Reactions of Serine Palmitoyltransferase with Serine and Molecular Mechanisms of the Actions of Serine Derivatives as Inhibitors†

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Received September 21, 2003; Revised Manuscript Received November 21, 2003

ABSTRACT: Serine palmitoyltransferase (SPT) is a key enzyme in sphingolipid biosynthesis and catalyzes the decarboxylative condensation of L-serine and palmitoyl coenzyme A to 3-ketodihydrosphingosine. We have succeeded in the overproduction of a water-soluble homodimeric SPT from *Sphingomonas paucimobilis* EY2395^T in *Escherichia coli*. The recombinant SPT showed the characteristic absorption and circular dichroism spectra derived from its coenzyme pyridoxal 5′-phosphate. On the basis of the spectral changes of SPT, we have analyzed the reactions of SPT with compounds related to L-serine and product, and showed the following new aspects: First, we analyzed the binding of L-serine and 3-hydroxypropionate and found that the spectral change in SPT by the substrate is caused by the formation of an external aldimine intermediate and not by the formation of the Michaelis complex. Second, various serine analogues were also examined; the data indicated that the α-carboxyl group of L-serine was quite important for substrate recognition by SPT. Third, we focused on a series of SPT inhibitors, which have been used as convenient tools to study the cell responses caused by sphingolipid depletion. The interaction of SPT with myriocin suggested that such product-related compounds would strongly and competitively inhibit enzyme activity by forming an external aldimine in the active site of the enzyme. *β*-Chloro-L-alanine and L-cycloserine were found to generate characteristic PLP-adducts that produced inactivation of SPT in an irreversible manner. The detailed mechanisms for the SPT inactivation were discussed. This is the first analysis of the inhibition mechanisms of SPT by these compounds, which will provide an enzymological basis for the interpretation of the results from cell biological experiments.

Sphingolipids are ubiquitous constituents of membrane lipids in mammalian cells, and are also widely distributed in other animals, plants, and microbes (*1*). In eukaryotes, sphingolipids, such as sphingosine, sphingosine-1-phosphate, and ceramide, are known to play important roles as second messengers in various cellular events including proliferation, differentiation, senescence, apoptosis, and immune response (*2*). Serine palmitoyltransferase (SPT: EC 2.3.1.50)[1] catalyzes a pyridoxal 5′-phosphate (PLP)-dependent condensation reaction of L-serine with palmitoyl coenzyme A (CoA) to generate 3-ketodihydrosphingosine (KDS), which is shown in Scheme 1. This reaction is the first committed step in making a backbone structure called the long-chain base (LCB) in the sphingolipid biosynthesis. SPT is thought to be a key enzyme regulating the cellular sphingolipid content because its activity is lower than those of other enzymes involved in the sphingolipid biosynthesis (*3*, *4*). Genetic and biochemical studies of the yeast *Saccharomyces cerevisiae* and Chinese hamster ovary (CHO) cell mutants demonstrated that eukaryotic SPTs function as a heterodimer composed of two subunits, named LCB1 and LCB2 (*4*−*10*).

Various chemical compounds have been employed to study the intracellular functions of sphingolipids in model cell systems, such as yeast and mammalian culture cells. Many of them are specific inhibitors of the sphingolipid metabolic enzymes. For example, aureobasidin A inhibits phosphatidylinositol:ceramide phosphoinositol transferase (*11*), and fumonisin is a strong inhibitor of sphingosine (sphinganine) *N*-acyltransferase (ceramide synthase) (*12*). Natural compounds that inhibit SPT activity have also been discovered during the past decade (*13*−*16*). Sphingofungins (*13*), lipoxamycins (*14*), and viridiofungins (*15*) are specific and potent inhibitors of SPT, having a common structural feature related to sphingosine (Scheme 2), and were isolated as families of antifungal agents. Myriocin (*16*) also specifically and quite potently inhibits SPT activity, and among the sphingosine-like SPT inhibitors, this is the only compound commercially presently available. Moreover, analogues of L-serine, such as L-cycloserine and *β*-chloro-L-alanine (*β*CA), have also been used as SPT inhibitors to decrease sphingo-

† This work was supported by a Grant-in-Aid for Encouragement of Young Scientists (B) 13780488 (to H.I.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by a research grant from the "ONO Medical Research Foundation" (to H.I.), by a research grant from the "Uehara Memorial Foundation" (to H.I.), and by a Grant-in-Aid for Scientific Research on Priority Areas (B) 13125203 (to H.H.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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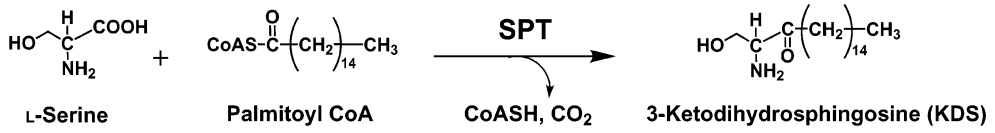
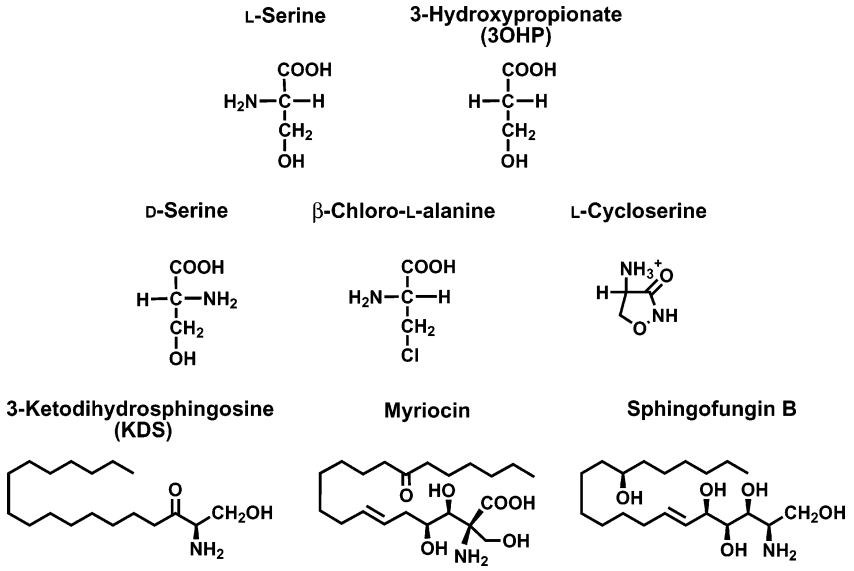
[1] Abbreviations: EY, Eiko Yabuuchi, Aichi Medical University; T, nomenclatural type strain for the species; PLP, pyridoxal 5′-phosphate; LCB, long chain base; SPT, serine palmitoyltransferase; CoA, coenzyme A; KDS, 3-ketodihydrosphingosine; CHO, Chinese hamster ovary; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethansulfonic acid; IPTG, isopropyl 1-thio-*β*-D-galactoside; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; CD, circular dichroism; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; *β*CA, *β*-chloro-L-alanine; 3OHP, 3-hydroxypropionate.

