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Pyridoxal 5′-Phosphate Binding of a Recombinant Rat Serine:Pyruvate/Alanine:Glyoxylate Aminotransferase[1]

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Serine:pyruvate/alanine:glyoxylate aminotransferase in the liver is a class IV aminotransferase. The present study was undertaken to characterize the pyridoxal 5′-phosphate (PLP) binding to a recombinant rat serine:pyruvate/alanine:glyoxylate aminotransferase (SPT_{10}), which is a homodimer of 44.4 kDa subunits. Purified SPT_{10} exhibited absorption maxima at ~330 nm in addition to a 278 nm protein peak and a ~420 nm peak of PLP bound *via* Schiff base, and contained 0.56-0.69 mol of PLP per mol of subunit. Apo-SPT_{10} without measurable bound PLP did not exhibit the absorbance at ~420 nm, but still showed the ~330 nm peak. Upon reconstitution, 0.73-0.79 mol of PLP per mol of subunit was bound to apo-SPT_{10} with an apparent K_d of ~0.1 μM, resulting in a holo-SPT_{10} preparation whose specific activity and $A_{\sim 420}/A_{\sim 330}$ absorbance ratio were higher than those of the original SPT_{10}. On SDS/PAGE of BrCN-cleavage peptides of $NaBH_4$-reduced SPT_{10}, 22-23 kDa fragments migrated as a pair of bands. On amino acid sequencing, the ~22 and ~23 kDa pair gave the same sequence, except that Lys was released only from the ~22 kDa band material in the cycle corresponding to Lys209. NaB^3H_4-treated SPT_{10} also migrated as a pair of 44-45 kDa bands and ^3H was incorporated only into the ~45 kDa band. It appears that SPT_{10} has the capacity to bind 1 mol of PLP to Lys209 of every subunit, but usually binds less PLP in a Schiff base structure, probably due to the presence of a 330 nm-absorbing chromophore.

Key words: absorption spectrum of a B_6 enzyme, pyridoxal 5′-phosphate, pyridoxal 5′-phosphate binding Lys residue, SDS/PAGE after $NaBH_4$ reduction, serine:pyruvate/alanine:glyoxylate aminotransferase.

Serine:pyruvate aminotransferase [EC 2.6.1.51] was discovered in 1956 in dog liver (*1*). Alanine:glyoxylate aminotransferase [EC 2.6.1.44] was first purified approximately 5-fold from hog liver (*2*) and then extensively from human liver (*3*). These two enzymes had been studied individually, but later serine:pyruvate aminotransferase and isozyme 1 of alanine:glyoxylate aminotransferase were found to be the same enzyme (*4*). Recently, this enzyme was classified, together with phosphoserine aminotransferase [EC 2.6.1.52], as a class IV aminotransferase (*5*). Since both its serine:pyruvate and alanine:glyoxylate aminotransferase activities are believed to be of physiological importance, this enzyme is called serine:pyruvate/alanine:glyoxylate aminotransferase (SPT/AGT) in this paper.

SPT/AGT in the liver is an enzyme of species-specific dual organelle localization and presumably of dual function. In carnivores, this enzyme is located mostly in mitochondria (*6, 7*), while in rat liver, it is found in both mitochondria and peroxisomes (*8, 9*), and only the mitochondrial enzyme is markedly induced by glucagon (*8-10*). In the marmoset, SPT/AGT is also found in both mitochondria and peroxisomes in the liver (*7*), but in herbivores and man, this enzyme is entirely peroxisomal (*6, 7, 11*). Peroxisomes are the subcellular compartment where glyoxylate, an immediate precursor of oxalate, is mainly produced. It is well known that the calcium salt of oxalic acid is hardly soluble in water, and frequently forms calculi, especially in urinary tissues. SPT/AGT in peroxisomes has been assumed to be essential to remove glyoxylate through conversion to glycine and to prevent the harmful overproduction of oxalate in animals. In fact, primary hyperoxaluria type 1 (PH1), a lethal congenital metabolic disease characterized by increased production of oxalate and precipitation of calcium oxalate crystals in many tissues, is caused by a defect in SPT/AGT (*12*). We reported previously a $CRM^{\pm}/ENZ^{-}$ PH1 case caused by a point mutation of T to C in the coding region of the SPT/AGT gene, encoding a Ser to Pro substitution at residue 205 of SPT/

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Abbreviations: ε_{mM}, millimolar extinction coefficient; PLP, pyridoxal 5′-phosphate; SDS/PAGE, SDS/polyacrylamide gel electrophoresis; SPT activity, serine:pyruvate aminotransferase activity; SPT/AGT, serine:pyruvate/alanine:glyoxylate aminotransferase; SPT_{10}, a recombinant SPT/AGT from $pRspt_{10}$, a plasmid containing the full length cDNA for rat SPT/AGT.

AGT (*13*). Minatogawa *et al.* (*14*) found another CRM$^-$/ENZ$^-$ type of PH1 in which a Ser187Phe substitution was predicted to be caused by a C to T mutation. We had previously suggested, based on a comparison of the predicted secondary structure of rat SPT/AGT with those of other aminotransferases, that Lys209 is the pyridoxal 5'-phosphate (PLP) binding residue (*15*). If this is indeed the case, then the two point mutations associated with the CRM$^\pm$/ENZ$^-$ or CRM$^-$/ENZ$^-$ type of PH1 are located in the neighborhood of the PLP-binding Lys residue in the active site. In the present study we attempted to confirm that Lys209 is the PLP-binding residue.

We have also attempted, in collaboration with Dr. Kousuke Morikawa, Protein Engineering Research Institute, Osaka, Japan, an X-ray crystallographic analysis of SPT/AGT, but the crystals obtained have not been suitable for that purpose. Preliminary experiments suggested that the SPT/AGT preparations used were pure enough in terms of protein, but not necessarily uniform with respect to the PLP binding. Therefore, binding of the PLP-cofactor to SPT/AGT was also analyzed.

We used a recombinant SPT/AGT (SPT_{10}) from $pRspt_{10}$, a pUC8-derived plasmid containing the full length cDNA for rat SPT/AGT (*16*), for the following reasons: (1) a large quantity of purified SPT_{10} can be easily obtained: (2) The predicted amino acid sequences of rat and human SPT/AGTs exhibit 79.3% identity, and, when conservative amino acid substitutions are included, the similarity is as much as 92.6% (*17*): (3) SPT_{10} consists of the whole amino acid sequence of rat mitochondrial SPT/AGT with 9 extra amino acid residues at the N-terminal (*18*), but its catalytic and physical properties such as specific activity, substrate specificity, K_m for α-keto acid substrates, electric charge and quaternary structure, are all very similar to those of SPT/AGT purified from rat liver mitochondria (*18*). The amino acid residues of SPT_{10} are numbered, in this paper, from the Met corresponding to the N-terminal Met of the rat mitochondrial enzyme.

