Acceleration of the Substrate Cα Deprotonation by an Analogue of the Second Substrate Palmitoyl-CoA in Serine Palmitoyltransferase

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Serine palmitoyltransferase (SPT) is a key enzyme of sphingolipid biosynthesis and catalyzes the pyridoxal 5′-phosphate (PLP)-dependent decarboxylative condensation reaction of L-serine with palmitoyl-CoA to generate 3-ketodihydrophosphingosine. The binding of L-serine alone to SPT leads to a conformation change in the PLP-L-serine external aldimine. This is consistent with the accepted mechanism of the SPT reaction directly influencing cellular sphingolipid homeostasis (1). Therefore, the molecular mechanism of the SPT reaction has been attracting attention, since it has been shown that sphingolipid is a potent bioactive lipid mediator playing crucial roles in diverse aspects of cell structure and function (1–4).

Eukaryotic SPTs have been known to exist as membrane-bound heterodimers composed of two subunits called SPTLC1 (LCB1) (long chain base 1), which does not have a PLP-binding motif, and SPTLC2 (LCB2), which carries a lysine residue that forms the Schiff base with PLP (5–10). The identification of a new third SPT subunit, SPTLC3, and an octameric SPT structure model have been proposed (11, 12). However, further studies of the SPT reaction mechanism have not been carried out, because the instability and the hydrophobic nature of eukaryotic SPTs have precluded obtaining a sufficient amount of the enzyme for detailed observations of the reaction (13).

Recently, we isolated several SPT genes from the sphingolipid-containing bacteria, such as Sphingomonas paucimobilis, Sphingobacterium multivorum, Sphingobacterium spiritivorum, and Bdellovibrio stolpii (14–16). These bacterial SPTs show about 30% identity in the amino acid sequence with eukaryotic SPT subunit proteins and conserve the amino acid residues of eukaryotic SPTs assumed to be involved in catalysis. All of the bacterial SPTs were successfully overproduced in Escherichia coli and purified as water-soluble active homodimers. Bacterial SPTs are considered to be a prototype of the eukaryotic membrane-bound enzymes, and this system was a major breakthrough that provided the first detailed mechanistic studies of the SPT reaction. The recent elucidation of a high resolution crystal structure of the S. paucimobilis SPT is expected to boost the structural/functional analysis of the catalytic reaction of this enzyme (17).

Fig. 1 shows the proposed reaction mechanisms of SPT. The first step of the SPT reaction cycle is the binding of the

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3 The abbreviations used are: SPT, serine palmitoyltransferase; PLP, pyridoxal 5′-phosphate; KDS, 3-ketodihydrophosphingosine; AONS, 8-amino-7-oxononanoate synthase; KBL, 2-amino-3-ketobutyrate ligase; ALAS, 5-aminolevulinic acid synthase.
amino acid substrate L-serine to the enzyme and the forma-
tion of the external aldimine (II). In a previous paper (18), we
spectroscopically and kinetically analyzed this step using the
purified SPT, L-serine, and its analogues. Through the study,
it was found that the α-carboxyl group of the substrate L-ser-
ine is important for the recognition by the enzyme, and the
Michaelis complex of SPT and L-serine readily undergoes
transaldimination to form the external aldimine. As gener-
ally observed for the PLP-dependent enzymes, the formation
of the quinonoid intermediate (carbanion) from the external
aldimine is the critical step in the reaction cycle. In the early
studies using a yeast cell free system, two contradictory
mechanisms for the KDS formation have been proposed: for-
mation of the quinonoid intermediate (A-III) by decarbox-
ylation of L-serine of the external aldimine intermediate (II),
followed by the acylation reaction to form the final product KDS. Mechanism B, the former quinonoid intermediate (B-III) is formed by the
deprotonation of the Cα position of the L-serine moiety of II. KDS is produced via the latter quinonoid intermediate (B-V), which is formed by the acylation and
the decarboxylation of B-III. The SPT-KDS external aldimine (VI) is a common intermediate in both mechanisms. B-V can be formed in mechanism A through VI by the principle of microscopic reversibility; therefore, even if the SPT reaction proceeds via mechanism A, the proton derived from the solvent can be
incorporated into the KDS.

In this paper, we specifically studied the regulation of the
α-deprotonation of L-serine, followed by acylation and sub-
sequent decarboxylation (B) (19–21). According to the
report by Krisnangkura and Sweeley (21) that deuterium was
introduced into the α-position of KDS during the reaction in
D₂O using rat liver microsomes, mechanism B has been
thought to be more appropriate. However, considering the
microscopic reversibility at the equilibrium between VI and
B-V, the introduction of deuterium into the C2 position of
KDS is possible even if the reaction proceeds via mechanism
A. Therefore, the above-mentioned deuterium exchange
experiment does not deny mechanism A, and it has been
elusive which mechanism operates in the formation of KDS.

In this paper, we specifically studied the regulation of the
α-deprotonation of the amino acid substrate L-serine and the
involvement of the second substrate palmitoyl-CoA. We inves-
tigated using ¹H NMR to study the exchange of the α-proton of
Substrate Synergism in Serine Palmitoyltransferase

L-serine with the solvent in the presence and absence of S-(2-oxoheptadecyl)-CoA, the structural analogue of palmitoyl-CoA. The results, together with the spectroscopic and kinetic studies, clearly demonstrated the presence of "substrate synergism," in which the α-proton of l-serine is activated by the binding of the second substrate palmitoyl-CoA. Molecular modeling of SPT complexed with l-serine and palmitoyl-CoA provided a plausible mechanism consistent with the experimental findings.

