Molecular cloning, expression and characterization of pyridoxamine–pyruvate aminotransferase

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Pyridoxamine–pyruvate aminotransferase is a PLP (pyridoxal 5′-phosphate) (a coenzyme form of vitamin B₆)-independent aminotransferase which catalyses a reversible transamination reaction between pyridoxamine and pyruvate to form pyridoxal and L-alanine. The gene encoding the enzyme has been identified, cloned and overexpressed for the first time. The *mlr6806* gene on the chromosome of a symbiotic nitrogen-fixing bacterium, *Mesorhizobium loti*, encoded the enzyme, which consists of 393 amino acid residues. The primary sequence was identical with those of archaeal aspartate aminotransferase and rat serine–pyruvate aminotransferase, which are PLP-dependent aminotransferases. The results of fold-type analysis and the consensus amino acid residues found around the active-site lysine residue identified in the present study showed that the enzyme could be classified into class V aminotransferases of fold type I or the AT IV subfamily of the α family of the PLP-dependent enzymes.

Analyses of the absorption and CD spectra of the wild-type and point-mutated enzymes showed that Lys¹⁹⁷ was essential for the enzyme activity, and was the active-site lysine residue that corresponded to that found in the PLP-dependent aminotransferases, as had been suggested previously [Hodsdon, Kolb, Snell and Cole (1978) *Biochem. J.* **169**, 429–432]. The K_d value for pyridoxal determined by means of CD was 100-fold lower than the K_m value for it, suggesting that Schiff base formation between pyridoxal and the active-site lysine residue is partially rate determining in the catalysis of pyridoxal. The active-site structure and evolutionary aspects of the enzyme are discussed.

Key words: active-site structure, *Mesorhizobium loti*, molecular cloning, pyridoxal 5′-phosphate-independent aminotransferase, pyridoxamine–pyruvate aminotransferase (PPAT).

INTRODUCTION

PPAT (pyridoxamine–pyruvate aminotransferase) (EC 2.6.1.30), which is involved in a degradation pathway for PN (pyridoxine) [1], one of six natural vitamin B₆ compounds, is a PLP (pyridoxal 5′-phosphate)-independent aminotransferase that catalyses the transfer of an amino group between PM (pyridoxamine) and pyruvate in the forward reaction, and between PL (pyridoxal) and L-alanine in the reverse reaction. Because this reaction serves as a model for a half-reaction of the overall transamination reaction catalysed by general PLP-dependent aminotransferases, many studies have been performed on its enzymatic [2,3], kinetic [4–6] and molecular [7,8] properties. However, neither its primary structure nor the *ppat* gene encoding the enzyme has been reported. Thus the locations of amino acid residues that are essential for enzyme activity in the protein have not been identified, although a lysine residue, which forms a Schiff base with PL, and the adjacent cysteine residue have been suggested to be essential ones [8]. The cysteine residue has been pointed out to be an essential cysteine residue with a reactive thiol group that is protected from reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) by PL [9]. Kinetic studies have shown that the mechanism of the PPAT reaction is similar to that for PLP-dependent enzymes [4].

The large and diverse group of PLP-dependent enzymes involved in amino acid metabolism has been classified into four families [10] or four fold types [11] based on the amino acid sequence or secondary- and tertiary-structural identity respectively.

Although there are many PLP-dependent enzymes, there are only a few PLP-independent enzymes that catalyse the same reactions as those of PLP-dependent enzymes. Histidine decarboxylase (EC 4.1.1.22) [12], aspartate decarboxylase (EC 4.1.1.11) [13] and S-adenosylmethionine decarboxylase (EC 4.1.1.50) [14] are pyruvoyl enzymes. L-Serine dehydratase (EC 4.2.1.17) [15] has an iron–sulphur cluster. Glutamate racemase (EC 5.1.1.3) [16] and aspartate racemase (EC 5.1.1.13) [17] each have an active-site cysteine residue. These PLP-independent enzymes do not exhibit amino acid sequence identity with PLP-dependent enzymes. Only two enzymes, PPAT and pyridoxamine-5′-phosphate-2-oxoglutarate aminotransferase (EC 2.6.1.54) [18], among the large number of aminotransferases are PLP-independent. These enzymes use vitamin B₆ compounds as substrates instead of as cofactors. Thus it would be interesting to determine whether or not they show amino acid sequence identity with PLP-dependent enzymes, and to determine their structural and functional relationship to PLP-dependent aminotransferases.