10.1021/bi035706v CCC: $27.50
Published on Web 01/07/2004

Scheme 1: Reaction Catalyzed by Serine Palmitoyltransferase



Scheme 2: Analogues of Serine and KDS



lipids in intact cells (*17*−*20*). Although there are studies on SPT inhibitors by Hanada et al., where a series of sphingofungins and their stereoisomers were synthesized and their inhibitory activities were examined in vivo and in vitro (*21*−*23*), the molecular mechanisms of the SPT inactivation still remain elusive. Detailed studies, including a three-dimensional structural analysis of the SPT−inhibitor complexes, should not only be essential to understand the cellular effects of these inhibitors, but will also be helpful in designing new drugs directed at SPT. The latter would be of particular interest because of the recent discovery that the human hereditary sensory neuropathy type I is caused by mutations in the gene encoding the Lcb p1 subunit of SPT (*23*, *25*). However, it has been very difficult to obtain a sufficient amount of the purified enzyme for a detailed analysis because eukaryotic SPTs are unstable membrane-bound proteins of extremely low cellular contents (*26*).

Previously, we reported the purification, characterization, and molecular cloning of SPT from *Sphingomonas paucimobilis* EY2395^T (*27*). This bacterial enzyme shows about 30% homology with the enzymes of the α-oxamine synthase family, and amino acid residues supposed to be involved in catalysis are conserved. *Sphingomonas* SPT is a prototype of eukaryotic enzymes. Unlike the eukaryotic enzymes, *Sphingomonas* SPT is a water-soluble homodimeric enzyme, and therefore, is expected to provide a simple model system to study the enzyme reaction without a detergent micelle or lipid membrane. The SPT protein overproduced in *Escherichia coli* (*E. coli*) was found to be fully active (*27*). Thus, we could obtain a sufficient amount of the purified enzyme for spectroscopic studies. In the present study, we spectroscopically analyzed the reactions of the recombinant SPT with L-serine and its analogues, and examined the molecular basis of activation of the substrate(s) and inhibition by substrate analogues.

MATERIALS AND METHODS

Chemicals. Sphingofungin B was a kind gift from Dr. Kentaro Hanada of the National Institute of Infectious Diseases in Tokyo, Japan. L-Serine and D-serine were obtained from Nacalai Tesque (Kyoto, Japan). Palmitoyl CoA was from Funakoshi (Tokyo, Japan). Myriocin, *O*-phospho-L-serine, *β*-chloro-L-alanine (*β*CA), L-cycloserine, D-sphingosine, and isopropyl 1-thio-*β*-D-galactoside (IPTG) were from Sigma. α-Methyl-L-serine was from Acros Organics (New Jersey, USA). Serinol and serinamide were from Aldrich. 3-Hydroxypropionic acid (3OHP) was from Tokyo Kasei Kogyo (Tokyo, Japan). Dihydrosphingosine was from Biomol (Pennsylvania, USA). 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF) and lactate dehydrogenase (rabbit muscle) were from Roche Molecular Biochemicals. *β*-NADH was from Oriental Yeast (Tokyo, Japan). PD-10 and Sephacryl S-200 HR were from Amersham Biothech. DEAE-Toyopearl 650M, and Butyl-Toyopearl 650M were from Tosoh (Tokyo, Japan). *E. coli* BL21(DE3) pLysS and the plasmid pET21b were from Novagen. All other chemicals were of the highest grade commercially available.

Expression of the SPT Gene in Escherichia coli. The internal *Nde*I restriction site (^{334}ATGCAT) of *SPT1* was changed to ATGCAC without changing the codons by site-directed mutagenesis, and the new restriction sites, *Nde*I and *Hin*dIII, were introduced into *SPT1* at the translation initiation and termination sites, respectively, by PCR. The modified *SPT1* (*SPT1*$_{mod}$) was ligated into pET21b, and the recombinant plasmid (pET21b/*SPT1*$_{mod}$) was used to transform the *E. coli* BL21 (DE3) pLysS cells. The protein expression was induced with 0.1 mM IPTG and continued for 4 h.

Purification of Recombinant SPT. All purification procedures were performed at 4 °C. The purification buffer

containing 0.1 mM EDTA, 5 mM DTT, 0.1 mM AEBSF (a protease inhibitor), and 20 μM PLP was used in all the following procedures. The cells (10 g of wet weight) were suspended in 150 mL of 20 mM potassium phosphate buffer (pH 7.3) and sonically disrupted (Branson Sonic Power, Sonifier model 450) at 20 kHz for 9 min. The intact cells and debris were removed by centrifugation (100000*g*, 60 min). The supernatant solution was applied to a DEAE-Toyopearl 650M column (2.5 × 20 cm) equilibrated with the same buffer. The proteins were eluted with 1 L of a linear gradient from 0 to 500 mM NaCl. The fractions containing the SPT activity were collected. $(NH_4)_2SO_4$ was added to 30% saturation, and the solution was applied onto a Butyl-Toyopearl 650M column (2.5 × 20 cm) equilibrated with the same buffer containing 30%-saturated $(NH_4)_2SO_4$. The enzyme activity was eluted with 1 L of linearly decreasing $(NH_4)_2SO_4$ concentrations (30 to 0%). The pooled active fractions were concentrated and then applied to a Sephacryl S-200 HR column (1.6 × 80 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.3) containing 0.1 mM EDTA and 150 mM NaCl. The active fractions were combined, concentrated to 5 mL, filtered, and stored at 4 °C. The final sample contained 70 mg of a pure preparation of SPT.

Spectrometric Measurements. The absorption and fluorescence spectra of SPT were recorded with a Hitachi spectrophotometer U-3300 and a Hitachi spectrofluorometer model 850, respectively, at 25 °C. The circular dichroism (CD) spectra of SPT were recorded with a Jasco spectropolarimeter J720-WI at 25 °C. The stopped-flow spectrophotometry was performed on an Applied Photophysics SX.18MV system (Leatherhead, U.K.) at 25 °C. The dead time was 2.3 ms under a gas pressure of 500 kPa. The exponential absorption changes were analyzed with the control software provided with the apparatus. The apparent rate constants obtained were then analyzed for their dependency on the substrate concentration by nonlinear regression with the software Igor Pro (Ver. 4.01, WaveMatrics, Lake Oswego, OR). The time-resolved spectra were collected on the SX.18MV system equipped with a photodiode array accessory and the XScan (version 1.08) control software. Extrapolation of the spectra to $t = 0$ or to infinite concentration of L-serine was carried out by global fitting analysis using the kinetic parameters obtained from the single wavelength experiment described above and the program Pro-KII (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.

Other Methods. The SPT activity was measured according to the previously described methods (*27*). The protein concentration during the purification procedure was determined with a BCA protein assay kit (Pierce Chemical) using bovine serum albumin as a standard. The protein concentration of purified SPT was determined spectrophotometrically using a molar extinction coefficient of 2.83×10^4 M^{-1} cm^{-1} at 280 nm for the PLP form of the enzyme (*27*). SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out with the SDS-Tris system using 10% polyacrylamide gel according to the procedure described by Laemmli (*28*).

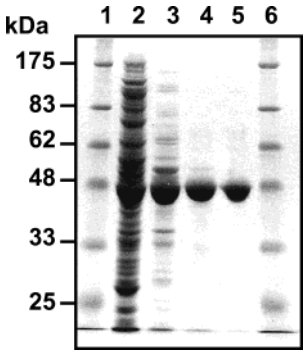


FIGURE 1: SDS−polyacrylamide gel electrophoresis at various steps in the SPT purification. Lanes 1 and 6, molecular mass standard (prestained protein marker, broad range, from New England Biolabs); lane 2, crude extract after sonication and centrifugation at 100000*g* (20 μg of protein); lane 3, DEAE-Toyopearl column (5 μg of protein); lane 4, butyl-Toyopearl column (5 μg of protein); lane 5, Sephacryl S-200 column (1 μg of protein). The samples (10 μL) were analyzed by SDS−polyacrylamide gel electrophoresis on a 10% gel and stained with Coomassie Brilliant Blue R-250.

