine (77 mM), H159A SPT is not completely saturated with 200 mM of L-serine. Therefore, although the \(A\) of Equation 3 is largely the external aldimine, it
contains a small fraction of the internal aldime. This, however, does not affect the kinetic parameters and the absorption spectra of the intermediates in Equation 3 except for $K_d$ and the spectrum of $A$, both of which are irrelevant to the following discussion.

A similar kinetic analysis was carried out for the reaction of H159A SPT with [α-2H]l-serine and palmitoyl-CoA, to detect the isotopically sensitive step. The time-resolved spectra are shown in Fig. 4C. Accumulation of the quinonoid intermediate was less than that observed for the reaction with l-serine and palmitoyl-CoA. The global analysis of the time-resolved spectra gave, however, intermediate spectra essentially identical to those obtained from the reaction with l-serine and palmitoyl-CoA (Fig. 4D). Among the kinetic parameters, only $k_{+2}$ showed a high kinetic isotope effect: $0.064 \pm 0.004 \text{ s}^{-1}$ for [α-2H]l-serine compared with $0.46 \text{ s}^{-1}$ for l-serine (Table 2). The kinetic isotope effect value of 7.2 is very close to the value of 7.3 for the $\alpha$-prototropic shift in aminotransferases (19), which also involves deprotonation at Coα. This strongly indicates that $k_{+2}$ really represents the $\alpha$-deprotonation and supports that C in Equation 3 is the quinonoid intermediate formed by deprotonation of the external aldime with l-serine (III in Scheme 1).

Further support for the model of Equation 3 came from the direct observation of V in Scheme 1. When H159A SPT was reacted with KDS, a new absorption band appeared at 505 nm, indicating the formation of the quinonoid intermediate (Fig. 5A). The shape and position of the quinonoid intermediate closely matched those of D obtained by global fitting (Fig. 4B). The spectrum of H159A SPT saturated with KDS was not obtained because of the water insolubility of KDS and the spectral deterioration caused by the glycerol used to dissolve KDS (indicated by the increase in the baseline absorption). Using the $K_d$ value of 13 μM ($k_{+2}/k_{-2}$) for KDS and the concentration of KDS used here (6 μM), the molar absorptivity of the quinonoid species generated from KDS is calculated to be 34,800 M$^{-1}$ cm$^{-1}$, comparable with that of D. These results support the idea that D is the deprotonated species of the PLP-KDS external aldime, i.e. D is equivalent to V in Scheme 1. The absorbance spectra of WT SPT with KDS showed the formation of the external aldime, but the quinonoid intermediate is only slightly generated (Fig. 5B).

The spectrum of the quinonoid intermediate formed by the reaction of H159A SPT with KDS was not stable, and the intensity of the 505-nm absorption gradually decreased with a concomitant increase in the absorption at 332 nm (Fig. 5C). The increase in the 332-nm band was a biphasic process, in which the fast phase corresponded to the decrease in the 505-nm absorption band. These results are interpreted as the decay of the quinonoid intermediate proceeding through a relatively fast protonation to yield the ketimine, followed by a slow hydrolysis to PMP and a ketone.

### DISCUSSION

**Binding of PLP**—H159A SPT was obtained as an apoenzyme, and H159F, H159Y, and H159W SPTs as a partial apoenzyme. However, the ability of SPT to bind PLP was not significantly affected by changing His$^{159}$ to Ala or aromatic amino acid residues; the affinity of H159F was even higher than WT SPT by 10-fold (Table 1). Thus, we concluded that His$^{159}$ has no significant role in holding the coenzyme PLP at the active site of SPT. Considering the fact that all of the His$^{159}$ mutant enzymes studied here catalyzed the abortive transamination, and among them, H159A had the highest transamination activity and the highest fraction of the apoenzyme, we may think that the His$^{159}$ mutant enzymes underwent abortive transamination in the...
**Multifunctional Histidine Residue**

*E. coli* cells or in the crude extract and became apoenzymes by losing the transamination product PMP.