EXPERIMENTAL PROCEDURES

Chemicals—Palmitoyl-CoA was obtained from Funakoshi (Tokyo, Japan). Isopropyl 1-thio-β-d-galactoside and coenzyme A were from Sigma. The PD-10 columns were from Amersham Biosciences/GE Healthcare. E. coli BL21(DE3) pLysS and plasmid pET21b were from Novagen. All other chemicals were of the highest commercially available grade.

Expression and Purification of SPT—By the PCR amplification using pET21b/SPT1_mod (18) as the template, the eight amino acid residues from Ala2 to Pro9 of the SPT gene were deleted, and new restriction sites, NdeI and HindIII, were introduced into the gene at the translation initiation and termination sites, respectively. The mutation of His159 to phenylalanine (H159F) was introduced into the SPT gene by two-step PCR using the following mutagenic primers: 5′-GACTGTACGGAATTGCTATAGCAGCCGCTCAGCCCCACGC-3′ (forward1), 5′-CCTCGACCGGACA-GCTTTGCCGTGACATGACAGC-3′ (forward2), 5′-CGTCATAGATCGACGCAAAGCTGTGCGGTGCGAG-3′ (reverse1), 5′-GACTGTACGTCGACTACGGCAGTGAAGCCGACC-GCGCGG-3′ (reverse2) (mutated bases are italicized). As the first step, the two DNA fragments encoding Met1–Asp164 and Leu154–Gly420 of SPT were PCR-amplified using the primer pairs of forward1-reverse1 and forward2-reverse2, respectively, against pET21b/SPT1_mod. Then the full-length SPT gene having the H159F mutation and the restriction site of NdeI or HindIII at each end was synthesized by the second PCR amplification against the mixture of former PCR products using the primer pair of forward1-reverse2. Each modified SPT gene was treated with both NdeI and HindIII, purified, and ligated into pET21b. Then these recombinant plasmids were used to transform the E. coli BL21 (DE3) pLysS cells. Expression of the protein was induced with 0.1 mM isopropyl 1-thio-β-d-galactoside and continued for 6 h at 37 °C. The recombinant enzyme was purified as previously described (14). The expression level of the N-terminal deleted construct was lower than that of the full-length SPT, but the activity and the physical characteristics of the purified enzyme were not significantly changed as summarized in Table 1.

Synthesis of S-(2-Oxoheptadecyl)-CoA—1-Chloroheptadecan-2-one was synthesized from tetradecyl bromide and 1-chloro-2-methoxy-ethane. S-(2-Oxoheptadecyl)-CoA (Fig. 2) was synthesized from 1-chloroheptadecan-2-one and coenzyme A according to the method described by Rudnick et al. (22). The rough product (70% purity) was purified using a Megabond Elute C18 cartridge (Varian), and then the eluate of the synthesized compound was confirmed by mass spectroscopy and 1H NMR: electrospray ionization-mass spectrometry (on ThermoQuest LCQ) 1044 [M + H]+; fast atom bombardment-mass spectrometry (on JEOL SX102A) 1044[M + H]+, 1038[M+Li+2H]+, 1032[M+2Li+3H]+; 1H NMR (D2O, 400 MHz on a Varian UNITY 400) δ 0.70, 0.88 (6H, –CH(OM)–CH2–); 0.86 (3H, CH3); 3.42 (2H, –S–CH2–); 6.09 (1H, C1′H of adenosine), 8.05 (1H, C2), 8.46 (1H, C8-H of purine).

TABLE 1
Kinetic parameters of purified SPT at 25 °C

|        | kcat(Ser) | kcat(palmitoyl-CoA) | kcat/mM CoA |
|--------|-----------|---------------------|-------------|
| Wild type SPT | 6.2 ± 0.6 | 1.0 ± 0.1 | 41.5 ± 1.7 |
| SPT_del9 | 3.5 ± 0.6 | 1.2 ± 0.1 | 48.2 ± 2.6 |

FIGURE 2. Structures of S-(2-oxoheptadecyl)-CoA (A) and palmitoyl-CoA (B). The methylene bridge between the acyl carbonyl and the CoA sulfur (arrow) makes this palmitoyl-CoA analogue resistant to nucleophilic substitution reactions.
Spectrometric Measurements—The absorption spectra of SPT were recorded by a Hitachi U-3300 spectrophotometer at 25 °C. The stopped-flow spectrophotometry was performed using an Applied Photophysics SX.18MV system (Leatherhead, UK) at 25 °C. The dead time was 2.3 ms under a gas pressure of 500 kilopascals. The time-resolved spectra were collected by the SX.18MV system equipped with a photodiode array accessory and the XScan (version 1.08) control software. The absorption changes were analyzed using the program ProK-II (Applied Photophysics). The buffer solution for the spectrometric measurements contained 50 mM HEPES-NaOH (pH 7.5), 150 mM KCl, and 0.1 mM EDTA. SPT was equilibrated with this buffer by gel filtration using a PD-10 (Sephadex G-25) column prior to the measurements.

NMR Samples and Spectral Measurements—For the NMR spectroscopy, all exchangeable hydrogens of the enzyme, the ligands, and the buffer base were replaced with deuterium. Known amounts of the buffer base were dissolved in D₂O and freeze-dried. After repeating this treatment twice, the buffer solutions were reconstituted in D₂O. L-Serine, D-serine, and S-(2-oxoheptadecyl)-CoA were also subjected to the same pretreatment. They were each dissolved in 50 mM potassium pyrophosphate buffer (D₂O, pH 7.5). SPT was equilibrated twice with deuterium-substituted buffer using a PD-10 column.