MATERIALS AND METHODS

Purification of SPT_{10}—Escherichia coli DH1 transformed with $pRspt_{10}$ was cultured and collected as described previously (*18*). SPT_{10} was purified from 20–30 g (wet weight) of packed bacteria by a modification of the method described previously (*18*). All the purification procedures were carried out at 0–4°C except where otherwise indicated. The bacteria were suspended in 3 volumes (w/v) of an extraction medium (pH 7.5) consisting of 5 mM K-phosphate, 1 mM EDTA, 1 mM pyruvate, 0.1 mM PLP, 0.01% (v/v) Tween 80, 0.5 mM phenylmethylsulfonyl fluoride, 0.001% (w/v) E-64C, and 0.1% (v/v) ethanol, and then subjected twice to disruption with a French Press (French Pressure Cell Press, SLM-Aminco, Urbana, IL, USA) with a working pressure of 15,000 psi. The bacterial extract obtained on centrifugation at 40,000 rpm for 60 min was then subjected to brief heat treatment (15 s) at 65°C, immediately followed by rapid cooling to about 5°C in an ice-salt bath. The supernatant obtained by centrifugation at 14,000 rpm for 15 min was applied to a phosphocellulose (P-11) cation exchange column (2.4×40 cm) which had been equilibrated with 5 mM K-phosphate (pH 7.5) containing 1 mM EDTA, 1 mM pyruvate, 0.1 mM PLP, and 0.01% (v/v) Tween 80, and the enzyme was eluted with a linear gradient formed from 750 ml each of 5 and 200 mM K-phosphate (pH 7.5) in the equilibration buffer. The active fractions were concentrated in a dialysis tube to approximately 10 ml against polyethylene glycol 20,000, and then 3-ml portions were subjected to gel filtration chromatography on a Sephadex G-50 (fine) column (1.5×46 cm) with 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA as the equilibration and elution buffer. The enzyme was almost pure at this stage of purification, as shown in Fig. 1, and as much as 100 mg of purified SPT_{10} was obtained from 20 g of packed bacteria (Table I).

Determination of Serine:Pyruvate Aminotransferase Activity—The activity of SPT_{10} was determined as the amount of hydroxypyruvate produced from L-serine, essentially as described previously (*10*), except that reactions were carried out in 0.4 ml of incubation mixture. One unit of the enzyme was defined as the amount which catalyzed 1 μmol of hydroxypyruvate formation per min. Under the standard assay conditions, the rate of hydroxypyruvate formation was proportional to the enzyme concentration up to 45 milliunits per 0.4 ml reaction mixture.

Preparation of apo-SPT_{10}—Two milliliters of the purified SPT_{10} preparation from which free PLP had been removed by Sephadex G-50 gel filtration was concentrated to approximately 0.2 ml with the aid of a Centricon-30 (Amicon/Grace, Danvers, MA, USA). To the concentrated enzyme, 1.0 ml of 80 mM K-phosphate (pH 7.5) containing 1 mM EDTA and 0.8 ml of 1 M L-serine (pH 8.0) were

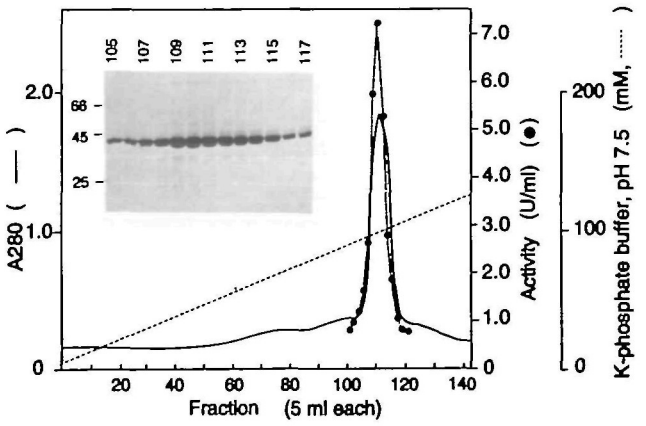

Fig. 1. **P-Cellulose (P-11) column chromatography of SPT_{10}.** The procedures are described in detail under "MATERIALS AND METHODS." —, absorbance at 280 nm (A_{280}); ●, SPT activity; ····, concentration of K-phosphate (pH 7.5). Inset: 5 μl-aliquots of active fractions (fractions 105–117) were subjected to SDS/PAGE.

TABLE I. **Purification of SPT_{10}.** *E. coli* DH1 transformed with $pRspt_{10}$, a plasmid containing the full length cDNA for rat SPT/AGT, was cultured, and SPT_{10} was purified from 20 g of packed bacteria as described under "MATERIALS AND METHODS."

	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Extract	106	1,036	2,383	0.43	100
Heat	85	729	877	0.83	70
P-11	60	400	110	3.64	39
Conc. P-11	10.7	509	117	4.35	49
Sephadex G-50	54.4	462	102	4.62	45

added, and after incubation at room temperature for 10 min, the mixture was concentrated to approximately 0.2 ml as above. The same procedure was repeated, and the resultant concentrated enzyme was mixed with 1.8 ml of 1 M K-phosphate (pH 6.0). The mixture was incubated at room temperature for 10 min and then concentrated to approximately 0.2 ml as above. The same procedure was repeated, and the concentrated enzyme solution finally obtained was made up to 2.0 ml with 80 mM K-phosphate (pH 7.5) containing 1 mM EDTA. The recovery of protein through the whole procedure for the preparation of apo-SPT_{10} was approximately 90%.

Determination of Protein—Determination of protein during the course of the SPT_{10} purification was carried out by the method of Lowry *et al.* (*19*) with bovine serum albumin as a standard. The concentration of purified SPT_{10} from which free PLP had been removed was also determined spectrophotometrically in 6 M guanidine-HCl at 278 nm. SPT_{10} had been shown to be a homodimer of 44.4-kDa subunits (*18*). The concentration of the SPT_{10} subunit was calculated from the absorbance at 278 nm by using a millimolar extinction coefficient (ε_{mM}, light path = 1.0 cm) of 48.4 ($A^{1mg/ml} = 1.09$) for each subunit of the PLP-form of holo-SPT_{10}, and of 46.4 ($A^{1mg/ml} = 1.045$) for each subunit of apo-SPT_{10}. The ε_{mM} values for the apo-SPT_{10} and the PLP-form of holo-SPT_{10} subunit were calculated according to Kuramitsu *et al.* (*20*) on the basis of 5 tryptophan and 12 tyrosine residues (*15, 18*) and 1 mol of PLP binding to each mole of the SPT_{10} subunit. The ε_{mM} of 46.4 calculated for apo-SPT_{10} agreed well with the value calculated according to Gill and Hippel (*21*) from the deduced amino acid data. The absorbance at 278 nm of native SPT_{10} at pH 7.5 was approximately 75% of that in 6 M guanidine-HCl. Therefore, a value of ε_{mM} at 278 nm of 36.3 ($A^{1mg/ml} = 0.82$) was used for routine estimation of the concentration of native holo-SPT_{10}. The value obtained with the routine spectrophotometric method agreed well with that determined by Lowry's method.