In the present study, the gene encoding PPAT in *Mesorhizobium loti*, which has a metabolic pathway for PN [19], was identified and cloned. The enzyme was similar to PLP-dependent aspartate aminotransferase from *Methanobacterium thermoformicum* [20]. On comparison of the activities of the recombinant and point-mutated enzymes, it was found that Lys¹⁹⁷ was essential for the enzyme activity, but that Cys¹⁹⁸ was not. Structural and functional insights into PPAT are discussed based on the results of sequence alignment and spectroscopic studies.

Abbreviations used: LB, Luria–Bertani; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5′-phosphate; PN, pyridoxine; PPAT, pyridoxamine–pyruvate aminotransferase.

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The nucleotide sequence data have been deposited in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession number AB195265.

EXPERIMENTAL

Bacterial strains, plasmids and cultivation

M. loti MAFF303099 was obtained from the MAFF (Ministry of Agriculture, Forestry and Fisheries) DNA Bank (Tsukuba, Japan). *M. loti* was grown in a natural TY medium [21], pH 6.8, consisting of 0.5 % tryptone, 0.3 % yeast extract and 0.1 % $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. For preparation of partially purified *M. loti* PPAT, the bacterium was grown in a PM synthetic medium [19] supplemented with 0.1 % PM instead of PN. *Escherichia coli* strains JM109 and BL21(DE3) were purchased from TaKaRa Bio and Novagen respectively. *E. coli* transformant cells were cultured in LB (Luria-Bertani) medium (1 % polypeptone, 0.5 % yeast extract and 1 % NaCl) containing ampicillin (50 $\mu\text{g}/\text{ml}$). Cloning and expression vectors, pNEB205A and pET21a, were obtained from New England Biolabs and Novagen respectively.

Partial purification and N-terminal amino acid sequencing of PPAT from *M. loti*

The enzyme was partially purified from *M. loti* cells (5.0 g) grown in the PM medium. Cells suspended in 50 ml of buffer A (20 mM potassium phosphate buffer, pH 8.0, and 1 mM EDTA) containing 1 mM PMSF were sonicated on ice for 5 min (ten bursts of 30 s at 1 min intervals) with a Heatsystems Ultrasonicator W-220. A supernatant was obtained by centrifugation for 9000 g for 20 min at 4 °C. The precipitate was resuspended in 22 ml of buffer A, and the suspension was sonicated and centrifuged again. The combined supernatant solution (77 ml) was used as the crude extract. The crude extract was fractionated with $(\text{NH}_4)_2\text{SO}_4$, and the precipitate obtained upon centrifugation of the 30–70 % saturated solution was dissolved in 30 ml of buffer A. This solution, after dialysis against buffer A, was applied to a QA52 column (2.0 cm $\times$ 15 cm; Whatman) equilibrated with buffer A. The enzyme fraction eluted with buffer A containing 0.1 M KCl was subjected to heat treatment (70 °C for 10 min) in the presence of 10 mM PN. After centrifugation at 9000 g for 20 min at 4 °C, the supernatant solution was applied to a butyl-Toyopearl column (0.6 cm $\times$ 1.5 cm; Tosoh) equilibrated with buffer A containing 3 M KCl. The enzyme fraction eluted with the buffer containing 2 M KCl was used as the partially purified enzyme preparation. The partially purified enzyme was subjected to SDS/PAGE (10 % gel), and the protein component on the SDS/polyacrylamide gel exhibiting a subunit molecular mass corresponding to that of *Pseudomonas* PPAT [7] was transferred on to a PVDF membrane using a Trans-blot cell system (Bio-Rad). The transferred enzyme was subjected to N-terminal amino acid sequencing with an Applied Biosystems 492 protein sequencer.