The ¹H NMR spectra were measured in Wilmad 5-mm NMR tubes kept at 23 °C by a Varian UNITY 400 spectrometer operating at 400 MHz. A flip angle of 20° with a relaxation delay time of 5 s was used. To obtain a better signal-to-noise ratio, an exponential window function for 0.2- or 0.5-Hz line-broadening factor was applied to the free induction decays of NMR measurements. Chemical shifts are expressed as ppm relative to an external standard of 3-(trimethylsilyl)propionic acid-d₄.

Structural Modeling—The modeling of S. paucimobilis SPT on the crystal structure of Rhodobacter capsulatus 5-amino-2-methylvaline acid synthase (ALAS) complexed with succinyl-CoA (Protein Data Bank code 1BWO) was carried out using MOE (version 2005.06; Chemical Computing Group, Montréal, Canada) according to the manufacturer’s instructions. The amino acid sequence of SPT (GenBank™ number AB055142) was aligned to that of R. capsulatus ALAS (GenBank™ P18709) using the BLOSUM62 substitution matrix with the gap start and gap extend penalties set to 180 and 90°, respectively, so that the conformations of the external aldime of most PLP enzymes that activates the α-proton. The aliphatic group of palmitoyl-CoA was extended from C3 of succinyl-CoA using the Molecular Builder of MOE. Two structures, one with the PLP-α-serine aldime and the other with the PLP-α-serine aldime plus palmitoyl-CoA, were energy-minimized using the MMFF94x force field with all of the atoms of SPT and the ligands allowed to move.

Other Methods—The SPT activity was measured according to the previously described methods with minor modification (14). Aliquots of the reaction mixture were spotted on silica gel 60 HPTLC plate (Merck), and [3-¹⁴C]KDS was separated from L-[3-¹⁴C]serine with a solvent system of chloroform, methanol, 2 N NH₄OH (40:10:1, v/v/v). The radioactivity of each KDS spot was quantified by a BAS2500 image analyzer (Fuji Film, Tokyo, Japan), using the intensity of the authentic L-[3-¹⁴C]serine. The protein concentration during the purification procedure was determined with a BCA protein assay kit (Pierce) using bovine serum albumin as the standard. The protein concentration of the purified SPT was spectrophotometrically determined using the molar extinction coefficient of 2.83 × 10⁴ M⁻¹ cm⁻¹ at 280 nm for the PLP form of the enzyme (14). SDS-PAGE was carried out using the SDS-Tris system with a 10% polyacrylamide gel according to the procedure described by Laemmli (23).

RESULTS

Generation of the Quinonoid Intermediate from the External Aldime by S-(2-Oxoheptadecyl)-CoA—Sphingomonas SPT has absorption bands at 338 and 426 nm, which arise from the enolimine and ketoenamine tautomers, respectively, of the PLP-L-serine aldime and the other with the PLP-α-serine aldime plus palmitoyl-CoA, were energy-minimized using the MMFF94x force field with all of the atoms of SPT and the ligands allowed to move. The SPT activity was measured according to the previously described methods with minor modification (14). Aliquots of the reaction mixture were spotted on silica gel 60 HPTLC plate (Merck), and [3-¹⁴C]KDS was separated from L-[3-¹⁴C]serine with a solvent system of chloroform, methanol, 2 N NH₄OH (40:10:1, v/v/v). The radioactivity of each KDS spot was quantified by a BAS2500 image analyzer (Fuji Film, Tokyo, Japan), using the intensity of the authentic L-[3-¹⁴C]serine. The protein concentration during the purification procedure was determined with a BCA protein assay kit (Pierce) using bovine serum albumin as the standard. The protein concentration of the purified SPT was spectrophotometrically determined using the molar extinction coefficient of 2.83 × 10⁴ M⁻¹ cm⁻¹ at 280 nm for the PLP form of the enzyme (14). SDS-PAGE was carried out using the SDS-Tris system with a 10% polyacrylamide gel according to the procedure described by Laemmli (23).

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Substrate Synergism in Serine Palmitoyltransferase

The buffer system was 50 mM HEPES–NaOH (pH 7.5) containing 150 mM KCl and 0.1 mM EDTA. The measurements were done at 25 °C. The enzyme concentration was 5 μM. A, absorption spectra of SPT. Lines 1 (dotted line), the substrate-free form of SPT. Lines 2–7 were taken in the presence of 45 mM L-serine and 0, 0.04, 0.11, 0.29, 0.70, and 1.34 mM, respectively, of S-(2-oxoheptadecyl)-CoA, and, therefore, the analogue cannot undergo the Claisen-type condensation reaction with the carboxiyl Cα of the quinonoid intermediate.