RESULTS

Purification of Recombinant SPT from E. coli. The SPT produced in *E. coli* amounted to about 10−20% of the total protein in the crude extract of the *E. coli* cells (Figure 1). The growth rate of *E. coli* was not inhibited after the protein expression was induced. When the *E. coli* cells expressing SPT were incubated with [^{14}C]-serine, the accumulation of [^{14}C]-3-ketodihydrosphingosine (KDS), the reaction product of SPT, in the host cells was detected. This result indicates that the overproduced SPT was catalytically active. It also implies that KDS is not toxic to the *E. coli* host. The enzyme was purified to homogeneity by column chromatography in three steps. The purified SPT showed a single protein band with an apparent M_r of about 50 000 on SDS−PAGE (Figure 1). About 70 mg of the protein could be obtained from a 2-L culture. As previously reported, the catalytic properties of the recombinant SPT was the same as those of the native SPT; there was no significant difference between the two enzymes with respect to their steady-state kinetic parameters (*27*); K_m(ser) = 10 mM, K_m(palmitoyl CoA) = 0.86 mM, and k_{cat} = 180 s^{-1} for the recombinant SPT.

Absorption, CD, and Fluorescence Spectra of Recombinant SPT. The absorption spectrum of SPT showed maxima at 338 and 426 nm (Figure 2A, line 1). The absorption spectrum did not change in the pH range of 6.0 and 8.0, indicating that the two absorption bands (426- and 338-nm bands) do not represent different protonated states of the PLP aldimine of SPT. Instead, these molecular species are considered to be the enolimine and ketoenamine forms, respectively, of the aldimine formed between PLP and a lysine residue, Lys265, of SPT (*27*, *31*). There is another possibility that the 338-nm species is a substituted aldamine. It is known that the enolimine structure gives a maximum emission either at around 510 or 410 nm, whereas the substituted aldamine emits very strong fluorescence with maximum intensity at around 390 nm when excited at 330 nm (*29*, *30*). A fluorescence emission maximum was observed at 510 and 400 nm upon excitation at 330 nm (Figure 3, line 1). When the fluorescence was monitored at 510 nm, the fluorescence excitation spectrum (Figure 3, line 3) was similar to the absorption spectrum in Figure 2, having the maxima at

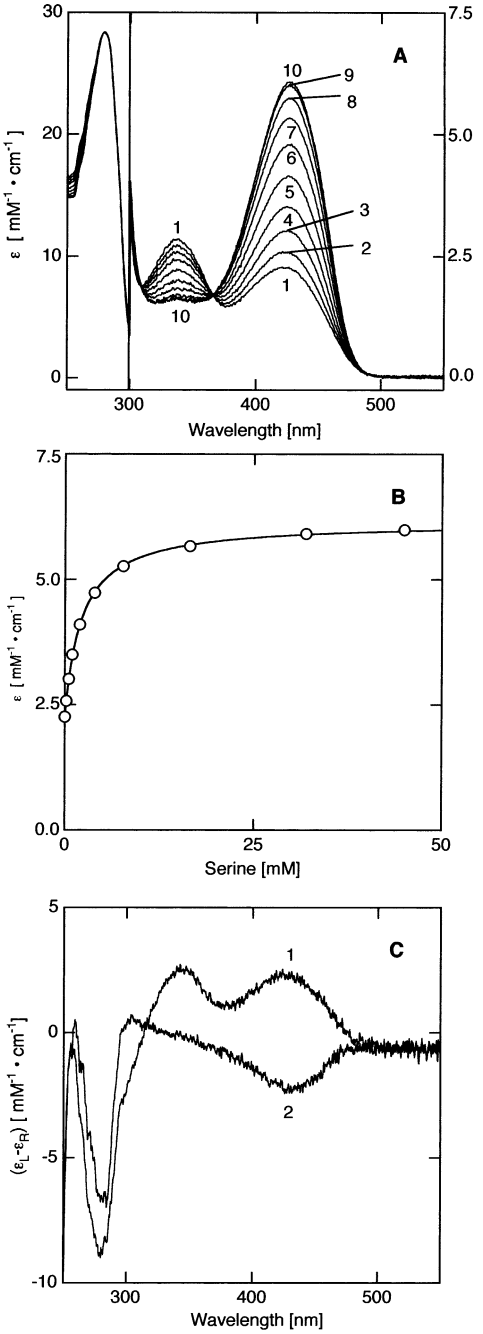


FIGURE 2: Absorption and CD spectra of SPT in the presence of L-serine. The buffer system was 50 mM HEPES−NaOH (pH 7.5) containing 150 mM KCl and 0.1 mM EDTA. The measurements were done at 25 °C. The enzyme concentration was 10 μM. (A) Absorption spectra in the presence of 0, 0.20, 0.50, 1.0, 2.0, 3.9, 7.7, 17, 32, and 45 mM of L-serine (lines 1−10, respectively). (B) Titration curve of the molar extinction coefficient at 422 nm. (C) CD spectra in the absence (line 1) and presence (line 2) of 45 mM L-serine.

wavelengths corresponding to the absorption maxima of SPT. These observations indicate that the enolimine tautomeric form of the aldimine is responsible for the 338-nm species. The CD spectrum of SPT showed positive bands at 338 and 426 nm, corresponding to the absorption spectrum (Figure 2C, line 1).

Interaction of SPT with L-Serine. The absorption and CD spectra of SPT were measured in the presence of various concentrations of the substrate, L-serine. The absorption at 426 nm increased upon the binding of L-serine with a concomitant decrease at 338 nm (Figure 2A). These spectral changes showed a hyperbolic dependency on the concentrations of L-serine (Figure 2B), and the apparent dissociation constant (K_d^{app}) for L-serine was calculated to be 1.4 mM (Table 1). The CD spectrum of SPT in the presence of a saturating amount of L-serine showed a negative band at 426 nm (Figure 2C).

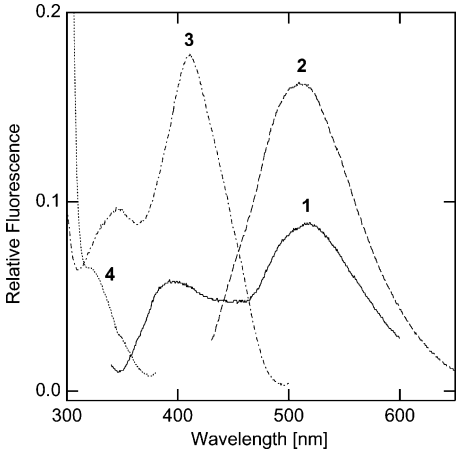


FIGURE 3: Fluorescence spectra of SPT. The buffer system was 50 mM HEPES−NaOH (pH 7.5) containing 150 mM KCl and 0.1 mM EDTA. The measurements were done at 25 °C. The enzyme concentration was 10 μM. Line 1 (solid), fluorescence emission spectra upon excitation at 330 nm; line 2 (dashed), fluorescence emission spectra upon excitation at 420 nm; line 3 (dashed−dotted), fluorescence excitation spectra upon emission at 510 nm; line 4 (dotted), fluorescence excitation spectra upon emission at 400 nm.

Table 1: Dissociation Constants of SPT

compounds	K_d^{app} (mM)
L-serine	1.4 ± 0.1
3-hydroxypropionate	120 ± 9
α-methyl-L-serine	61 ± 4
O-phosphoserine	>100[a]
L-serinol	>100[a]
L-serinamide	38 ± 2
L-serine methyl ester	25 ± 1
D-serine	10 ± 2
L-threonine	3.8 ± 1.0
myriocin (ISP-1)	0.0013 ± 0.0002
sphingofungin B	>0.02[b]

[a] The obtained K_d^{app} values for *O*-phosphoserine and L-serinol under the experimental condition significantly exceeded 100 mM. [b] The K_d^{app} value for sphingofungin B could not be determined because of the low solubility of this compound in water.