Cloning and expression of the *mlr6806* (PPAT) gene

The *mlr6806* gene was amplified by PCR with chromosomal DNA of *M. loti* MAFF303099 prepared with an Aquapure genomic DNA isolation kit (Bio-Rad). The reaction mixture (50 μl) for PCR consisted of GC buffer I, 0.2 mM dNTPs, 2.5 mM MgCl_2 , template DNA, 20 pmol of each primer and 1.25 units of LA Taq polymerase (TaKaRa Bio). The primers contained deoxyuridine (U), 5'-GGAGACAUGGATCCGAGCTGATGT-ACTCGCAGCACAT-3' (F1), which corresponds to the sequence from positions -91 to -69, counting A of the start codon (ATG) of the *mlr6806* gene as +1 (the underlining indicates a BamHI site), and 5'-GGGAAAGUTCAGGCGTCGCGTCA-3' (R1), which corresponds to a complementary strand comprising positions +1163 to +1179 (the bold letters indicate a termination codon). The PCR conditions were as follows: heating to 94 °C for

5 min; 30 cycles of 94 °C for 1 min, 57.5 °C for 1 min and 72 °C for 2 min, followed by a final extension for 5 min. The amplified DNA fragment was ligated into the linearized pNEB205A vector with a USER (Uracil-Specific Excision Reagent) friendly cloning kit (New England Biolabs). The constructed plasmid, pNEB6806, was introduced into and extracted from *E. coli* JM109 cells. After the sequence of the introduced fragment had been verified by DNA sequencing with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), pNEB6806 was digested with BamHI and EcoRI, and then the digested DNA fragment was inserted into the BamHI/EcoRI sites of pET21a. The EcoRI site existed in the pNEB205A vector. The constructed plasmid, designated as pET6806, was introduced into *E. coli* BL21(DE3) cells.

The transformed cells were aerobically grown in 5 ml of LB medium containing ampicillin (50 $\mu\text{g}/\text{ml}$) at 30 °C until the D_{600} reached 0.8. The broth was then added to 200 ml of the same medium, and the inoculated cells were grown at 23 °C for 24 h. The cells were harvested by centrifugation at 9000 g for 20 min at 4 °C, and were then washed with 0.9 % NaCl.

Site-directed mutagenesis of PPAT

The genes encoding K197L and C198A mutant enzymes were prepared using the two-step PCR method. The first PCR for K197L was performed with pNEB6806 as a template and the combination of primers 5'-GCCCCAACGCATGCTGG-3' (sense) and R1, or that of primers 5'-CCAGGCATGCGTTGGGGC-3' (antisense) and F1. The bold letters indicate the mismatched site. The second PCR step was performed with the two amplified DNA fragments as the templates, and primers F1 and R1. The first PCR for C198A was performed with pNEB6806 as a template and the combination of primers 5'-CCAACAAAGCCCTGGGCG-3' (sense) and R1, or that of primers 5'-CGCCCAGGGCTTTGTTGG-3' (antisense) and F1. DNA sequencing confirmed that the desired mutation was present and that no other mutations were present.