Determination of PLP—Enzyme-bound PLP was determined by both a spectrophotometric method (*22*) and the phenylhydrazine method of Wada and Snell (*23*). For the spectrophotometric method, the coenzyme was released from holo-SPT_{10}, that had been passed through a Sephadex G-50 column to remove free PLP, by adding an equal volume of 0.6 N $HClO_4$. After removal of the precipitated protein by centrifugation, the supernatant was neutralized and made up to 0.1 N with respect to NaOH, and then the concentration of released PLP was determined from the absorbance at 388 nm using the reported ε_{mM} of 6.6 (*24*). For the phenylhydrazine method, 1.9 ml of a deproteinized sample in 0.3 N $HClO_4$ was mixed with 100 μl of 2% (w/v) phenylhydrazine hydrochloride in 10 N H_2SO_4. Samples were allowed to stand for 10 min at room temperature, and then the absorbance at 410 nm was read. Enzyme-bound PLP was also determined as the absorbance at 388 nm of SPT_{10} in 0.1 N NaOH according to Matsuo and Greenberg (*25*).

BrCN Cleavage—A large excess (above 1,000-fold molar excess) of $NaBH_4$ powder was added to 1.2-3 mg (27-68 nmol of subunit) of holo-, apo-, or reconstituted SPT_{10} in 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA. After 30 min at 4°C, the mixture was dialyzed overnight at 4°C against 50 mM NH_4HCO_3 (pH 8.5) containing 1 mM EDTA. The dialyzed enzyme was transferred to the main chamber of a Thunberg tube. The side arm of the tube contained 4-vinylpyridine and tri-*n*-butylphosphine in an amount representing 50-120-fold molar excess each over protein sulfhydryl or half-cystine groups. Air in the Thunberg tube was immediately replaced by N_2. Then the enzyme was mixed with the 4-vinylpyridine/tri-*n*-butylphosphine, and the mixture was kept on ice overnight under N_2 in the dark. The alkylated SPT_{10} was dialyzed overnight at 4°C against 50 mM NH_4HCO_3 (pH 8.5) and then lyophilized. The $NaBH_4$-reduced and pyridylethylated SPT_{10} was dissolved in 70% HCOOH to obtain a solution of about 10 mg protein/ml, and BrCN cleavage was carried out in a sealed vessel with a 30- to 100-fold molar excess of BrCN with respect to the calculated number of Met residues. After 24-48 h in the dark at room temperature, the BrCN-cleavage reaction was terminated by 10-fold dilution with H_2O, and the sample was dried in a SpeedVac concentrator (Savant, Farmingdale, NY, USA). The dried residue was dissolved in H_2O to a protein concentration of approximately 3 mg/ml, and then 10-μl aliquots were subjected to SDS/12% PAGE according to the method of Laemmli (*26*). Protein bands were stained with Coomassie Brilliant Blue or transferred to a PVDF membrane using 25 mM Tris containing 195 mM glycine, 15% methanol, and 0.05% SDS as the transfer buffer. Protein bands transferred to a PVDF membrane were visualized with 0.5% Ponceau S in 1% acetic acid to confirm their positions (*27*), and then the materials were subjected to automatic amino acid sequencing with the aid of an ABI protein sequencer, model 476A. For all SDS/PAGEs, a mixture of marker substances (bovine serum albumin [66 k], ovalbumin [45 k], chymotrypsinogen A [25 k], and cytochrome *c* [12.5 k]) (Pharmacia-Biotech, Tokyo) was electrophoresed simultaneously for molecular weight calibration.

Other Methods—All spectrophotometric measurements were carried out with a recording spectrophotometer (Union-Giken, SM-401, Hirakata). SDS/PAGE of ^{3}H-labeled SPT_{10} was carried out with Rainbow™ [^{14}C]methylated proteins (ovalbumin [46 k], carbonic anhydrase [30 k], trypsin inhibitor [21.5 k], and lysozyme [14.3 k]) (Amersham Life Science, Tokyo) as molecular weight markers, and the radioactivity was detected by indirect fluorography according to Chamberlain (*28*).

Materials—*Bacillus stearothermophilus* D-amino acid aminotransferase was kindly donated by Drs. K. Soda and T. Yoshimura, Institute for Chemical Research, Kyoto University. Pig heart cytosolic aspartate aminotransferase was obtained from Boehringer Mannheim (Tokyo), tryptone and yeast extract from DIFCO Laboratories (Detroit, MI, USA), ampicillin, Tween 80, phenylmethylsulfonyl fluoride, polyethylene glycol 20,000, tri-*n*-butylphosphine, and BrCN from Wako Pure Chemical (Osaka), P-11 from Whatman BioSystems (Maidstone, Kent, UK), Sephadex G-50 (fine) from Pharmacia (Uppsala, Sweden), spinach glyoxylate reductase from Sigma (St. Louis, MO, USA), Tricine, PLP, $NaBH_4$, phenylhydrazine hydrochloride, Ponceau S, and Coomassie Brilliant Blue from Nacalai Tesque (Kyoto), Na-salicylate from Katayama Chemical Industries (Osaka), and 4-vinylpyridine from Aldrich (Milwaukee, WI, USA). NaB^3H_4 (37.0 GBq/mmol) was a product of DuPont/NEN (Wilmington, DE, USA). E-64C was kindly donated by Dr. K. Hanada, Research Labora-

tories, Taisho Pharmaceutical (Saitama). Other chemicals were of the highest grade commercially available.