Kinetic Analysis of the Spectral Transition of the SPT-L-Serine Complex upon Reaction with S-(2-Oxoheptadecyl)-CoA—In order to elucidate the process of the quinonoid formation in more detail, a transient kinetic analysis was carried out on the reaction described above. SPT (42 μM) was saturated in advance with 30 mM L-serine and reacted with S-(2-oxoheptadecyl)-CoA, and the spectral changes were followed in a stopped-flow spectrophotometer. A time-dependent increase in the 493 nm absorbance was observed (Fig. 4, A and B). The reaction was followed at the S-(2-oxoheptadecyl)-CoA concentrations of 10, 20, 40, 80, 160, and 320 μM. At each concentration, the spectral change was a nearly single exponential process. However, since the S-(2-oxoheptadecyl)-CoA concentrations were of the same order as the enzyme concentration, a numerical analysis using the Pro-KII program was necessary. The spectra generated according to the following scheme were well fit by a global analysis to the experimentally obtained time-resolved spectra,

\[
E_S + S_2 \rightleftharpoons E_S S_2 \rightleftharpoons E Q S_2
\]

Scheme 1

where \( E \) represents SPT, \( S_1 \) is L-serine, \( E S \) is the SPT-L-serine external aldimine complex, \( S_2 \) is S-(2-oxoheptadecyl)-CoA, \( E S S_2 \) is the ternary complex, and \( E Q S_2 \) is the SPT-L-serine quinonoid intermediate complexed with S-(2-oxoheptadecyl)-CoA. The kinetic parameters were obtained as follows: \( K_{d_{analog}}^0 = 31.7 \pm 1.1 \mu M \), \( k_{+2} = 2.6 \pm 4.8 \text{ s}^{-1} \), and \( k_{-2} = 64.4 \pm 4.2 \text{ s}^{-1} \). That the S.D. value of \( k_{-2} \) exceeds the mean value is the result of the Levenberg-Marquardt minimization and not that of the experimental errors. The calculated absorption spectrum of \( E_S S_2 \) was very similar to the spectrum of \( E_S \) and had no absorption at 493 nm (Fig. 4C, lines 1 and 2). On the other hand, the calculated absorption spectrum of \( E Q S_2 \) showed an intense absorption at around 493 nm with a shoulder at around 460 nm. The apparent dissociation constant (\( K_{d_{pp}}^0 \)) obtained from the static spectroscopic analysis (Fig. 3C) is related to the above kinetic parameters as follows,

\[
K_{d_{pp}}^0 = \frac{k_{-2}}{k_{+2} + k_{-2}} K_{d_{analog}}^0 \quad \text{(Eq. 1)}
\]

The calculated value of \( K_{d_{pp}}^0 \) using the transient kinetic parameters obtained as above was 30 μM, which was very close to the experimental value of 35 μM, thus supporting the validity of the above kinetic analysis. The apparent equilibrium constant for the \( E_S S_2 \rightleftharpoons E Q S_2 \) reaction, \( k_{+2}/k_{-2} \), is 0.04. This small value explains why the quinonoid peak in Fig. 3 is small.
S-(2-Oxoheptadecyl)-CoA Enhances the Exchange Rate of the $\alpha$-Proton of L-Serine—$^1$H NMR was used to determine the extent of the SPT-catalyzed hydrogen-deuterium exchange at C$^\beta$H$_9251$ of L-serine. In D$_2$O buffer at pH 7.5, L-serine gives four resonance peaks centered at 3.85 ppm and eight resonance peaks around 3.98 ppm, corresponding to three protons with the ABX spin system. The former peaks come from the $\alpha$-proton, and the latter come from the $\beta$-methylene protons (Fig. 5A).

The spectrum was essentially unchanged when only SPT was added to the solution. However, the further addition of S-(2-oxoheptadecyl)-CoA to the solution caused a time-dependent decrease in the $\alpha$-proton signal without altering the intensity of the $\beta$-protons (Fig. 5, B–E). This clearly shows that the binding of S-(2-oxoheptadecyl)-CoA to the external aldimine enhances $\alpha$-deprotonation of L-serine and is consistent with the observation that S-(2-oxoheptadecyl)-CoA causes accumulation of the quinonoid intermediate (Fig. 3 and 4).
hand, when \( S-(2\text{-oxoheptadecyl})\text{-CoA} \) was added to the reaction solution, \( t_{1/2} \) decreased to 0.75 ± 0.03 h, much shorter than the above cases.

A minimal kinetic scheme for the SPT-catalyzed hydrogen-deuterium exchange at the \( \alpha \)-C of \( L\)-serine is represented as follows,

\[
E + S_1 \xrightarrow{k_{a+}} ES_1 \xrightarrow{k_{-2}} EQ
\]

where \( E \) represents SPT, \( S_1 \) is \( L\)-serine, \( ES_1 \) is the SPT-\( L\)-serine external aldimine complex, and \( EQ \) is the quinonoid intermediate. Assuming that the rate of disappearance of the \( \alpha \)-proton of \( L\)-serine is equal to the rate of the \( \alpha \)-hydrogen abstraction from \( ES_1 \), the following differential rate equation is obtained,

\[
\frac{df[S_1]}{dt} = f k_{-2}[ES_1] = f k_{-2}[E]_t
\]

(Eq. 3)

Using the values \([E]_t = 5 \mu M\), \([S_1] = 10 \mu M\), and \( k_{a+} = 2.56 \times 10^{-4} \text{s}^{-1}\), \( k_{-2} \) is calculated to be 0.51 s\(^{-1}\) from Equation 4. This value roughly coincides with the \( k_{-2} \) value of 2.2 s\(^{-1}\) obtained from the analysis of the time-resolved spectra on the SPT ternary complex formation, showing the consistency of the spectroscopic and NMR analyses described above.