Purification of recombinant and mutant enzymes

Step 1: harvested cells (4.5 g obtained from 800 ml of the above-mentioned culture broth) were suspended in buffer A containing 1 mM PMSF. The cell suspension was sonicated on ice for 5 min (ten bursts of 30 s at 1 min intervals) at 4–15 °C. The supernatant (26 ml) obtained by centrifugation at 9000 g for 20 min at 4 °C was used as the crude extract. Step 2: 10 mM PN was added to the crude extract, and then the mixture was heat-treated at 70 °C for 10 min. The solution was cooled immediately on ice and then centrifuged at 9000 g for 20 min at 4 °C to obtain a supernatant. Step 3: 3.5 M KCl was added to the supernatant (25 ml), and then the mixture was applied to a butyl-Toyopearl column (1.7 cm $\times$ 9.0 cm) equilibrated with buffer A containing 3.5 M KCl. The column was washed with buffer A containing 3.5 M KCl until the A_{280} of the eluate reached less than 0.1. Then, the column was eluted with a linear gradient of KCl (3.5–0 M). Step 4: the eluted enzyme solution (44 ml) was dialysed thoroughly against buffer A. The dialysed solution (52 ml) was applied to a QA52 column (1.7 cm $\times$ 8.5 cm) equilibrated with buffer A. The column was washed with buffer A as described above. Then, the column was eluted with a linear gradient of KCl (0–0.3 M). Step 5: the eluted enzyme solution (33 ml) was concentrated by 70 % saturated $(\text{NH}_4)_2\text{SO}_4$ precipitation, and the concentrated solution was applied to a Sephacryl S-200 High Resolution column (2.3 cm $\times$ 84 cm; Amersham Biosciences) equilibrated with buffer A containing 0.1 M KCl.

The mutant enzymes were purified in essentially the same way as for purification of the recombinant enzyme as described above.

Because the K197L enzyme showed no activity, SDS/PAGE (10% gel) was performed to identify fractions that contained it during the purification steps.

Enzyme and protein assays

PPAT activity was assayed during its purification by the phenylhydrazine method [22] as follows. The reaction mixture (0.4 ml) consisted of 0.1 M borate/KOH, pH 9.0, 3.33 mM sodium pyruvate, 3.33 mM PM and the enzyme. The reaction was performed at 30°C for 10–30 min and was stopped by the addition of 66 µl of 9 M H₂SO₄. The initial velocity of the enzyme reaction was determined by measuring the initial increase or decrease in A₃₉₁ due to PL produced or consumed at 30°C in 1 ml of a reaction mixture consisting of 0.1 M borate/KOH, pH 9.0. Kinetic parameters were determined using curve-fitting software (KaleidaGraph) as described previously [23]. One unit of enzyme was defined as the amount that catalysed the formation of 1 µmol of PL per min.

Protein was measured by the dye-binding method with BSA as the standard [24]. The protein concentration of the purified enzyme was determined from the molar absorption coefficient ($\epsilon_{280} = 52420 \text{ cm}^{-1} \cdot \text{M}^{-1}$ in subunit) determined based on the amino acid composition [25].

The effects of PLP, PMP (pyridoxamine 5'-phosphate) and various inhibitors of PLP-dependent enzymes were determined as follows. The enzyme solution in 0.1 ml of buffer A was incubated with or without (control) the compound in question at 30°C for 30 min, and then the incubated solution was applied twice to a Sephadex G25 spin column (Amersham Biosciences) to remove the compound. Then, the activity of the eluted enzyme was measured.

Other analytical methods

Absorption spectra were measured at 25°C with a Biospec-C1600 spectrophotometer (Shimadzu). CD spectra were measured at 25°C with a Jasco J-720 spectropolarimeter. The enzyme solutions were dialysed thoroughly against buffer A. The concentrations of enzymes, substrates and sodium borohydride used for these experiments are shown in the Figure legends.

The DDBJ, EMBL, GenBank® and GSDS Nucleotide and Protein sequence databases were searched for proteins that shared identity with PPAT by use of the BLAST algorithm. Multiple sequence alignment was carried out using ClustalX (version 1.83).

The molecular mass of the recombinant enzyme was determined by size-exclusion chromatography (Sephacryl S-200 high-resolution column, 2.3 cm × 84 cm). A calibration curve was created based on the elution pattern of catalase (240 kDa) (Amersham Biosciences), lactate dehydrogenase (142 kDa) (Oriental Yeast), alanine aminotransferase (110 kDa) (Boehringer-Mannheim), PN 4-oxidase (53 kDa) purified as described previously [26] and cytochrome *c* (12.4 kDa) (Sigma). The subunit molecular mass and purity of the enzyme were determined by SDS/PAGE (10% gel) [27] with the molecular-mass markers described previously [23].