Spectral Transition of SPT upon Reaction with L-Serine. The reaction of L-serine with SPT was analyzed using a stopped-flow spectrophotometer. When the SPT (86.6 μM) was reacted with L-serine, an increase in the 426 nm absorbance and a concomitant decrease in the 338 nm absorbance were observed (Figure 4A). The change in the spectrum was a single-exponential process. The reaction was followed by monitoring the absorbance at 426 nm at the L-serine concentration from 0.25 to 100 mM. The apparent rate constants, k_{app} for absorption change clearly showed saturation kinetics (Figure 4B), and the reaction could be analyzed by assuming the following scheme:

$$E + S \overset{K_d}{\rightleftharpoons} ES_1 \overset{k_{+2}}{\underset{k_{-2}}{\rightleftharpoons}} ES_2 \qquad (1)$$

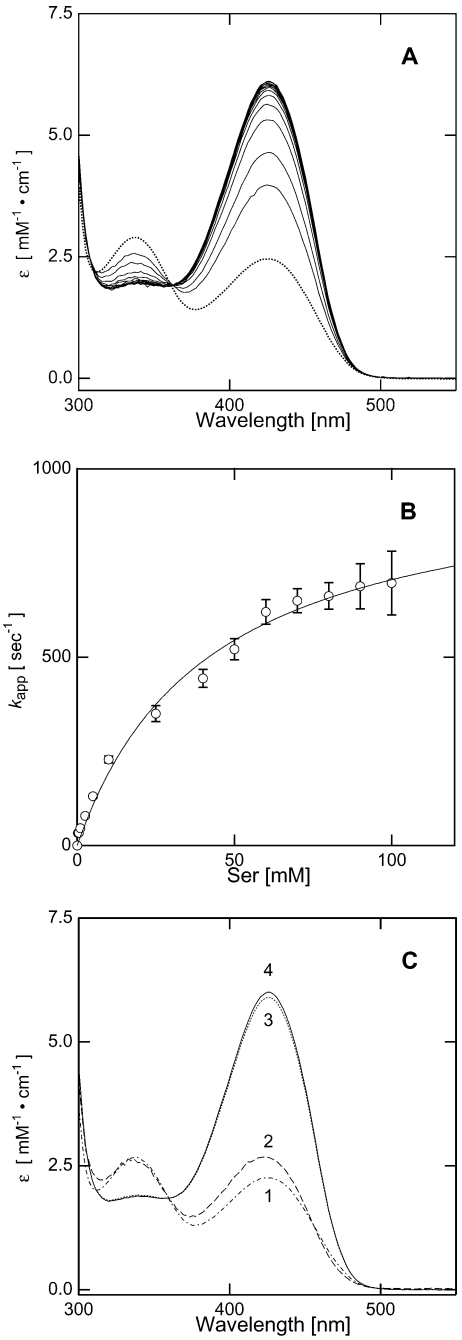


FIGURE 4: (A) Time-resolved spectra for the reaction of SPT and L-serine at pH 7.5. Enzyme (86.6 μM) and L-serine (10 mM) were reacted in 50 mM HEPES−NaOH (pH 7.5) containing 150 mM KCl and 0.1 mM EDTA at 25 °C. Bold dotted line represents the spectrum of SPT in the absence of L-serine. After the addition of L-serine, spectra were taken between 1.28 and 49.92 ms at 2.56-ms intervals. (B) Dependency on the L-serine concentration of the apparent rate constant (k_{app}) for the absorption change. The k_{app} values were obtained by fitting the absorption change at 426 nm to the single-exponential equation. The solid line represents the best fit curve using eq 2. (C) Calculated absorption spectra based on the analysis of the time-resolved spectra. Line 1, the initial spectrum of SPT; line 2, the spectrum of ES_1 obtained from extrapolation of the time-resolved spectra to $t = 0$ and [L-serine] $= \infty$; line 3, the spectrum of the equilibrium mixture of ES_2 and ES_3 obtained from extrapolation of the final spectra to [L-serine] $= \infty$; line 4, the spectrum of ES_3 calculated from lines 2 and 3 and the kinetic parameters (see text).

Here E denotes SPT, and S is L-serine, and ES_1 and ES_2 are complex species. K_d, k_{+2}, and k_{-2} denote the dissociation constant and the rate constants for the indicated reaction steps. The apparent rate constants, k_{app}, can be expressed using the kinetic parameters shown in eq 1 to be

$$k_{app} = \frac{[S]}{K_d + [S]} k_{+2} + k_{-2} \quad (2)$$

The kinetic parameters in eq 1were obtained by fitting the data of Figure 4B to eq 2: $K_d = 53.9 \pm 8.7$ mM, $k_{+2} = 1016.6 \pm 68.2$ s^{-1}, $k_{-2} = 32.6 \pm 11.2$ s^{-1}. The apparent dissociation constant, K_d^{app}, obtained from the static spectroscopic analysis (Figure 2A,B) is related to the above kinetic parameters as follows:

$$K_d^{app} = \frac{k_{-2}}{k_{+2} + k_{-2}} K_d \quad (3)$$

The calculated apparent dissociation constant, $K_d^{app} = 1.7$ mM, was close to the experimental value of 1.4 mM, thus supporting the validity of the above kinetic analysis. Each set of time-resolved spectra at different substrate concentrations was extrapolated to $t = 0$, and then extrapolated to [L-serine] $= \infty$ using $K_d = 53.9$ mM. This calculated absorption spectrum of ES_1 was very similar to the initial spectrum of SPT except for a slight blue shift in the ketoenamine peak position (Figure 4C, lines 1 and 2). On the other hand, extrapolation to [L-serine] $= \infty$ of the final spectra yields the spectrum of the equilibrium mixture of ES_1 and ES_2 (Figure 4C, line 3). The absorbance value of the mixture, designated as A_{mix} is expressed as follows:

$$A_{mix} = \frac{1}{k_{+2} + k_{-2}} (k_{-2} A_{ES_1} + k_{+2} A_{ES_2}) \quad (4)$$

where A_{ES_1} and A_{ES_2} are the absorbance values of ES_1 and ES_2, respectively. As A_{ES_1} and the kinetic parameters are already known, the absorption spectrum of ES_2 could be calculated using eq 4, and is shown in Figure 4C (line 4).

Interaction of SPT with 3OHP. As shown in Scheme 2, 3OHP is a desamino analogue of serine that cannot form an aldimine with PLP. The addition of 3OHP to SPT did not cause any changes in the absorption and CD spectra (data not shown). These findings indicate that 3OHP does not bind to SPT or that the binding of 3OHP does not cause any spectral changes. To distinguish the two possibilities, SPT was titrated with L-serine in the presence of various concentrations of 3OHP. 3OHP was found to competitively inhibit the binding of L-serine to SPT, and the inhibition constant (K_i) for 3OHP was calculated to be 120 mM (Table 1).