Similar experiments were carried out with \( D\)-serine. The exchange of the \( \alpha \)-proton of \( D\)-serine with the solvent was slow either when incubated with SPT only (\( t_{1/2} = 150 \pm 5 \text{ h} \)), SPT and palmitoyl-CoA (\( t_{1/2} = 456 \pm 99 \text{ h} \)), or SPT and \( S-(2\text{-oxoheptadecyl})\text{-CoA} \) (\( t_{1/2} = 1948 \pm 211 \text{ h} \)).

\text{Reaction with the True Substrate—}The reaction of the SPT-\( L\)-serine external aldimine complex with the true substrate palmitoyl-CoA was followed by \( ^1\text{H} \text{NMR} \) in the same way as the analogue. In the presence of 10 mM \( L\)-serine and 5 mM SPT, the addition of 1.5 mM palmitoyl-CoA resulted in stoichiometric decreases in the signal intensity of the \( \beta \)-methylene proton of \( L\)-serine (data not shown), showing that the reaction completely proceeded. The hydrogen-deuterium exchange at \( \alpha \)-C, as judged from the \( \alpha/\beta \) ratio, was 2% for 1.5 mM palmitoyl-CoA. These low levels of exchange indicate that the rate of the C–C bond formation from the quinonoid intermediate is significantly higher than the rate of reprotonation of the quinonoid intermediate (see “Discussion”).

\text{Models for the SPT-\( L\)-Serine and the SPT-\( L\)-Serine-Palmitoyl-CoA Complexes—}In order to structurally explain the activation of the \( \alpha \)-proton of the external aldimine, crystallographic analyses of the enzyme-substrate binary and ternary complexes are required. The crystal structure of the substrate-free form of \( S\). \textit{paucimobilis} SPT at 1.3 \( \AA \) has been reported (17). However, the crystals of the complex of this enzyme with substrates (or ana-
logues) have not been obtained yet. All other members of the α-oxamine synthase subfamily have been successfully crystallized, and structures of a number of the enzyme-ligand complexes have been determined that include the AONS-8-amino-7-oxononanate complex (24), KBL-2-amino-3-ketobutyrate complex (25), ALAS-glycine complex (26), and ALAS-succinyl-CoA complex (26). Therefore, based on the crystal structure of _R. capsulatus_ ALAS complexed with succinyl-CoA, structural models for the SPT-α-serine external aldime complex and the ternary complex of the SPT-α-serine external aldime and palmitoyl-CoA were constructed.

The active site structure of the SPT model was very similar to that of the crystal structure of ALAS; residues conserved between SPT and ALAS were essentially superimposable with each other (data not shown). These residues include His<sup>159</sup> located at the re face of the PLP-Lys internal aldime and stacking with the PLP pyridine ring, Asp<sup>231</sup> interacting with PLP N1, and His<sup>234</sup> interacting with PLP O3.<sup>3</sup>

In the initial model for the binary (external aldime) complex, the Ca–H bond of α-serine was perpendicular to the PLP pyridine ring (the dihedral angle C4'–Nα–Ca–H being 90°). After energy minimization, however, the carboxylate group of α-serine approached His<sup>159</sup> and formed a strong hydrogen bond/ionic interaction with Nε2 of His<sup>159</sup> (Fig. 7). As a result, the dihedral angle of C4'–Nα–Ca–H became 159° (149°; hereafter, the values in parenthesis are those of subunit 2). Thus, the Ca–H bond of α-serine deviated by 60–70° from the theoretically ideal direction for α-deprotonation.

The model for the ternary complex (SPT-α-serine external aldime plus palmitoyl-CoA) is shown in Fig. 8, A and B. Palmitoyl-CoA docks with SPT as a bent structure to insert its thioester into the active center of the enzyme. The adenosine 3′,5′-bisphosphate moiety of palmitoyl-CoA is seen near the entrance of the active site, and the acyl chain moiety of palmitoyl-CoA is suitably placed into the large pocket, which is rich in hydrophobic amino acid residues. Fig. 8C shows the active site view of the ternary complex. His<sup>159</sup> forms a hydrogen bond with the acyl carbonyl O (thioester O) of palmitoyl-CoA with a distance of 2.8 Å. The carboxyl group of α-serine loses its interaction with His<sup>159</sup> and, instead, forms two hydrogen bonds with the guanidino group of Arg<sup>390</sup>. The Ca–H bond takes a perpendicular orientation to the plane formed by the PLP pyridine ring and the imine of the Schiff base, which is suitable for the α-proton abstraction by Lys<sup>265</sup>.

**Substitution of His<sup>159</sup> of SPT by Phe Resulted in Inactivation of the Enzyme**—For the assessment of the role of His<sup>159</sup> in the catalysis of SPT, the residue was changed to Phe, which was chosen to conserve the aromaticity and the size of the side chain. H159F SPT was stably overexpressed and purified in the same way as the wild type enzyme. In the case of the wild-type enzyme, clear UV-visible and CD spectral changes were observed by the addition of the α-serine, indicating the external aldime formation. For H159F SPT, although the 420 nm peak increased slightly, the 330 nm peak did not decrease, and the CD spectrum did not change. The apparent _K_<sub>D</sub> value for α-serine was 11.1 mM, compared with 1.4 mM of the wild-type enzyme. These results suggest the formation of the Michaelis complex, but not the external aldime, on the reaction with α-serine. The accumulation of the quinonoid intermediate was not detected by the further addition of S-(2-oxoheptadecyl)-CoA or palmitoyl-CoA to α-serine-saturated H159F SPT. Furthermore, _K_<sub>D</sub> was not detected even after 2 μM H159F was incubated with 20 mM of α-serine and 2 mM of palmitoyl-CoA for 20 min at 25 °C. Under the same substrate concentration condition, the wild-type SPT showed the activity of ν/[E] = 0.4 s<sup>-1</sup>.