RESULTS

Absorption Spectra and PLP Content—SPT_{10} is a special product from $pRspt_{10}$, a plasmid encoding a 44.4-kDa protein (*18*). The molecular weight of native SPT_{10} was estimated to be approximately 80 K by gel filtration chromatography on a Sephadex G-150 column (*18*), and upon SDS/PAGE, SPT_{10} migrated as a single band corresponding to 44 kDa (Fig. 1, inset). These data suggest that SPT_{10} is a dimer of identical subunits. SPT_{10} was purified to near homogeneity in the presence of 0.1 mM PLP and 1 mM pyruvate, as described under "MATERIALS AND METHODS," and then freed of PLP and pyruvate by Sephadex G-50 gel filtration for determination of its absorption spectra. Removal of free PLP did not affect its specific activity (Table I) and the addition of PLP to the enzyme after gel filtration did not augment the activity. Every preparation of purified SPT_{10} obtained as described under "MATERIALS AND METHODS" was nearly homogeneous, as judged from SDS/PAGE, but the specific activity varied from preparation to preparation, ranging from 3.5 to 5.3 units/mg protein. The purified enzyme after gel filtration chromatography exhibited absorption maxima at ~330 and ~420 nm, in addition to one at 278 nm at pH 7.5 (Fig. 2). The $A_{\sim330}/A_{278}$, $A_{\sim420}/A_{278}$, and $A_{\sim330}/A_{\sim420}$ ratios of the purified preparation shown in Fig. 2 were 0.093, 0.088, and 1.057, respectively, but the ratios also varied from preparation to preparation. The range of variation in the $A_{\sim330}/A_{\sim420}$ ratio observed in this study was 0.7–1.5. There was a tendency that preparations exhibiting lower absorbance at ~330 nm exhibited higher specific activity. When the pH was increased in the range of 7.5–9.5, the peak at ~420 nm gradually shifted to 390–395 nm. The peak at ~330 nm shifted to a slightly longer wavelength between pH 8 and 9. Reduction with $NaBH_4$ affected both the absorption spectra and the activity. The enzyme treated with $NaBH_4$ as described under "MATERIALS AND METHODS" was dialyzed against a large excess of 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA for measurement of the absorption spectra and enzyme activity. The reduced and dialyzed SPT_{10} was catalytically inactive, even in the presence of exogenous PLP. Spectral measurement showed that the ~420 nm peak disappeared with an increase in the absorbance at ~330 nm, suggesting that $NaBH_4$ reduced the aldimine linkage (λ_{max} ~420 nm) formed between the 4-aldehyde group of PLP and an amino group of the protein to yield an aldamine bond (λ_{max} ~330 nm) (Fig. 3A) (*29*). The addition of L-serine to the enzyme solution at pH 7.5 caused an immediate decrease in the absorbance at ~420 nm and an increase in that at ~330 nm (Fig. 3B). In contrast to the case of $NaBH_4$ reduction, the decrease in the absorbance at ~420 nm caused by L-serine was limited to about 62%.

Upon the addition of NaOH (final 0.1 N) to purified SPT_{10}, the characteristic absorption peak at 388 nm of free PLP appeared (Fig. 2). The disappearance of the ~420 nm peak indicated that all the coenzyme is released by this treatment, and the PLP content of the purified SPT_{10} was determined from the absorbance at 388 nm to be 0.59 and 0.69 mol per mol of subunit for two separate enzyme preparations. The PLP content of purified SPT_{10} was also determined after deproteinization with $HClO_4$. From the absorbance at 388 nm in 0.1 N NaOH of the $HClO_4$ extract,

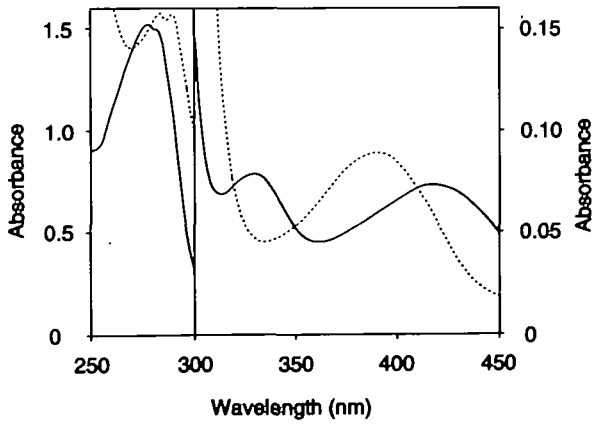

Fig. 2. **Absorption spectra of purified SPT_{10}.** Absorption spectra of purified SPT_{10} (1.83 mg/ml) were taken after removal of free PLP by Sephadex G-50 column chromatography. —, native enzyme in 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA; ·····, after addition of 1/100 volume of 10 N NaOH to a final concentration of 0.1 N.

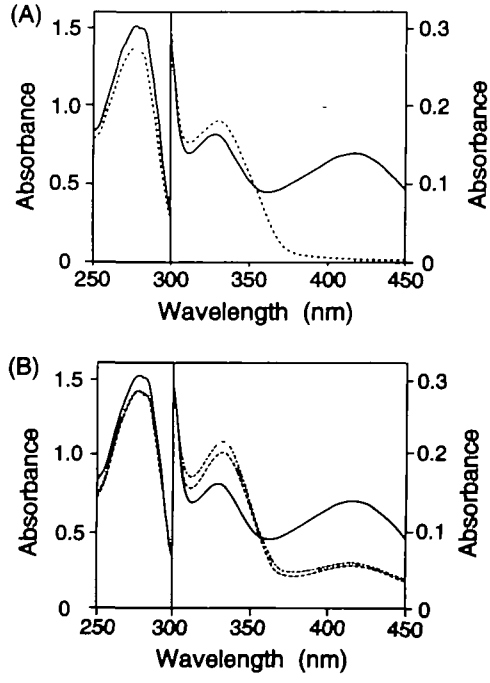

Fig. 3. **Absorption spectra of purified SPT_{10} after reduction with $NaBH_4$ (A) and in the presence of an amino donor (B).** In (A), 1.83 mg/ml of purified SPT_{10} was reduced with $NaBH_4$ as described under "MATERIALS AND METHODS" and then dialyzed overnight against 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA for measurement of absorption spectra. —, native SPT_{10}; ·····, $NaBH_4$-reduced SPT_{10} after dialysis. In (B), 100 μl of 1 M L-serine was added to 900 μl of purified SPT_{10} (1.83 mg/ml) in 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA, and absorption spectra were obtained immediately after the addition and 10 min later. —, native SPT_{10}; ·····, immediately after addition of L-serine; ——, 10 min later.

the PLP contents of three different purified preparations were determined to be 0.56, 0.59, and 0.68 mol per mol of subunit. With the same $HClO_4$ extracts, similar values (0.51, 0.64, and 0.71 mol of PLP per mol of subunit) were obtained with the phenylhydrazine method (*23*). There was again a tendency that preparations showing lower absorbance at ~330 nm had higher PLP contents.

These results altogether suggest that SPT_{10} has the capacity to bind 1 mol of the PLP cofactor per mol of 44.4 kDa subunit, but not every subunit necessarily contains the cofactor, at least through an aldimine linkage.