His$^{159}$ Accommodates the l-Serine Moiety of the External Aldimine—The SPT-l-serine complex predominantly exists as the external aldime (13). The apparent dissociation constant $K_d$ for l-serine increased by 55-fold with the H159A mutation and about 20-fold with the H159F, H159Y, and H159W mutations (Table 1). Consistent with this, the $K_d$ for l-serine increased by 10–20-fold by the H159A mutation. This clearly indicates that His$^{159}$ provides a binding site for the l-serine moiety of the external aldime. When *S. paucimobilis* SPT was modeled on the crystal structure of *Rhodobacter capsulatus* 5-aminolevulinate synthase, the carbonyl group of l-serine in the external aldime was expected to form a hydrogen bond with the Ne2 of His$^{159}$ (13). This structure has not been proven experimentally, because crystallization of *S. paucimobilis* SPT complexed with l-serine or its analogues has not been successful to date. However, a recent crystallographic study of a *Sphinogobacterium* SPT, which is closely related to *S. paucimobilis* SPT, has shown that it binds l-serine exactly in the same orientation as the model structure described above.4 Therefore, it is reasonable to consider that in *S. paucimobilis* SPT, His$^{159}$ contributes to fixing the l-serine moiety in the external aldime by forming a hydrogen bond with the carboxylate group. In this regard, it is important to point out that a similar hydrogen bond has been shown to be formed between the carboxylate group of the PLP-2-amino-3-ketobutyrate external aldime and His$^{156}$ of 2-amino-3-ketobutyrate CoA ligase, although 2-amino-3-ketobutyrate corresponds to the condensation product rather than the substrate of the SPT reaction (10). The lower $K_d$ for l-serine of H159F, H159Y, and H159W SPTs relative to H159A SPT may be considered to be due to some interactions such as van der Waals’ or hydrophobic interaction of the bulky aromatic intermediate (13) (illustrated as III in Scheme 2). Based on this model, we can consider that in the mutant SPTs in which His$^{159}$ was replaced by aromatic amino acid residues, the bulky aromatic ring would hamper the access of the carbonyl group of palmitoyl-CoA to the right position, because the hydrogen atoms of the aromatic ring do not undergo any favorable interaction with the carbonyl oxygen. On the other hand, H159A has no such aromatic ring at this position and allows access by the carbonyl group of palmitoyl-CoA to react with the l-serine moiety. This would explain why H159A SPT retained activity, but H159F, H159Y, and H159W SPTs lost their activity. This is also consistent with the finding that the quinonoid intermediate was not accumulated in the reaction of H159F with l-serine and palmitoyl-CoA (data not shown).

The rate constant of the $\alpha$-proton abstraction ($k_{\alpha}$) was estimated to be 0.46 s$^{-1}$ (Table 2), which was only 5.7-fold lower than the corresponding value of WT obtained by $^1$H NMR in the presence of a palmitoyl-CoA analogue (2.6 s$^{-1}$) (13). The decrease in the value by the H159A mutation may be considered to reflect a slight deviation from the ideal conformation of the external aldime. The $k_{\alpha}$ value of H159A SPT is, however, nonetheless much higher than the

---

4 H. Ikushiro, M. M. Islam, A. Okamoto, J. Hoseki, T. Murakawa, S. Fujii, I. Miyahara, and H. Hayashi, manuscript submitted for publication.
rate constant for the abortive transamination \((4.4 \times 10^{-4} \text{ s}^{-1})\). Therefore, it is reasonable to consider that, after the binding of palmitoyl-CoA, the Ca–H bond of the external aldimine is fixed to the orientation perpendicular to the imine-pyridine plane, which is favorable for deprotonation. The previous modeling study (13) suggested that the binding of palmitoyl-CoA induces the conformational change in the active site residues, and the carboxylate group of the L-serine moiety of the external aldimine forms a new hydrogen bond with the guanidinium group of Arg390. The present results strongly support this mechanism, and together with the results of the acceleration of the \(-\text{deprotonation by the palmitoyl-CoA analogue (13)}\), we conclude that the binding of palmitoyl-CoA causes the carboxylate group of L-serine to switch its hydrogen-bonding/ionic interaction partner from His159 to Arg390 to produce the conformation of the external aldimine favorable for deprotonation (Scheme 2, IIa → IIb).