PL was analysed by reversed-phase isocratic HPLC by means of the method described previously [28]. L-Alanine was determined by reversed-phase HPLC on a Nova-Pak C₁₈ column [3.9 mm × 300 mm, 60 Å (1 Å = 0.1 nm), 4 µm; Waters] using fluorescence detection (λ_{ex} , 344 nm; λ_{em} , 433 nm). The reaction product was derivatized at room temperature (25°C) for 2 min with 0.28 M borate/NaOH, pH 9.0, containing 0.2% *o*-phthalaldehyde and 0.2% *N*- α -t-butyloxycarbonyl-L-cysteine. The sample was applied and eluted at 40°C with a linear gradient

Figure 1 SDS/PAGE patterns of the partially purified enzyme from *M. loti* and preparations obtained at individual purification steps from recombinant *E. coli*

The partially purified enzyme (0.06 µg) was applied to lane PP. Lane C, the cell extract of BL21(DE3)/pET6806 (10 µg); lane H, the preparation after heat treatment (5 µg); lane B, butyl-Toyopearl (3 µg); lane Q, QA52 (2 µg); lane S, Sephacryl S-200 (2 µg). The K197L and C198A mutant enzymes (2 µg each) were also analysed. The standard proteins were applied to lane SP. SDS/PAGE was performed on a 10% gel. Molecular-mass sizes are given in kDa.

of 7–47% acetonitrile plus 3% tetrahydrofuran in 0.1 M acetate/NaOH buffer, pH 6.0, at a flow rate of 0.7 ml/min.

RESULTS

PPAT activity in *M. loti* cells, and partial purification and N-terminal amino acid sequence of PPAT

M. loti MAFF303099 grown in the TY natural medium showed very low (1.19 ± 0.22 m-units/mg) enzyme activity. When *M. loti* was grown in PM and PN synthetic media, the specific activity increased by approx. 18-fold (21.0 ± 2.6 and 21.6 ± 1.5 m-units/mg respectively). Although the enzyme may not be necessary for the utilization of PN, the induction of the enzyme by PN has been observed in *Pseudomonas* MA-1 [2]. We have found that the *ppat* gene (*mlr6806*) is located among the genes that encode the enzymes involved in the PN-degradation pathway on the chromosome of *M. loti* (T. Yagi, N. Yokochi, Y. Yoshikane, B. Yuan, J. Funami, M. Tadokoro, F. Ge, unpublished work). Thus PN and PM, and some of their metabolites, may directly or indirectly induce the expression of all of the genes that form the gene cluster for the degradation of PN. The partially purified enzyme preparation gave a main protein band corresponding to a molecular mass of 41 kDa, which is similar to that of the *Pseudomonas* enzyme (38 kDa) [7], and several minor bands on an SDS/PAGE gel (Figure 1). The N-terminal amino acid sequence of the main protein was Met-Arg-Tyr-Pro-Xaa-Xaa-Ala.

Identification, cloning and expression of the *ppat* gene

A search of the chromosomal genes of *M. loti* for the internal sequence [Ala-Asp-Ile-Tyr-Val-Thr-Gly-Pro-Asx-Lys-Cys-Leu(Pro₂, Gly₂, Ala₂, Met)(Thr, Leu₂)Gly-Val-Ser-Glu-Arg] reported by Hodsdon et al. [8] showed that it is identical with the internal amino acid sequence of a protein assigned as a deduced aspartate aminotransferase encoded by the *mlr6806* gene. We have found that the N-terminal amino acid sequence (Met-Arg-Tyr-Pro) encoded by the *mlr6806* gene was the same as that of PPAT determined as described above. Thus the *mlr6806* gene was cloned into the pET vector and an expression plasmid, pET6806, was prepared. *E. coli* BL21(DE3) cells transformed with the