**DISCUSSION**

**Acceleration of the α-Deprotonation of the External Aldimine by the Substrate Acyl-CoA Analogue**—The results presented above clearly show that the addition of the palmitoyl-CoA analogue, S-(2-oxoheptadecyl)-CoA, to the SPT-α-serine complex causes accumulation of the quinonoid intermediate, which is not detectable in the SPT-α-serine complex (Figs. 3 and 4).<sup>1</sup>H NMR studies indicated that S-(2-oxoheptadecyl)-CoA accelerates the α-deprotonation of the α-serine moiety in the external aldime by more than 100-fold (Fig. 6). Apparently, this enhancement in the deprotonation rate is considered to be the cause of the accumulation of the quinonoid intermediate upon the addition of S-(2-oxoheptadecyl)-CoA. Zhang and Ferreira (27) are the first who reported the acceleration of the substrate α-deprotonation by the acyl-CoA substrate; they found that the physiologic substrate succinyl-CoA and its analogue acetocacetyl-CoA increased the rate of glycine proton removal ~250,000- and 10-fold, respectively. The activation of the
of the quinonoid intermediate (28, 29). For the α-oxamine synthase subfamily enzymes, it had been an issue whether the quinonoid intermediate is formed by (A) α-decarboxylation or (B) α-deprotonation of the substrate amino acid. An early proposal was (A), which was simply assumed because the products lose the carboxyl group present in the substrate amino acids. However, subsequent studies on eukaryotic SPT (21), AONS (30), ALAS (31), and KBL (32) showed that the solvent deuterium is incorporated into the position of the substrate Cα and favored mechanism (B). The decarboxylation is considered to be catalyzed in the external aldimine formed just after the Claisen-type condensation reaction, in which the carboxyl group is expected to be activated not only by the PLP moiety but also by the carbonyl group derived from the substrate acyl-CoA and situated at the β position to the carboxyl group. This, on the other hand, raises the possibility that the incorporation of the deuterium into Cα can be a result of the hydrogen-deuterium exchange that occurred at the product-PLP aldimine (VI), since the proton should be activated like the carboxyl group. The present study, which uses an acyl-CoA analogue that stops the catalytic reaction at the step of the C–C bond formation, strongly suggests that the α-deprotonation is an event that occurs before the C–C bond formation and supports the notion that α-deprotonation is the prerequisite step for the Claisen-type condensation reaction.

α-Proton of the external aldimine by the binding of the second substrate acyl-CoA may therefore be a common feature among the α-oxamine synthase subfamily enzymes.

α-Deprotonation as the Prerequisite Step for the Claisen-type C–C Bond Formation—The catalytic reactions of all PLP-dependent enzymes acting on amino acids involve the formation when palmitoyl-CoA was added to the SPT-L-serine external aldimine. Therefore, it is difficult to carry out a kinetic analysis of the Cα deprotonation in the presence of palmitoyl-CoA. However, by combining the observation of the hydrogen-deuterium exchange of L-serine in the presence of palmitoyl-CoA and the result of the kinetic analysis of the reaction with S-(2-

**FIGURE 8.** Model structure of the ternary complex of SPT, L-serine, and palmitoyl-CoA. Modeling of SPT on the crystal structure of ALAS from *R. capsulatus* was carried out using MOE. A, overall structure of the SPT homodimer is shown as *ribbons* in a parallel stereo view. One monomer is shown in *pale cyan*, and the other is shown in *pale green*. Palmitoyl-CoA and PLP-L-serine aldimine are shown in *purple* and *yellow*, respectively. The molecules in the pale green monomer are shown in *light colors* for distinction. B, overall view of the SPT homodimer model from another angle. Object colors are the same as in A. C, close-up view of the active site region of a monomer (the left one of A). Shown with *balls* and *sticks* are the palmitoyl-CoA, PLP-L-serine aldimine, and catalytic residues, His159, Lys265, and Arg390. His159 formed a hydrogen bond with the C2 carbonyl oxygen of palmitoyl-CoA with a distance of 2.77 Å. The carboxyl group of L-serine, in turn, forms two new hydrogen bonds with the guanidino group of Arg390 with distances of 2.36 and 2.37 Å. The α-proton of L-serine (green) took a perpendicular orientation to the plane formed from the PLP-pyridine ring and the double bond of the Schiff base, suitable for the α-proton abstraction by the Lys265 ε-amino group located at a distance of 3.14 Å. Panels were generated using PyMOL version 0.99.
oxoheptadecyl)-CoA, we can gain some understanding of the reaction of the SPT-L-serine external aldimine with palmitoyl-CoA.