**Role of His159 as an Acid Catalyst in the Claisen-type Condensation**—The H159A mutation resulted in the decreased \(\alpha\)-deprotonation rate by 5.7-fold. Despite this, accumulation of the quinonoid intermediates, which had not been observed even transiently in the reaction of WT SPT, was observed during the turnover reaction of H159A SPT (Fig. 4A). This can be explained by the observation that the rate constant \((k_+3)\) for the Claisen-type condensation reaction (Scheme 2, III → IV), estimated to be \(\geq 75 \text{ s}^{-1}\) for WT SPT (13), was decreased to 4.1 \(\text{s}^{-1}\) by H159A mutation. A chemical consideration suggests that the carbonyl oxygen of palmitoyl-CoA should be protonated when it is attacked by the carbanionic \(\alpha\) of the quinonoid intermediate. The fact that \(\text{Ne}2\) of His159 is expected to be protonated and form a hydrogen bond with the carbonyl oxygen of palmitoyl-CoA (Scheme 2, IIb) makes it highly probable that His159 donates the proton to the carbonyl oxygen during the nucleophilic attack (Scheme 2, III). Thus, His159 may be the general acid catalyst for the condensation reaction, as has been indicated by Hunter et al. (20) for the role of the corresponding His residue of 5-aminolevulinate synthase in the condensation reaction of glycine and succinyl-CoA.

**Other Roles of His159**—As discussed above, the H159A mutation decreased the rate of the Claisen-type condensation step (III → IV). In Scheme 2, if we assume that His159 does not affect
the step after IV, only III and not Vb is expected to be observed as the quinonoid intermediate in the time-resolved spectra. Therefore, the accumulation of Vb raises the possibility that His^{159} plays some important role in the step after Vb.

For discussing this point, the results of the spectroscopic analysis of the binding of the product KDS to SPT provides some clues. When KDS is added to the enzyme solutions, H159A SPT shows an intense absorption of the quinonoid intermediate (Fig. 5, A and B). Because KDS does not bear a carboxylate group, the accumulated quinonoid species is considered to be Vb in Scheme 2. In the crystal structure of the 8-amino-7-oxononanoate synthase-8-amino-7-oxononanoate complex, a hydrogen bond between O7 of 8-amino-7-oxononanoate and His^{133} is formed (9). In a similar way, His^{159} may form the hydrogen bond with the acyl carboxyl O of KDS in the complex of WT SPT with KDS (Scheme 2, VI). Clearly, this hydrogen bond destabilizes Vb relative to Va, because the quinonoid intermediate requires a planar structure spanning from the pyridine ring to Cβ of the KDS moiety. This explains why Vb is stabilized in the H159A SPT. Supporting this, the transamination starting from KDS was observed only with H159A SPT (Fig. 5C). The transamination requires the ketimine structure, which has a planar structure of the KDS moiety and therefore would be unfavorable for forming the hydrogen bond between the carbonyl O of KDS and His^{159}. Accordingly, we can speculate that the protonation to Va is promoted in WT SPT because of the formation of Va that is fixed by the hydrogen bond between His^{159} Ne2 and the carbonyl O of the KDS moiety. The structure of Va is also important for preventing the unwanted abortive transamination of the KDS product.

Considering the association of the carbonyl group of palmitoyl-CoA and KDS with His^{159}, it is also reasonable to assume that a similar carbonyl-His^{159} interaction is formed in IV of Scheme 2. In IV, the Co–COO− bond is 30° out of the imine-pyridine plane. This conformation, however, is not ideal if the decarboxylation of IV is catalyzed by the imine-pyridine conjugate system. Rather, the Ca–COO− bond would be cleaved by the action of the carbonyl group, because the bond is expected to be more perpendicular to the plane of the carbonyl group. Again, this is analogous to the mechanism proposed for the 5-aminolevulinate synthase-catalyzed decarboxylation to form 5-aminolevulinate (20), and His^{159} may contribute to the decarboxylation if it donates a proton to O in the carbonyl group.

Conclusion—The multifunctional role of His^{159} in the reaction mechanism was experimentally proposed by the analysis of the His^{159} mutant SPTs. His^{159} may be the residue that recognizes t-serine, which fixes the conformation of the external aldime, thereby preventing it from undergoing the abortive transamination. His^{159} also seems to enhance the Claisen-type condensation and decarboxylation by functioning as an acid catalyst. Furthermore, His^{159} may avoid accumulation of the quinonoid intermediate of SPT with KDS by shifting the equilibrium toward the external aldime. These proposals will be verified in the future by combined crystallographic and kinetic studies using a series of substrate analogues designed to mimic the intermediates.

Acknowledgment—We thank Professor S. Fujii (Kansai Medical University) for measuring of the ^1H NMR spectra.