Figure 2 Nucleotide sequence, deduced amino acid sequence, part of the deduced secondary structure, and active site and consensus amino acid residues

The secondary structure was deduced by the method of Ito et al. [29]. The active-site lysine residue is shown as a large letter. The consensus amino acid residues around the active-site lysine residue are shown as underlined letters. α -Helix and β -sheet structures are shown as cylinders and arrows respectively.

expression plasmid showed very high PPAT activity (410 m-units/mg) compared with cells transformed with the pET vector as a control, which showed specific activity of 0.18 ± 0.03 m-unit/mg. The crude extract of BL21(DE3)/pET6806 cells gave a dense protein band corresponding to a molecular mass of 41 kDa (Figure 1). The N-terminal amino acid sequence of the protein in this dense band determined with the protein sequencer was Met-Arg-Tyr-Pro-Glu-His-Ala-Asp-Pro-Val, which was identical with that deduced from the nucleotide sequence of *mlr6806*. Thus the *mlr6806* gene was identified as the *ppat* gene. The nucleotide sequence of the *ppat* gene and the deduced amino acid sequence of PPAT are shown in Figure 2. The enzyme consisted of 393 amino acid residues encoded by 1179 nucleotides. Its molecular mass was 41 589 Da.

Purification and some properties of recombinant and mutant enzymes

The recombinant enzyme was purified to homogeneity from BL21(DE3)/pET6806 cells through four steps, i.e. heat treatment and three column chromatography steps (see Table S1 at <http://www.BiochemJ.org/bj/396/bj3960499add.htm>). The purified enzyme showed specific activity of 6.2 and 10.5 units/mg with the phenylhydrazine method and the initial velocity measurement assay respectively. The purified enzyme gave a single band corresponding to a molecular mass of 41 ± 1 kDa on SDS/PAGE (Figure 1). This molecular mass was in good agreement with that predicted from the deduced amino acid sequence. The molecular mass of the native enzyme was found to be 154 ± 7.2 kDa on size-exclusion chromatography. These results showed that the enzyme is a homotetrameric protein. The optimum pH estimated based on the K_m and k_{cat} values was pH 9.5 (see Figure S1 at <http://www.BiochemJ.org/bj/396/bj3960499add.htm>). The optimum temperature for the enzyme activity was 70 °C when 100 mM borate/KOH buffer, pH 9.0, was used; 17 and 3 % of the maximal activity

Table 1 Kinetic parameters of PPAT

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
PM	0.044 ± 0.004	28 ± 0.5	640 000
PL	0.059 ± 0.005	41 ± 0.8	690 000
Pyruvate	0.34 ± 0.02	29 ± 0.6	85 000
2-Oxobutyrate	7.1 ± 0.1	24 ± 0.2	3400
L-Alanine	11 ± 0.6	41 ± 0.7	3700
(S)-2-Aminobutyrate	20 ± 0.9	9.0 ± 0.2	450

was observed at 30 °C and 80 °C respectively (see Figure S2 at <http://www.BiochemJ.org/bj/396/bj3960499add.htm>). The activation energy calculated from an Arrhenius plot was 10.2 ± 0.1 kcal/mol (1 kcal = 4.184 kJ).

The reaction product derived from PM was co-eluted with standard PL on isocratic reversed-phase HPLC and thus was identified as PL (see Figure S3 at <http://www.BiochemJ.org/bj/396/bj3960499add.htm>). The reaction product derived from pyruvate was co-eluted with L-alanine upon HPLC and was thus identified as L-alanine (see Figure S4 at <http://www.BiochemJ.org/bj/396/bj3960499add.htm>). The reaction products, PL and L-alanine, were produced in equimolar amounts. PL and L-alanine were substrates in the reverse reaction.