Resolution into the Apoenzyme and Reconstitution— Dissociation of the PLP cofactor from purified SPT_{10} was carried out by incubation with a high concentration of L-serine, followed by ultrafiltration in the presence of 0.9 M K-phosphate (pH 6.0), as described under "MATERIALS AND METHODS." The enzyme thus treated exhibited almost no activity unless PLP was added exogenously. Figure 4A shows the absorption spectra of apo-SPT_{10}. The apoenzyme exhibited an absorption peak at 325-330 nm, but no absorbance at ~420 nm at pH ~6.75. The presence of the 325-330 nm peak for apo-SPT_{10}, whose activity is almost entirely dependent on exogenously added PLP, suggests that the 325-330 nm chromophore in apo-SPT_{10} is not involved in the catalytic activity. When a solution of apo-SPT_{10} was made up to 0.1 N NaOH, no absorption peak appeared at 388 nm and the 325-330 nm peak became a shoulder. The PLP cofactor was also shown to be undetectable in apo-SPT_{10}, after deproteinization with $HClO_4$, as to both the absorbance at 388 nm and the phenylhydrazine method.

For reconstitution of holo-SPT_{10}, apo-SPT_{10} was incubated with 1 mM PLP for 60 min at 37°C, followed by removal of free PLP by Sephadex G-50 (fine) chromatography with 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA as the equilibration and elution buffer. The specific activity of the resultant reconstituted SPT_{10} was 136% of that of the original SPT_{10}. The reconstituted enzyme exhibited absorption maxima at ~330 and ~420 nm, in addition to one at 278 nm. The $A_{278} : A_{\sim 330} : A_{\sim 420}$ ratio of the reconstituted preparation was 1 : 0.074 : 0.107 (Fig. 4B), while the absorbance ratio of the original SPT_{10} was 1 : 0.095 : 0.087 (Fig. 2). It is noteworthy that the relative absorbance at ~330 nm decreased by 22% and that at ~420 nm increased by 23% with the resolution and reconstitution procedures, in seeming agreement with the increase in the specific activity. The PLP content of the reconstituted

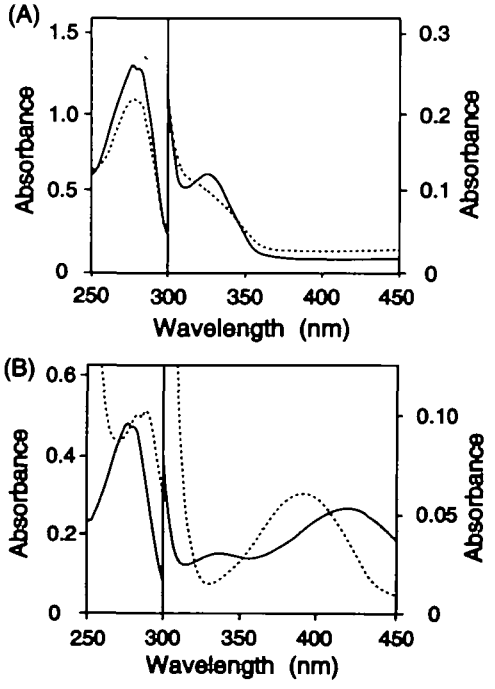

Fig. 4. **Absorption spectra of apo- (A) and reconstituted-SPT_{10} (B).** The concentrations of apo- and reconstituted-SPT_{10} were determined from the absorbance at 278 nm to be 1.67 and 0.60 mg/ml, respectively. —, absorption spectra of apo-SPT_{10} in 190 mM K-phosphate (pH ~6.75) containing 0.9 mM EDTA (A) or reconstituted SPT_{10} in 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA (B); , after addition of 1/100 volume of 10 N NaOH to a final concentration of 0.1 N.

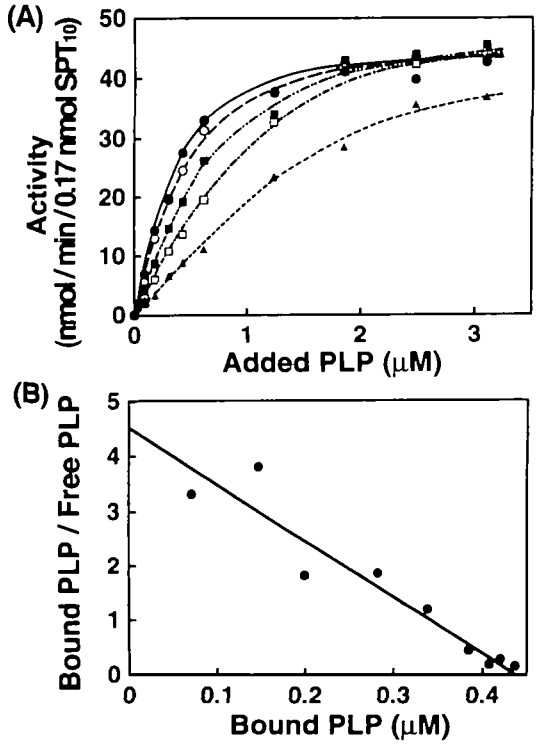

Fig. 5. **Reconstitution of holo-SPT_{10} as a function of the PLP concentration.** Apo-SPT_{10} (0.17 nmol as subunit) was incubated at 37°C for 20 min to 5 h in a mixture (0.35 ml) comprising 40 μmol of Tricine/HCl (pH 8.0), 4 μmol of Na-pyruvate, and various concentrations of PLP. Fifty microliters of 1 M L-serine (pH 8.0) was then added, and the reaction for determination of the SPT activity was carried out as described under "MATERIALS AND METHODS." In (A), reaction velocity (v) obtained is plotted against the concentration of PLP in the preincubation mixture. The preincubation was carried out for 20 min (▲), 40 min (□), 60 min (■), 3 h (○), or 5 h (●). V_{max} calculated from the double-reciprocal plot ($1/v$ *vs.* 1/[PLP]) where the concentration of PLP added exceeded that of apo-SPT_{10} subunit was 50 and 46 nmol/min after the 3-h and 5-h incubation, respectively. In (B), the Scatchard plot analysis was carried out using the data obtained from the 5-h preincubation. For this analysis, the concentration of reconstituted SPT_{10} at the respective PLP concentrations was calculated from the ratio of v to V_{max}. Then the concentration of free PLP was calculated as described in the text.

SPT_{10} was estimated to be 0.72, 0.73, and 0.79 mol per mol of subunit from the absorbance at 388 nm of the enzyme in 0.1 N NaOH and that of the $HClO_4$ extract in 0.1 N NaOH, and by the phenylhydrazine method, respectively.