Interaction of SPT with Other Serine Analogues and Natural Amino Acids. The addition of α-methyl-L-serine, *O*-phosphoserine, or L-serinol to SPT caused spectral changes similar to those observed with L-serine; the 426-nm peak increased with the substrate concentration but the 338-nm peak did not change its intensity (data not shown). The K_d^{app} for α-methyl-L-serine was 61 mM, and those for *O*-phosphoserine and L-serinol exceeded 100 mM (Table 1). L-Serinamide and L-serine methyl ester having an α-amino group did not show obvious spectral changes, but they competitively inhibited the binding of L-serine to SPT with inhibition constants of 38 and 25 mM, respectively (Table

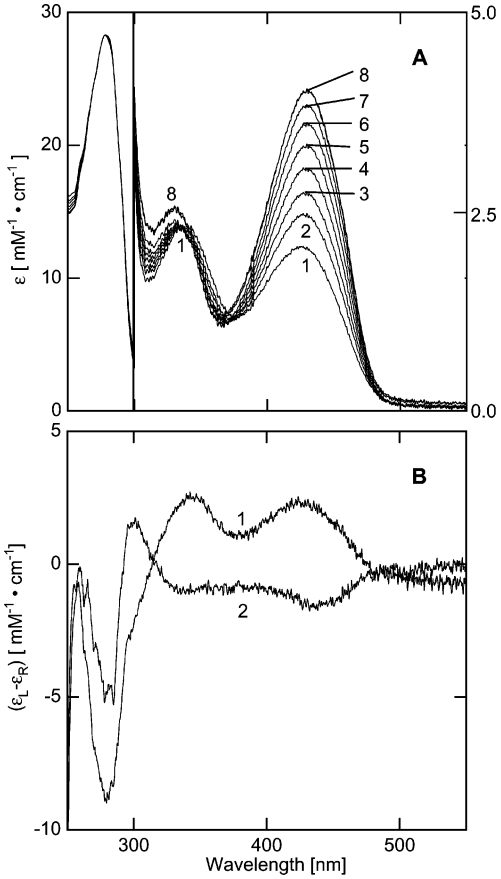


FIGURE 5: Absorption and CD spectra of SPT in the presence of D-serine. The conditions were the same as those in Figure 2. (A) Absorption spectra in the presence of 0, 2.5, 5.0, 9.9, 20, 48, 91, and 130 mM of D-serine (lines 1−8, respectively). (B) CD spectra in the absence (line 1) and presence (line 2) of 130 mM D-serine.

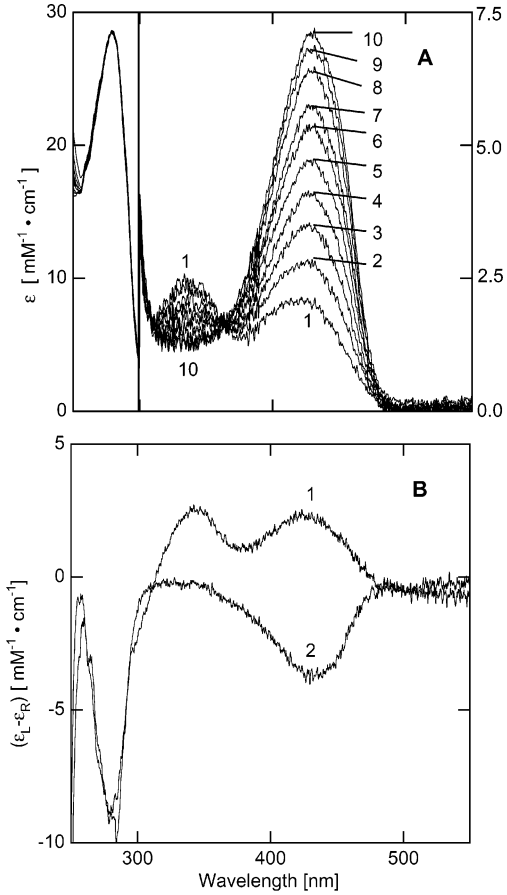


FIGURE 6: Absorption and CD spectra of SPT in the presence of myriocin. The conditions were the same as those in Figure 2. The concentration of the SPT was 0.8 μM. (A) Absorption spectra in the presence of 0, 0.25, 0.50, 0.74, 0.99, 1.2, 1.5, 2.0, 2.9, and 4.8 μM of myriocin (lines 1−10, respectively). (B) CD spectra in the absence (line 1) and presence (line 2) of 4.8 μM myriocin.

1). L-Threonine caused remarkable spectral changes with high affinity (K_d^{app} = 3.8 mM, Table 1). L-Cysteine caused a spectral shift to 330 nm, suggesting the formation of the inactive complex, thiazolidine, with PLP of the enzyme. Other natural amino acids did not cause remarkable spectral changes of SPT in range of the examined concentration (≤10 mM).

Interaction of SPT with D-Serine. A previous study has shown that D-serine was not metabolized by *Sphingomonas* SPT (*27*). However, inhibition of the SPT activity in CHO cells by D-serine has been reported and it was postulated that D-serine binds to the SPT active site to form a Schiff base (*4*, *21*, *22*). Therefore, we examined the interaction of SPT with D-serine using the purified bacterial enzyme. The absorption and CD spectra of the SPT showed significant changes in the presence of D-serine (Figure 5). The binding of D-serine to SPT increased the absorption at 426 nm with a K_d^{app} value of 10 mM (Table 1). The absorption at 330 nm also slightly increased. The complex of SPT with D-serine showed negative CD bands at around 420 and 330 nm.

Interaction of SPT with Myriocin, Sphingofungin B, and Other Product Analogues. Myriocin caused spectral changes in SPT basically similar to those observed with L-serine (Figure 6A). The K_d^{app} value for myriocin obtained by spectrophotometric titration using a low concentration of SPT was 1.3 μM (Table 1). The complex of SPT with myriocin showed a negative CD at 426 nm, as in the case of the SPT−L-serine complex (Figure 6A). Sphingofungin B, another analogue, also caused similar spectral changes (data not shown). In this case, the absorbance at 426 nm linearly increased up to 20 μM. Thus, the K_d^{app} value ≫ 20 μM (Table 1), although the exact value could not be determined because of the low solubility of this compound. Both 22.5 μM D-sphingosine and 10 μM dihydrosphingosine caused slight spectral changes in SPT like sphingofungin B, but further analysis was impossible because of the insolubility of these compounds.

Reaction of SPT with β-Chloro-L-Alanine. When 5 mM β-chloro-L-alanine (βCA) was added to SPT, the 426-nm peak decreased, and the 338-nm peak increased (Figure 7A). The absorbance below 300 nm also significantly increased. The absorption band around 330 nm indicates the formation of a PLP adduct. Because the absorbance below 300 nm is not derived from either βCA or PLP, it is considered that βCA was metabolized by SPT to give rise to a reaction product that shows the observed absorbance. To ascertain the formation of the PLP adduct and identify the reaction product, the reaction mixture was separated into the protein fraction and the small-molecule fraction using the VIVASPIN ultrafiltration device with a molecular weight cutoff of 10 000 (Sartorius). The protein fraction retained the absorption spectrum in the region above 300 nm after the removal of small molecules (Figure 7B, line 1), implying that the changes in PLP caused by βCA are irreversible and the PLP adduct is still bound to the enzyme. The PLP adduct was