PLP and PMP (each 10 μ M) did not enhance or reduce the enzyme activity. Hydroxylamine (10 mM), phenylhydrazine (1 mM) and sodium borohydride (1 mM), typical inhibitors of PLP-dependent enzymes, did not inactivate the enzyme at all.

Table 1 shows the substrate specificity of the enzyme with PM as the amino donor. The K_m and k_{cat} values for PM were determined with pyruvate as the amino acceptor, which was the best amino acceptor. 2-Oxobutyrate was also used as a substrate. The enzyme showed <0.5 % of the activity of pyruvate towards

2-oxoglutarate, 3-hydroxypyruvate, indol-pyruvate, phenylpyruvate, 3-methyl-2-oxobutyrates and 4-hydroxyphenylpyruvate when they were used at 10 mM. The enzyme showed <0.5 % of the activity of PM towards PMP (3.3 mM) with pyruvate as the amino acceptor.

Table 1 also shows the amino donor specificity of the enzyme with PL as the amino acceptor. The K_m and k_{cat} values for PL were determined with L-alanine as the amino donor, which was the best amino donor. (S)-2-Aminobutyrate was a poor substrate. The enzyme showed <0.5 % of the activity of L-alanine towards D-alanine, 3-aminopropionate, L-valine, L-leucine, L-isoleucine, L-methionine, L-tryptophan, L-phenylalanine, glycine, L-serine, L-threonine, L-cysteine (5 mM), L-tyrosine (1 mM), L-asparagine, L-glutamine, L-lysine, L-histidine, L-arginine, L-aspartate, L-glutamate, L-ornithine and (R)-2-aminobutyrate (each 20 mM). The enzyme showed <0.5 % of the activity of PL toward PLP (0.6 mM) with L-alanine as the amino donor.

The mutant enzymes were purified to homogeneity, as shown in Figure 1. The K197L enzyme showed no activity, but the C198A enzyme showed the same specific activity as that of the wild-type enzyme. The mutant enzymes were each eluted as a single peak when they were subjected to size-exclusion chromatography, and they showed the same native molecular mass corresponding to a tetrameric form as that of the native enzyme. The CD spectra of the mutant enzymes are shown in Figure S5 (<http://www.BiochemJ.org/bj396/bj3960499add.htm>), together with that of the wild-type enzyme. The CD spectra of the K197L and C198A enzymes were the same as that of the wild-type enzyme, showing that the mutated enzymes kept the same conformation as the wild-type one. The α -helix contents of the enzymes were estimated to be 31.6 % [30].

Spectrophotometric analysis and reactions with PL

The wild-type and mutant enzymes each exhibited a single absorption maximum at 280 nm when their absorption was monitored from 250 to 500 nm (Figure 3), showing the absence of chromophores such as PL and/or PM in the enzyme. The addition of PL resulted in the appearance of a new absorption maximum at 412 nm for the wild-type and C198A enzymes, indicating the formation of a Schiff base. The enzymes also showed a shoulder peak at 320 nm. The K197L enzyme did not show the absorption peak at 412 nm, but did show one at 320 nm. Because free PL showed an absorption peak at 316 nm under the same conditions, the absorption at 320 nm may be attributable to PL bound to an active site of the enzyme proteins. Reduction of the enzymes with a Schiff base by sodium borohydride shifted the absorption maximum from 412 nm to 330 nm. The absorption was not lost when the reduced enzyme had been dialysed thoroughly (Figure 3), showing that the pyridoxyl residue was fixed on the enzyme protein. On the contrary, PL exhibiting absorption at 320 nm in the K197L enzyme was not fixed upon sodium borohydride reduction, and on dialysis it was lost. These results show that Lys¹⁹⁷ is the active-site lysine residue and can form a Schiff base with PL. In contrast, the thiol group of Cys¹⁹⁸ is not required for the interaction of PL with the active-site lysine residue.