For determination of the apparent K_d of the binding of PLP to apo-SPT_{10}, apo-SPT_{10} was incubated at 37°C for 20 min to 5 h with various concentrations of PLP, and then the SPT activity was determined under conditions with which the enzyme activity was strictly proportional to the concentration of the active enzyme. As shown in Fig. 5A, the conversion of apo-SPT_{10} to holo-SPT_{10} was a slow process, especially when the concentration of added PLP was low, requiring more than 5 h to reach equilibrium. Since SPT_{10} and PLP may not be entirely stable in the incubation for hours, and in fact, V_{max} obtained with a 5-h preincubation was approximately 8% lower than that with a 3-h preincubation, an apparent K_d of the PLP binding to apo-SPT_{10} was calculated from the data with a 5-h preincubation. In this calculation the V_{max} was considered to represent the activity when the binding of PLP to apo-SPT_{10} is maximum. Assuming that the ratio of enzyme activities obtained at various concentrations of PLP to V_{max} represents the proportion of existing PLP-bound enzyme, 0.22-0.28 μM PLP was found to cause half-maximum reconstitution of holo-SPT_{10}. Since low concentrations of PLP were used in this experiment, the PLP-binding to apo-SPT_{10} may have significantly affected the concentration of free PLP. Therefore, the concentrations of free and bound PLP were calculated based on the binding of 1 mol of PLP to 1 mol of SPT_{10} subunit. In this calculation, since the PLP content of reconstituted SPT_{10} was approximately 0.75 mol/mol subunit (see Fig. 4B), the concentration of apo-SPT_{10} which is potentially convertible to the holo-form was assumed to be 75% of that of apo-SPT_{10} added, and the apparent K_d of the PLP binding under the experimental conditions used was determined from the Scatchard plot to be approximately 0.1 μM (Fig. 5B).

Identification of the PLP-Binding Lys Residue—In order to determine the Lys residue to which the PLP cofactor binds, purified SPT_{10} was subjected to $NaBH_4$ reduction, followed by reductive pyridylethylation and BrCN cleavage. Lys209 of SPT_{10}, the previously predicted PLP-bind-

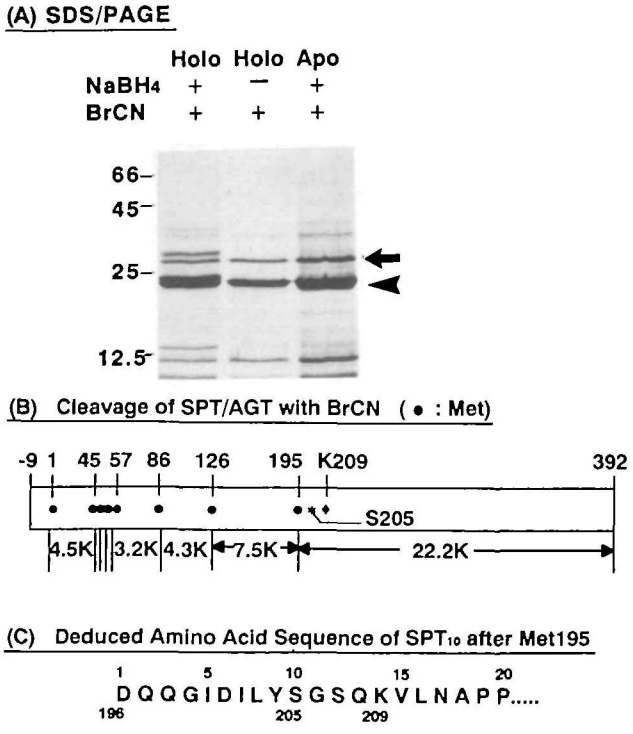

Fig. 6. **Cyanogen bromide cleavage of holo- and apo-SPT_{10}.** (A) Three milligrams each of purified SPT_{10} (Holo) and Apo-SPT_{10} (Apo) was subjected to BrCN cleavage after $NaBH_4$ reduction and pyridylethylation, as described under "MATERIALS AND METHODS." Another 3-mg aliquot of purified SPT_{10} was subjected to pyridylethylation and BrCN cleavage without the $NaBH_4$ reduction. The BrCN cleavage products corresponding to 90 μg of the starting SPT_{10} preparation were then subjected to SDS/PAGE and stained with Coomassie Brilliant Blue. The arrow and arrowhead indicate the 29-30 and 22-23 kDa pairs of stained bands, respectively. In (B), Met residues, Ser205 and Lys209 of SPT_{10} are indicated by ●, *, and ◆, respectively. The deduced amino acid sequence of SPT_{10} after Met195 is shown in (C) using the one-letter notation.

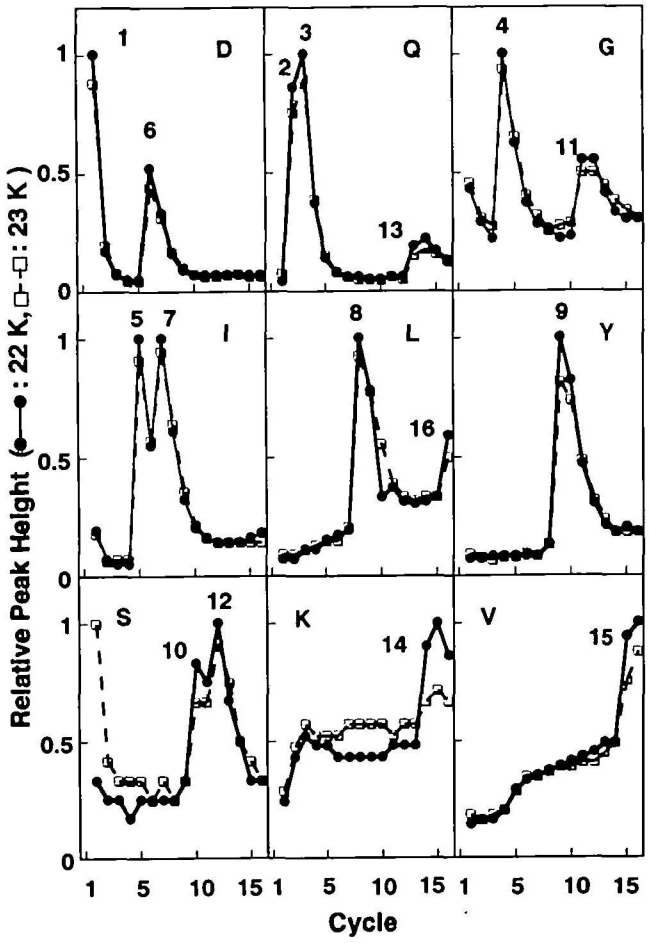

Fig. 7. **Edman degradation of the ~22 and ~23 kDa BrCN cleavage products.** The ~22 kDa (●) and ~23 kDa (□) BrCN cleavage products derived from 90 μg of purified SPT_{10} were transferred to a PVDF membrane and then subjected to automatic amino acid sequencing, as described under "MATERIALS AND METHODS." The peak heights of 9 phenylthiohydantoin amino acids recovered in each cycle are presented as relative values to the highest peak of each amino acid in ~22 kDa product, except that Ser of the ~23 kDa product is presented relative to the peak at the 12th cycle. The amino acid peaks that agree with the deduced amino acid sequence of SPT_{10} after Met195 (Fig. 6C) are numbered.