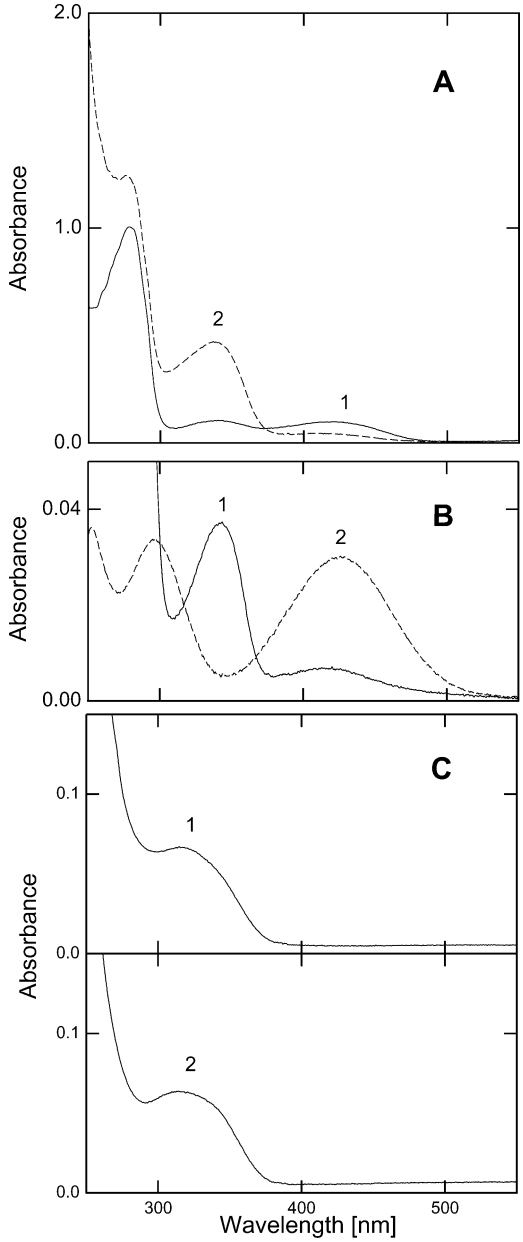


FIGURE 7: Reaction of SPT with βCA. The conditions were the same as those in Figure 2. (A) Absorption spectra of SPT in the absence (line 1) and presence (line 2) of 5 mM βCA. (B) Absorption spectra of PLP adduct before (line 1) and after (line 2) the alkaline treatment. (C) Absorption spectra of the authentic pyruvate (line 1) and the small-molecule fraction after the reaction with βCA (line 2).

released from the enzyme by alkaline treatment, and its absorption spectrum had maxima at 295 and 420 nm (Figure 7B, line 2). On the other hand, the small-molecule fraction had an intense absorbance below 300 nm with a peak at 320 nm (Figure 7C, line 2). This absorption spectrum was very similar to that of pyruvate (Figure 7C, line 1). To confirm this assignment, NADH and lactate dehydrogenase were added to the small-molecule fraction, and the conversion of NADH to NAD^+ was spectrophotometrically monitored and quantified. Under the present experimental conditions, 1.1 mM pyruvate was accumulated when 3.7 μM SPT was completely inactivated by 5,10 or 20 mM βCA.

Reaction of SPT with L-*Cycloserine.* When 2 mM L-cycloserine was added to SPT, the following absorption changes occurred over 10 min (Figure 8): The peak at 426

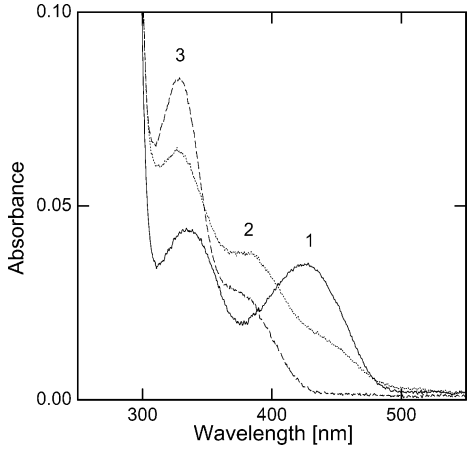


FIGURE 8: Reaction of SPT with L-cycloserine. The conditions were the same as those in Figure 2. Absorption spectra of SPT before addition (line 1), after 5 min (line 2), and 10 min (line 3) after the addition of 5 mM L-cycloserine.

nm decreased, and the 338-nm peak concomitantly increased. During the course of the reaction, an absorption band transiently appeared at 380 nm.

DISCUSSION

Overproduction of SPT for Detailed Spectroscopic Analysis. Sphingomonas SPT overproduced in *E. coli* was active both in vivo and in vitro. The catalytic and spectroscopic properties of the recombinant SPT were indistinguishable from those of the native SPT (*27*, Figure 2A). The establishment of this system enabled us to do detailed analyses of the reactions of SPT with inhibitors.

L-Serine Forms a Stable External Aldimine with SPT Which Is Accompanied by a Large Conformational Change of the Coenzyme. The internal aldimine of SPT exists as the mixture of approximately equal amounts of the enolimine and ketoenamine tautomers (Figure 2A). The binding of L-serine produced a spectrum that shows the presence of an aldimine with ketoenamine as the dominant form. This indicates that the PLP aldimine is placed in an environment that is more polar than that around the internal aldimine. Two possibilities are considered as the cause of this change in the polarity. First, L-serine forms a Michaelis (adsorption) complex with the enzyme, and placement of the hydrophilic amino acid beside the internal aldimine causes the equilibrium shift to the ketoenamine form. Second, L-serine forms an aldimine with PLP to form the external aldimine complex, and, as the serine-PLP aldimine carries hydrophilic carboxylate and hydroxyl groups, the environment of the aldimine is more polar than that of the internal aldimine.

To resolve the two possibilities, we carried out two experiments. The first is a transient kinetic analysis of the reaction of SPT with L-serine. Two intermediates, which are interpreted to be the Michaelis complex and the external aldimine, were obtained, and the kinetic parameters connecting the species were determined. The equilibrium between the Michaelis complex and the external aldimine is shifted far toward the external aldimine ($k_{+2}/k_{-2} = 31$). This indicates that the external aldimine occupies essentially the entire part of the complex of SPT with L-serine. The second is the binding study with 3OHP. This compound retains hydroxyl and carboxylate groups of L-serine, but lacks the

amino group necessary to form an aldimine with PLP. The 3OHP−SPT complex is expected to mimic the Michaelis complex of SPT with L-serine. Compared to the binding affinity for L-serine (K_d^{app} = 1.4 mM), that for 3OHP (K_d^{app} = 120 mM) is similar to the K_d value (54 mM) for Michaelis complex formation of L-serine with SPT obtained by the transient kinetic analysis. Furthermore, the observation that the absorption spectrum of SPT does not essentially change upon the binding of 3OHP is consistent with the result that the obtained spectrum of the Michaelis complex with L-serine shows only little change from that of the unliganded SPT (Figure 4C). On the basis of these results, we concluded that the spectra of SPT in the presence of a saturating concentration of L-serine represent the spectra of the external aldimine.

The CD spectra provide important information on the structure of the external aldimine. In aspartate aminotransferase, the transaldimination reaction from the internal aldimine to the external aldimine causes rotation of the PLP ring and change in the conformation (especially the imine-pyridine torsion angle) of the PLP aldimine (*32*). The CD spectrum of the unliganded enzyme shows a positive Cotton effect, but during the transaldimination, it shows inversion of the Cotton effect (*31*). Thus, the change in the orientation and/or the conformation of the PLP aldimine is reflected in the CD spectrum. A quite similar change in the CD spectrum is observed for the binding of L-serine to SPT (Figure 2), whereas no CD spectral change is observed for binding of 3OHP. These findings strongly suggest that, upon the formation of the external aldimine with L-serine, the PLP aldimine of SPT undergoes structural changes similar to those observed for aspartate aminotransferase. The spectroscopic measurements described above can be considered to be a convenient and reliable method to detect the formation of the external aldimine.