For the hydrogen-deuterium exchange reaction of L-serine in the presence of palmitoyl-CoA, Scheme 2 is rearranged into Scheme 3,

\[
\begin{align*}
\text{E} &\xrightleftharpoons{K_{\text{d}}^{\text{PalCoA}}} \text{ESh} \xrightleftharpoons{K_{\text{d}}^{\text{PalCoA}}} \text{EShP} \\
\text{ESh} &\xrightleftharpoons{K_{\text{d}}^{\text{PalCoA}}} \text{EShP} \xrightleftharpoons{K_{\text{d}}^{\text{PalCoA}}} \text{EShK} \\
\text{ESh} &\xrightleftharpoons{K_{\text{d}}^{\text{PalCoA}}} \text{EShP} \xrightleftharpoons{K_{\text{d}}^{\text{PalCoA}}} \text{EShK} \\
\end{align*}
\]

where SH and SD represent the hydrogen and deuterium forms of L-serine, respectively, P is palmitoyl-CoA, and K is KDS. Hydrogen removed as a proton from E-SH-P is considered to be rapidly diluted by the solvent D2O, and, for that reason, there would be no protonation of Q to regenerate E-SH-P. The \(K_{\text{d}}^{\text{PalCoA}}\) value (1.4 mM) is taken from the previous study (18). The other parameters were not experimentally obtained; therefore, we made the following assumptions. The \(K_{\text{d}}^{\text{PalCoA}}, k_{+1}^{\text{H}}, \) and \(k_{-2}^{\text{H}}\) values were considered to be the same as the corresponding \(K_{\text{d}}^{\text{d}}, k_{+3}^{\text{d}},\) and \(k_{-2}^{\text{d}}\) values obtained from the reaction with S-(2-oxoheptadecyl)-CoA. The kinetic deuterium isotope effect for the Ca deprotonation of the external aldmine was assumed to be 7.3, based on the report by Onuffer and Kirsch (33). Accordingly, the \(K_{\text{d}}^{\text{PalCoA}}, k_{+1}^{\text{H}}, k_{+2}^{\text{H}},\) and \(k_{-2}^{\text{H}}\) values were set to 40 \(\mu\text{M}\), 2.2 \(s^{-1}\), 0.31 \(s^{-1}\), and 8.9 \(s^{-1}\), respectively. Using these values and an arbitrarily set \(k_{+3}^{\text{H}}\) value and the initial concentrations of \([E] = 5 \mu\text{M}, [SH] = 10 \text{ mm},\) and \([P] = 1.5 \text{ mm},\) the time course of the concentration change of each species was simulated by Pro-KII.

The result showed that \([SD]/([SH] + [SD])\) (i.e. the extent of the hydrogen-deuterium exchange of L-serine) is highly dependent on the \(k_{+1}\) value; thus, increasing the \(k_{+1}\) value increased [SH] and decreased the extent of the exchange. It was found that in order to explain the less than 2% exchange, \(k_{+3}\) must have a value greater than 75 \(s^{-1}\). This value is much greater than the \(k_{+2}\) value (2.2 \(s^{-1}\)) and exceeds the value of \(k_{-2}\) (62 \(s^{-1}\)). This indicates that the quinonoid intermediate is rapidly converted into the product and is consistent with the finding that no apparent accumulation of the quinonoid intermediate was observed for the reaction of SPT with L-serine and palmitoyl-CoA. It also indicates that the Ca deprotonation is the rate-limiting step of the overall reaction of SPT.

\[\text{SPT} + \text{L-Serine} \rightarrow \text{SPT-L-Serine} \rightarrow \text{SPT-L-Serine} + \text{PalCoA} \]

\[\text{SPT} + \text{D-Serine} \rightarrow \text{SPT-D-Serine} \rightarrow \text{SPT-D-Serine} + \text{PalCoA} \]

**Structural Considerations Based on the Model**—Although the crystal structure for the SPT-substrate complex has not been obtained so far, the high homology of SPT to other enzymes of the \(\alpha\)-oxamine synthase subfamily enabled us to construct the models for the SPT-substrate binary and ternary complexes. The models shown in Figs. 7 and 8 provided a plausible mechanism that explains why the SPT-L-serine external aldimine is essentially inert to deprotonation, whereas the binding of the second substrate palmitoyl-CoA causes instantaneous \(\alpha\)-deprotonation.

**Substrate Synergism in Serine Palmitoyltransferase**

**FIGURE 9.** Proposed conformations of the pyridoxal aldimines of L-serine and D-serine as viewed along the Co-Na bond. The aldehyde carbon atom of PLP is shown as Cp, and the atoms of the pyridine ring are indicated by the rectangular box. In both cases of the SPT-L-serine external aldimine and the SPT-D-serine external aldimine, the \(\alpha\)-H bond of serine is not perpendicular to the imine-pyridine conjugate system; therefore, the \(\alpha\)-deprotonation is prevented. By the binding of the second substrate palmitoyl-CoA or its analogue S-(2-oxoheptadecyl)-CoA, the configuration of L-serine changes to the geometry appropriate for activation of the \(\alpha\)-proton (top). On the other hand, in the SPT-D-serine-acyl-CoA ternary complex, the Co-CH2OH bond, not the Co-H bond, becomes perpendicular to the imine-pyridine plane. Therefore, the acyl-CoA binding does not promote \(\alpha\)-deprotonation of D-serine (bottom).