ing Lys residue (*15*), was expected to be recovered, after BrCN cleavage, in a 22.2-kDa fragment as the 14th amino acid residue (Fig. 6, B and C), and in fact ^3H from NaB^3H_4 was incorporated into a 22-23 kDa fragment in a preliminary experiment. We then noticed that the 22-23 kDa and 29-30 kDa BrCN-cleavage products gave pairs of bands on SDS/PAGE (Fig. 6A). On amino acid sequencing, the 22-23 kDa pair (~22 and ~23 kDa band materials) gave the sequence DQQGIDILYSGSQ(K)VL...., except that Lys was released only from the smaller one (~22 kDa band) in the 14th cycle corresponding to Lys209 (Fig. 7). Since this sequence was exactly the same as the deduced amino acid sequence after Met195 (Fig. 6C), it was strongly suggested that both the ~22 and ~23 kDa bands correspond to the BrCN cleavage product, Asp196-Leu392, and that only the ~23 kDa band material contains the PLP cofactor bound to Lys209. The 29-30 kDa pair (~29 and ~30 kDa band materials) both gave the same sequence as the deduced amino acid sequence after Met126, IKKPGE..., suggesting that the 29-30 kDa pair correspond to the BrCN cleavage product, Ile127-Leu392 (*cf.* Fig. 6B). The same results were obtained in an experiment in which pyridylethylation and BrCN cleavage of $NaBH_4$-reduced SPT_{10} were carried out in the presence of 7 M guanidine hydrochloride.

When apo-SPT_{10} was used instead of purified-SPT_{10} or SPT_{10} was subjected to reductive pyridylethylation and BrCN cleavage without $NaBH_4$ reduction, these pairs of bands did not appear on SDS/PAGE, the ~22 and ~29 kDa bands being the only ones detectable in the 22-23 and 29-30 kDa regions, respectively (Fig. 6A).

$NaBH_4$-reduced SPT_{10} itself also gave a pair of bands, 44-45 kDa (~44 and ~45 kDa bands), on SDS/PAGE. On the other hand, apo-SPT_{10} migrated as a single band of ~44 kDa even after $NaBH_4$ treatment (Fig. 8). When NaB^3H_4 was used for reduction of the Schiff base between the ε-amino group of Lys209 and the PLP cofactor, ^3H was only incorporated into the ~45 kDa band material of holo-SPT_{10}. The ~44 kDa band material was not labeled, irrespective of whether its origin was holo-SPT_{10} or apo-SPT_{10}. All these data suggest that each pair of bands consists of the same protein or fragments of it, but only the slower-migrating band material contains ε-phosphopyridoxyl-Lys209. The observation that the densities of the respective bands in each pair were about the same for every SPT_{10} preparation used was consistent with the PLP content of 0.56-0.69 mol per mol of subunit.

In order to exclude the possibility that the faster-migrating band of each pair without a ε-phosphopyridoxyl-Lys residue is artificially formed due to the experimental procedures used, SPT_{10}, pig heart cytosolic aspartate aminotransferase and *Bacillus stearothermophilus* D-amino acid aminotransferase were subjected to SDS/PAGE with and without prior reduction with $NaBH_4$. Since SPT_{10} was purified, after the heat treatment step, in the presence of 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA, aspartate, and D-amino acid aminotransferases were used after Sephadex G-50 chromatography with 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA as a solvent. Confirming the results in Fig. 8, SPT_{10} migrated as a pair of bands, 44-45 kDa, after $NaBH_4$ reduction, but as a single band of ~44 kDa without the $NaBH_4$ treatment (Fig. 9). In contrast, both the $NaBH_4$-treated and untreated aspartate aminotransferase gave a single band of ~45 kDa, although the $NaBH_4$-untreated enzyme migrated a little faster. Essentially the same results as those with aspartate aminotransferase were obtained with D-amino acid aminotransferase.

These data altogether suggest that Lys209 of SPT_{10} is indeed the PLP-binding residue, and that the PLP cofactor is bound through an aldimine linkage to Lys209 of about half or a little more of the subunits.

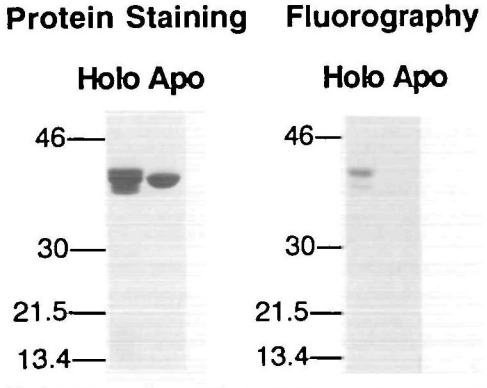

Fig. 8. **Reduction of purified SPT_{10} and apo-SPT_{10} with NaB^3H_4.** Purified SPT_{10} (Holo) and apo-SPT_{10} (Apo) (0.8 mg each in 0.5 ml of 100 mM K-phosphate containing 1 mM EDTA, pH 7.5) were separately mixed with approximately 0.1 mg of NaB^3H_4 (92.5 MBq), followed by the addition of 1.4 mg of non-labeled $NaBH_4$. The mixture was allowed to stand for 30 min at 4°C with gentle rotation, and was then dialyzed as described under "MATERIALS AND METHODS." Five microliter aliquots of the dialyzate were subjected to SDS/PAGE with Rainbow™ [^{14}C]methylated proteins as molecular weight markers. SDS/PAGE was carried out in duplicate, Coomassie Brilliant Blue staining being performed in one and indirect fluorography in the other, as described under "MATERIALS AND METHODS."

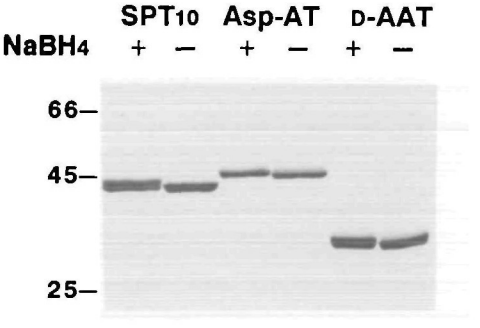

Fig. 9. **Effect of $NaBH_4$ reduction on the mobility on SDS/PAGE of aspartate and D-amino acid aminotransferases.** Pig heart cytosolic aspartate aminotransferase (Asp-AT) and *Bacillus stearothermophilus* D-amino acid aminotransferase (D-AAT) were first passed through a Sephadex G-50 column with 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA as the solvent, as described in the text. Then, SPT_{10}, Asp-AT, and D-AAT were dialyzed overnight against a large excess of 50 mM NH_4HCO_3 (pH 8.5), and diluted with the dialysis buffer to a protein concentration of 800-850 μg/ml. To 400-μl aliquots of the diluted enzymes, 3 mg of $NaBH_4$ powder was added, and after standing for 30 min at 4°C with gentle rotation, the samples were dialyzed again overnight against 50 mM NH_4HCO_3 (pH 8.5). Another 400 μl portion of the diluted enzymes was dialyzed without the $NaBH_4$ treatment. Ten microliters each of the dialyzed samples was subjected to SDS/PAGE, followed by staining with Coomassie Brilliant Blue.