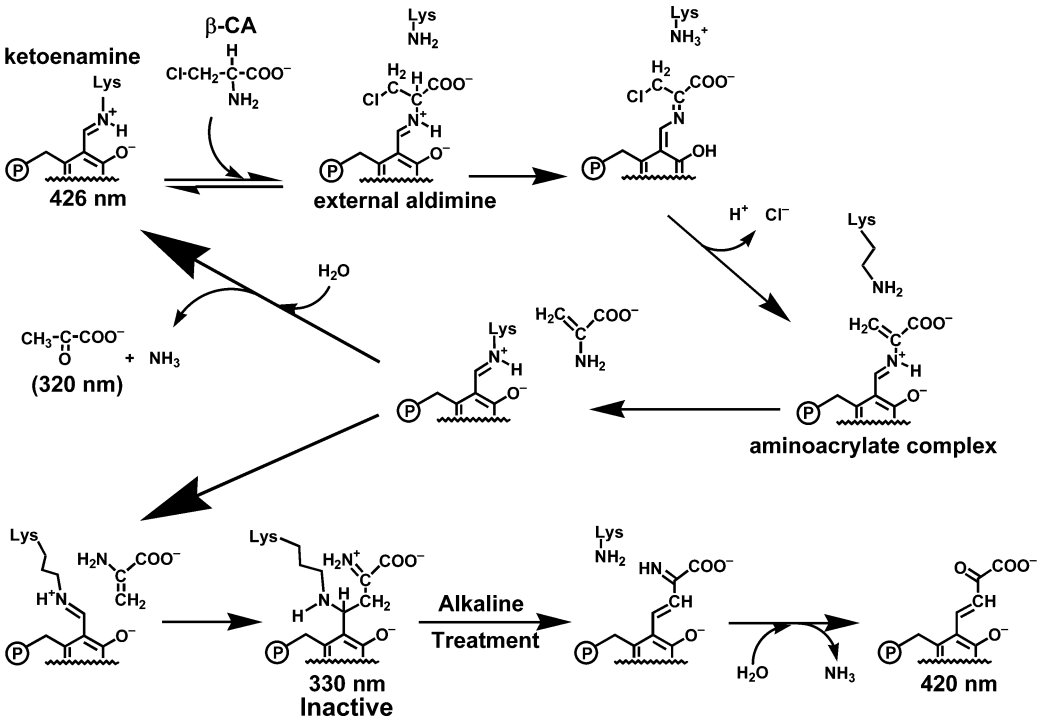
No Apparent Formation of the Quinonoid Species from L-Serine Alone. The catalytic reactions of most PLP enzymes, including SPT, involve the removal of α-H. However, for both the static (Figure 2A) and transient (Figure 4A) measurements, no apparent absorption is observed at around 500 nm, indicating that the α-deprotonated species (quinonoid intermediate) does not accumulate in the reaction of L-serine with SPT. Generally, the observation that the quinonoid intermediate is not detected indicates either that the species is not involved in the reaction, as has been observed for the reactions of cytosolic aspartate aminotransferase with aspartate (*33*) and dialkylglycine decarboxylase with aminoisobutyrate (*34*), or that the breakdown of the species is much faster than its formation. In the case of the reaction of SPT, which is a bisubstrate enzyme, there is another possibility that the binding of the second substrate, palmitoyl CoA, may cause alterations in the electronic nature or the conformation of the external aldimine, and generate the quinonoid species. If this is the case, we can also speculate that suppressing the quinonoid formation until the binding of the second substrate is a valuable mechanism to minimize side reactions, such as transamination, of L-serine.

Carboxylate Group Is Required for the Binding of Amino Acid Substrate. To determine the molecular aspects of the substrate recognition of SPT, the binding reactions of a series of serine analogues and natural amino acids to SPT were examined. The presence of a methyl group at Cα (α-methyl-L-serine) or Cβ (L-threonine) and phosphorylation of the hydroxyl group (*O*-phospho-L-serine) did not significantly impair the binding of the amino acid to SPT. On the other hand, modification of the carboxyl group of L-serine (L-serinol, L-serinamide, and L-serine methyl ester) resulted in a marked decrease in the binding ability. These results show that SPT has a site that specifically recognizes the α-carboxyl group of the amino acid substrate, whereas the site for the side chain of the amino acid is not very strict and allows significant modification. It is interesting to note that L-threonine binds to SPT more tightly than *O*-phospho-L-serine and rivals the natural substrate L-serine, but is not metabolized at all by the enzyme whereas *O*-phospho-L-serine is a weak substrate of SPT (*27*). This suggests that the β-branched structure of the amino acid causes an unfavorable interaction with the enzyme in the reaction steps after the external aldimine.

D-Serine Also Binds to SPT. D-Serine is physiologically found in the brain (*37*, *38*), where sphingolipids are abundant. Recently, Hanada et al. found that D-serine inhibited the KDS formation in CHO cells with an IC_{50} of ∼0.3 mM using a competition assay (*4*, *22*, *23*). To clarify the mechanism of inhibition, we studied the reaction of D-serine with *Sphingomonas* SPT. The addition of D-serine caused a shift in the absorption spectrum and an inversion of the CD spectrum similar to that of L-serine. Therefore, it is considered that D-serine forms the external aldimine. As shown above, the α-carboxyl group of the amino acid substrates is the critical structure for binding to SPT. Therefore, in the external aldimine, the α-carboxylate groups of L-serine and D-serine are considered to be fixed to the same active site residues. The hydroxymethyl group of D-serine would then occupy the place where the α-proton of L-serine normally exists, and conversely, the α-proton of D-serine in the place of the hydroxymethyl group of L-serine. The α-proton of L-serine of the external aldimine will be in a position favorable for activation, at least after the binding of the second substrate (see the previous discussion). Accordingly, it is expected that the α-proton of D-serine would not be in a position suitable for deprotonation (*32*). This explains the observation that D-serine is not the substrate of SPT (*22*, *23*, *27*). The larger fraction of the enolimine form of the SPT-D-serine complex as compared to that of the SPT−L-serine complex (Figure 5) suggests a difference in the pattern of the hydrogen bonds involving the substrate hydroxymethyl group and the catalytic amino group of Lys265, which affects the tautomerism of the external aldimine.

Myriocin and Sphingofungin B Are Strong Inhibitors of SPT That Will Mimic the Reaction Intermediate. Myriocin inhibited the SPT activity of the CTLL-2 cell line with an apparent K_i value of 0.28 nM (*16*). In addition, Hanada et al. reported that 1 μM myriocin almost completely inhibited the de novo synthesis of sphingolipids in the CHO cells (*21*). For *Sphingomonas* SPT, the myriocin bound to the enzyme forming the external aldimine with a K_d^{app} value of 1.3 μM (Figure 6, Table 1). Although the inhibition of SPT activity of the CTLL-2 cell line by myriocin has been considered to be noncompetitive (*16*), the binding reaction of L-serine to *Sphingomonas* SPT was competitively inhibited by myriocin. The affinity of myriocin is 10^3 fold higher than that of L-serine. Evidently, the presence of the large hydrophobic

Scheme 3: Proposed Reaction Mechanism of SPT with βCA, Based on the Spectral Characteristics of the PLP-adduct and the Studies on Aspartate Aminotransferase[a]



[a] Refs *39*−*42*.