The external aldimines of PLP and substrate amino acids in the PLP enzymes generally have a nearly planar structure, in which the protonated imine N makes an intramolecular hydrogen bond with O3' of PLP and the imine bond lies in the same plane as the pyridine ring. However, the bond around Na-Ca can freely rotate, and the external aldimine can take various conformations at this bond. The PLP enzymes exploit this partial conformational flexibility of the external aldimine to exert their reaction specificity. Among the three bonds around Ca (the bonds attached to Ca except Na-Ca), the bond that is most perpendicular to the imine-pyridine plane has the largest overlap of its HOMO with the LUMO of the \(\pi\)-orbital of the imine-pyridine conjugate system and is expected to be preferentially cleaved. This hypothesis, first proposed by Dunathan (34), has been proven to apply to all of the catalytic reactions of the PLP enzymes whose crystal structures have been so far elucidated (29). In the model for the SPT-L-serine external aldimine, the Ca-H bond approaches the imine-pyridine plane (Fig. 7). This conformation is quite unfavorable for cleaving the Ca-H bond (schematically shown in Fig. 9A). The observations that the binding of L-serine alone does not cause accumulation of the quinonoid intermediate (Fig. 3) and that the external aldimine exchanges \(\alpha\)-hydrogen with the solvent at a very slow rate can be understood by the low HOMO (Ca-H)-LUMO (PLP) interaction in this conformation of the external aldimine. The principal factor that makes the external aldimine take this
**Substrate Synergism in Serine Palmitoyltransferase**

conformation is the strong hydrogen bond/ionic interaction between the carboxyl group of L-serine and Ne2 of His159 (Figs. 7 and 9A).

In the ternary complex model (Fig. 8), however, the thioester O of palmitoyl-CoA forms a stable hydrogen bond with the Ne2 atom of His159, displacing the carboxyl group of L-serine. The L-serine carboxyl group, in turn, forms hydrogen bonds/ionic interaction with the guanidino group of Arg390. This interaction fixes the PLP-L-serine external aldimine to a new conformation, in which the Ca–H bond is almost perpendicular to the imine-pyridine plane and approaches the ε-amino group of Lys260 (schematically shown in Fig. 9A). Both factors are preferable for the α-deprotonation to proceed, and this properly explains the acceleration of the α-deprotonation of the external aldimine by palmitoyl-CoA.

Based on these models, the phenomenon that the exchange rate of α-proton of D-serine in both binary and ternary complex was extremely slow is explained as well (Fig. 9B). Assuming that the carboxyl group of D-serine is recognized by His159 in the SPT-D-serine binary complex and by Arg390 in the SPT-D-serine-acyl-CoA ternary complex, the Ca–H bond is not perpendicular to the imine-pyridine plane in either of the two complexes (Fig. 9B). These conformations are clearly unfavorable for the α-deprotonation.

Importantly, Webster et al. (24) have already presented a mechanism similar to this for the reaction of AONS. They interpreted the crystal structure of the AONS-product external aldime to imply that His133, which corresponds to His159 of SPT, makes a hydrogen bond/salt bridge to the carboxylate of the L-serine moiety of the external aldime and deviates the α-hydrogen from the ideal orientation, whereas the additional binding of the pimeloyl-CoA substrate would disrupt this interaction, allowing the Ca–H bond to be perpendicular to the imine-pyridine plane.

Comparison with Other α-Oxamine Synthase Subfamily Enzymes and the Role of the Active Site Residues—The above findings suggest the critical role of His159 in the regulation of the α-deprotonation of L-serine during the course of the catalytic reaction of SPT. This amino acid residue is conserved among all members of the α-oxamine synthase subfamily: His133 in AONS, His136 in KBL, and His142 in ALAS.

In most PLP enzymes belonging to the fold type I, which includes the α-oxamine synthase subfamily, the residue that exists at the re face of the PLP-Lys internal aldime is an aromatic amino acid, such as Trp, Tyr, and Phe. The presence of His instead of these aromatic residues may be reasonable, considering the ability of His to form hydrogen bonds with the carboxyl group of the amino acid substrate and the acyl carbonyl O of the acyl-CoA substrate and its role in the precise control of the α-proton activation. Our finding that H159F SPT does not seem to form the external aldime with L-serine and lose its catalytic activity supports the idea that His159 is important as an anchoring residue of the α-carboxylate of the L-serine moiety in the external aldime. However, Trp has an N atom (Ne1) corresponding to Ne2 of His and still is not found in the α-oxamine synthase subfamily enzymes. The advantage of His over Trp is that it can carry a dissociable proton at Ne2. This proton can activate the thioester bond by simply migrating to the acyl carbonyl O during the nucleophilic attack of the carbanion of the quinonoid intermediate (Fig. 10). It can also stabilize the carboxyl group of the amino acid substrate by neutralizing the negative charge. Since the carboxylate group is almost perpendicular to the imine-pyridine plane and is expected to be activated in the external aldime, this interaction may be important for avoiding decarboxylation of the substrate. In fact, it has recently been reported that, if the alanine moiety of the external aldime of AONS is replaced by trifluorolalanine, which has more electron-withdrawing substituent, the external aldime undergoes decarboxylation, indicating the potential lability of the Ca–COO− bond (35).

Most recently, on the mouse ALAS2 isozyme, Hunter et al. (36) reported the possibility that His207, which corresponds to His142 of Rhodobacter ALAS, would act as an acid catalyst at the decarboxylation step of the α-amino-β-ketoaspartate intermediate to form 5-aminolevulinate by carrying out the single and multiple turnover rapid scanning stopped-flow experiments.

In summary, the present results clearly demonstrate that the α-deprotonation of the SPT-L-serine external aldime does occur before the Claisen-type C–C bond formation and is strictly controlled by the presence/absence of the second substrate palmitoyl-CoA. The significance of this mechanism is that SPT escapes abortive transamination by minimizing the accumulation of the quinonoid intermediate, which, by accidental protonation at C4′, yields ketimine and subsequently pyridoxal 5′-phosphate and hydroxypropionate. Furthermore, the model building study strongly suggested that His159 plays important roles in this control mechanism. The proposed mechanism will be tested in future crystallographic and mutagenesis studies.
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Substrate Synergy in Serine Palmitoyltransferase