DISCUSSION

It was noteworthy in this study that $NaBH_4$-reduced SPT_{10} and its 22-23 and 29-30 kDa BrCN cleavage products migrated on SDS/PAGE as a pair of bands of about the same density. Available evidence indicated that the two bands in each pair are the same protein or peptide, except that only the slower-migrating band material contains an ε-phosphopyridoxyl-Lys residue. SPT_{10} is believed to be a homodimer of 44.4 kDa subunits. Therefore, if one considers the comparable densities of the two paired bands, it may be supposed that the PLP cofactor is bound through an aldimine linkage to a Lys residue of only one of the two subunits. If this is the case, binding of 1 mol of PLP to a subunit may be considered to cause a conformational change which prevents further binding of PLP to the other subunit (half-site-binding). On investigation of the PLP content of purified SPT_{10}, however, more than 1 mol of PLP was consistently detected per mol of dimer, although the determined values varied somewhat from preparation to preparation and with the method of measurement used. This suggests that SPT_{10} has the capacity to bind 1 mol of PLP per mol of subunit, but only about half or a little more than half of the subunits binds PLP through an aldimine linkage. The PLP content of purified SPT_{10} was determined after gel filtration through a Sephadex G-50 column. Some bound PLP may have been dissociated during the gel filtration. However, the apparent K_d of the PLP-binding to apo-SPT_{10} was determined to be approximately 0.1 μM (Fig. 5B) and the binding of PLP to apo-SPT_{10} was a slow process even at 37°C, especially when the concentration of added PLP was low, requiring more than 5 h before reaching equilibrium (Fig. 5A). In fact, when a Sephadex G-50 eluate containing 41.9 μM SPT_{10} was dialyzed at 4°C for 2 d against a large excess of 50 mM NH_4HCO_3 (pH 8.5) containing 1 mM EDTA with a change of the medium after 1 d, the decrease in bound PLP was only 22% as judged from A_{388} in 0.1 N NaOH (data not shown). Since the Sephadex G-50 gel filtration took only approximately 1 h at 4°C, the dissociation of bound PLP during the process may have been minimal, if any. In accordance with this, the addition of PLP to the enzyme after the gel filtration did not appreciably augment the activity.

Purified SPT_{10} exhibits absorption maxima at ~330 and ~420 nm, besides protein absorption at 278 nm (Fig. 2). Most PLP-containing enzymes exhibit an absorption peak in the range 400-440 nm. It is generally agreed that the absorption in this region represents an internal Schiff base of PLP with the active site Lys residue. On the other hand, the occurrence of an absorption peak at around 330 nm has been reported for some PLP-containing enzymes, such as leucine aminotransferase (*30*), aromatic L-amino acid decarboxylase (*31, 32*), *Bacillus sphaericus* D-amino acid aminotransferase (*33*), *Methanobacterium* aspartate aminotransferase (*34*), and so on. Three possible structures have been proposed for the 330-nm chromophore; an enolimine tautomer of a PLP-Lys Schiff base (*29, 35, 36*), a substituted aldamine structure (*37*), and an unprotonated PLP-Lys Schiff base (*38*). In the case of SPT_{10}, the ~330 nm-absorbing species has not been identified, but it appears that the chromophore is not involved in the catalytic reaction, at least at a comparable rate with the ~420 nm-absorbing bound PLP. First, apo-SPT_{10} prepared in this study exhibited only negligible activity in the absence of added PLP and contained no measurable ~420 nm-absorbing bound PLP, but still showed an absorption peak at ~330 nm. Second, the specific enzyme activity and the absorbance at ~420 nm of the reconstituted SPT_{10} increased to 136 and 123% of those of the original SPT_{10}, respectively, whereas the absorbance at ~330 nm decreased to about 78% with the resolution and reconstitution procedure (Fig. 4B). We feel that the PLP-binding site of the subunit without PLP is occupied by the ~330 nm-absorbing compound, which binds to the enzyme without forming a Schiff base and therefore is not covalently linked to the enzyme on $NaBH_4$ reduction. SPT_{10} is a recombinant rat enzyme expressed in *E. coli*, but SPT/AGT freshly prepared from rat liver mitochondria also showed an absorption peak at ~330 nm, besides a protein peak at 278 nm and a bound PLP peak at 400-420 nm (data not shown).

We reported previously a primary hyperoxaluria type 1 case caused by a point mutation of T to C in exon 6 of the SPT/AGT gene encoding a Ser to Pro substitution at residue 205 of SPT/AGT (*13*). A remarkable feature of the SPT/AGT deficiency in this primary hyperoxaluria type 1 case was that the mutant SPT/AGT appeared to be actively synthesized in the liver, but the enzyme level was very low with respect to not only activity, but also protein detectable on Western blot analysis due, at least in part, to its ATP-dependent degradation (*39*). In the present study, Lys209 was identified as the PLP-binding residue of SPT_{10}. Since human and rat SPT/AGTs show very similar amino acid sequences, and the sequence from Gln198 to Lys209 is exactly the same (*17*), it is likely that Lys209 of human SPT/AGT may also be the PLP-binding residue. As judged from the primary structure around the PLP-binding Lys of a number of enzymes, the partial structure, -Ser-X-X-Lys-, seems to be a structural feature common to pyridoxal enzymes as a whole (*40*). When amino acid sequence comparison is restricted to class IV aminotransferases and other proteins which are related to SPT/AGT in terms of the primary structure (*5, 41*), the sequence around the Lys residue corresponding to Lys209 of SPT/AGT is -(S/T/A)-G-(S/A)-Q-K-. The residues corresponding to Ser205 and Ser207 of SPT/AGT are Ala in phosphoserine aminotransferase, and Ser or Thr in *Methanobacterium* aspartate aminotransferase and the *Synechococcus hydrogenase* small subunit. At present, it is difficult to predict the structural and functional significance of individual amino acid residues in the sequences of class IV enzymes. However, it is possible that the conformation of the active site and thus the function of SPT/AGT are seriously affected by the mutation of Ser205 to a rigid, constraint-imposing Pro in the neighborhood of the PLP-binding Lys residue. Whether the signal by which the mutant SPT/AGT is recognized as an abnormal protein to be degraded is the mutation in the neighborhood of the PLP-binding Lys itself or is due to secondary changes derived therefrom remains to be determined.

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