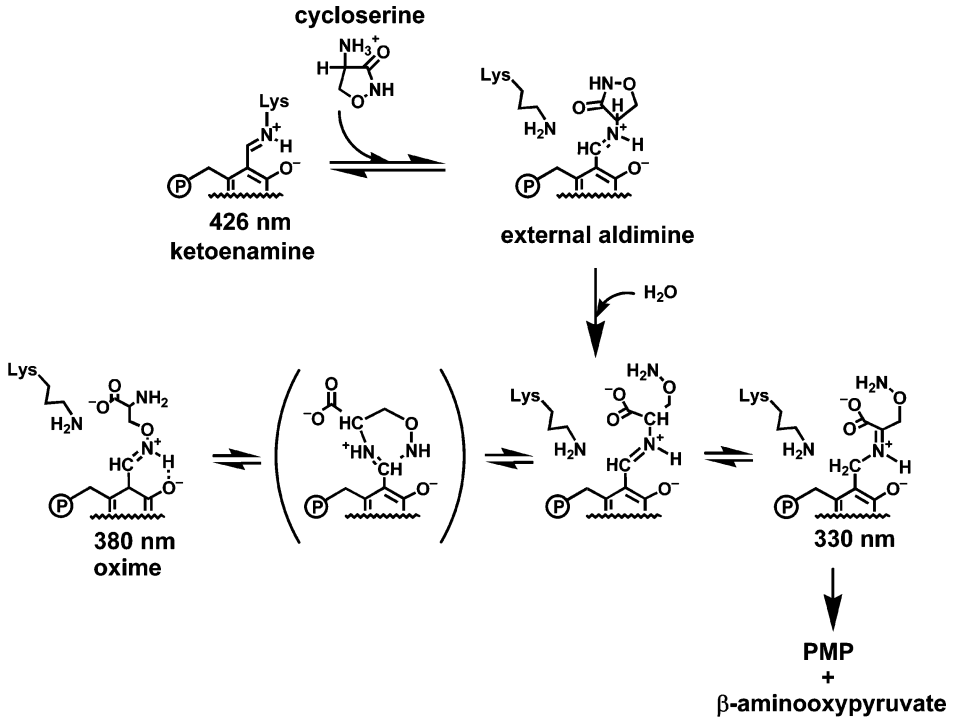
chain of myriocin, which would interact with the palmitoyl group-binding site of SPT, produces the tight binding of myriocin. The chemical structure of myriocin is analogous to the direct condensation product of L-serine and palmitoyl CoA, and differs in the presence of hydroxyl and keto groups and an unsaturated bond at the aliphatic side chain, and reduction of the β-keto group. The present study did not determine whether myriocin in the external aldimine undergoes decarboxylation. However, the absence of the β-keto group is considered to be a drawback to the decarboxylation.

The K_d^{app} value of the bacterial SPT for myriocin was greater than the apparent K_i (0.28 nM) or IC_{50} (15 nM) value of the eukaryotic enzymes (*16*). This finding might simply reflect the differences in the character of the proteins from different sources. Alternatively, the true dissociation constants of the eukaryotic SPTs for myriocin might be greater than the apparent ones. Because the experiments on the eukaryotic enzymes were done using whole culture cells, membrane preparations such as microsomes, or the reconstituted micelle system, myriocin, a very hydrophobic compound, would be enriched in the lipid bilayer, where the enzymes reside.

Similar to myriocin, sphingofungin B inhibits yeast SPT activity with an IC_{50} of 20 nM (*13*). Although the K_d^{app} value for sphingofungin B could not be determined, its affinity for *Sphingomonas* SPT was lower than that for myriocin.

βCA Causes Suicide Inactivation of SPT. βCA and L-cycloserine have been frequently used as inhibitors of SPT in the experiments involving a cell culture system (*17*−*20*), even though the inhibition mechanisms of SPT by these compounds are unclear and they are known to inactivate other PLP-dependent enzymes as well as SPT (*39*, *40*, *41*). Medlock and Merrill have investigated the effects of βCA on SPT activity in vitro with rat liver microsomes and in vivo with intact CHO cells and concluded that βCA acted on SPT as a "suicide" inhibitor (*20*). We showed that SPT was irreversibly inactivated by βCA to produce a PLP adduct and pyruvate using the purified bacterial enzyme (Figure 7). Similar phenomena were found in the reactions of aspartate aminotransferase with various β-substituted amino acids including βCA, and mechanisms have been proposed to explain these reactions (*39*−*42*). On the basis of the spectral characteristics of the PLP-adduct and the studies on aspartate aminotransferase, the reaction mechanism of SPT with βCA is proposed as shown in Scheme 3. First, the external aldimine intermediate is formed between βCA and the PLP of SPT. After the α-proton of βCA is subtracted by Lys265 of SPT, elimination of the chloride ion occurs to form the aminoacrylate-PLP aldimine. Aminoacrylate is released and the internal aldimine is regenerated by transaldimination. There are two possible pathways after this step. One is the regeneration of the active enzyme via dissociation of the aminoacrylate, which is hydrolyzed to pyruvate and ammonia in aqueous solvent. The other is the irreversible inactivation via a nucleophilic attack of the bound aminoacrylate on the internal aldimine. The partition coefficient between the two pathways was calculated as follows: [pyruvate accumulated]/[enzyme in the reaction mixture] = 1.1 mM/3.7 μM = 300. This means that one SPT molecule is inactivated in 300 cycles of the pyruvate-forming reaction. Although the reaction of L-serine does not accumulate the quinonoid intermediate, the deprotonated product from the external aldimine, the formation of pyruvate from βCA indicates that SPT actually catalyzes the deprotonation at the αC of βCA. This strongly suggests that the substrate L-serine is fixed to SPT like βCA, and the α-proton is subject to catalysis of the active site residue(s) and PLP in the same way as βCA.

Scheme 4: Proposed Reaction Mechanism of SPT with L-cycloserine, Based on the Spectral Characteristics of the PLP-adduct and the Studies on Aspartate Aminotransferase[a]



[a] Refs *39*, *40*, and *43*.

The reactions of aspartate aminotransferase with various cyclic amino acids have also been investigated (*39*, *40*, *43*). L-Cycloserine irreversibly inactivated SPT via a transient intermediate with a characteristic absorption peak at 380 nm (Figure 8). This intermediate is thought to be an oxime formed between the coenzyme and L-cycloserine (*43*). A possible reaction mechanism is shown in Scheme 4: The first step is the formation of the external aldimine between L-cycloserine and PLP of SPT. Decyclization of the isoxazolidone ring of L-cycloserine then takes place with the liberation of an aminooxy group and the carboxylate group. The preferable interaction of the librated carboxylate group with the enzyme may promote this step. This molecular species is either intramolecularly rearranged to an oxime of PLP with β-aminooxyserine or hydrolyzed to pyridoxamine 5′-phosphate and β-aminooxypyruvate. The hydrolysis proceeds more slowly than the rearrangement, leading to the transient accumulation of the oxime.

As demonstrated above, *Sphingomonas* SPT has been proven to be an ideal system for studying this enzyme. Although there are some differences between the prokaryotic and eukaryotic SPTs in the subunit composition or intracellular localization, it is expected that their active site structures and reaction mechanisms are very similar to each other. In this study, we showed that some of the examined compounds could mimic the reaction intermediate by forming a complex with SPT. The X-ray crystallographic analysis of *Sphingomonas* SPT is now in progress by our group. Therefore, by carrying out the structural analysis for the SPT-substrate/product analogue complex, we expect to obtain the molecular basis of the reaction mechanism of SPT soon. The elucidation of the structure and function of this enzyme would facilitate our understanding of the sphingolipid metabolism.

ACKNOWLEDGMENT

We thank Dr. K. Hanada of the National Institute of Infectious Diseases for the kind gift of sphingofungin B. We also thank Dr. T. Yano for the helpful discussions and critical reading of the manuscript, and Professor S. Kominami of Hiroshima University for his valuable suggestions concerning this research.

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BI035706V