Spectrophotometric titration of the wild-type enzyme, based on the A_{412} , showed that it bound 4 mol of PL per mol of enzyme. Thus each subunit binds one PL, as does the *Pseudomonas* enzyme [5].

Figure 4 shows CD spectra of PPAT in the absence and presence of various concentrations of PL and PM. PPAT itself showed no Cotton effect between 300 and 500 nm. The addition of PL or PM, which are achiral compounds and do not exhibit CD, showed positive Cotton effects at approx. 415 nm (PL) and 330 nm (PM).

Figure 3 Effects of PL and reduction on absorption spectra of the recombinant wild-type and mutated PPAT

The absorption spectra of the enzymes (each 5 μ M) in buffer A were monitored from 250 to 500 nm. To the enzyme solution (—), 20 μ M PL was added (---). Then, 1 mM sodium borohydride was added (.....). The reduced enzyme was dialysed thoroughly against buffer A and then the absorbance spectrum was measured (-.-.-.-.-).

This indicates that the bound substrates are in an asymmetric environment. The torsion angle around the C4-C4' bond, which is part of the π -electron conjugate system, of the PL-Lys¹⁹⁷ Schiff base may contribute to the large Cotton effect observed for the binding of PL [31].

As the CD analyses specifically indicates the specific binding of PL and PM to PPAT, the K_d values of PL and PM were then determined by fitting the following equation to the data, the equation being derived from the definition of the dissociation constant:

$$\Delta\epsilon = \frac{\Delta\epsilon_{\max}}{2[E]_0} \left\{ [E]_0 + [S]_0 + K_d - \sqrt{([E]_0 + [S]_0 + K_d)^2 - 4[E]_0[S]_0} \right\} \quad (1)$$

where $[E]_0$ and $[S]_0$ represent the total enzyme and substrate (PL or PM) concentrations. The K_d values were determined to be $0.77 \pm 0.25 \mu$ M for PL and $31 \pm 10 \mu$ M for PM. The value for

Figure 4 CD spectra of PPAT in the presence of various concentrations of PL and PM

CD spectra of the enzyme (10 μ M in subunit) were determined in buffer A at 25 °C. **(A)** Spectra in the presence of 0, 5, 10, 20, 40 and 60 μ M PL. The inset shows the concentration-dependency of the CD spectra at 415 nm. The CD values obtained at 414–416 nm with 0.2 nm intervals were averaged. The curve shows the theoretical line fitted using eqn 1. **(B)** Spectra in the presence of 0, 2, 5, 10, 50 and 100 μ M PM. The inset shows the concentration-dependency of the CD spectra at 335 nm in the same way as in **(A)**.

PM was similar to the K_m value for PM, but the value for PL was 100-fold lower than the K_m value for PL.

Sequence comparison

M. loti PPAT showed the highest identity (59 %) with a probable L-serine–pyruvate aminotransferase/archaeal aspartate aminotransferase in *Rhodospirillum rubrum*. Aspartate aminotransferase from *Methanobacterium thermoformicicum* [20] and rat serine–pyruvate aminotransferase [32] showed 30 % and 25 % identity respectively. Lys¹⁹⁷ in the *M. loti* enzyme was the active-site lysine residue corresponding to that in the general aminotransferases that forms a Schiff base with PLP. The *M. loti* enzyme has been deduced to have β -sheets in front of and behind the active-site lysine residue, as shown in Figure 2, indicating that the enzyme belongs to fold type I. Furthermore, the *M. loti* enzyme has consensus amino acid residues around the active-site lysine residue found in aminotransferase class V (PROSITE). The results showed that the *M. loti* PPAT could be classified as a fold type I aminotransferase class V protein, although it does not use PLP as a coenzyme.

In order to obtain structural and functional insights into *M. loti* PPAT, the primary structure of *M. loti* PPAT was compared with those of L-serine–pyruvate aminotransferase/archaeal aspartate aminotransferase of *Rhodospirillum rubrum*, *Methanobacterium thermoformicicum* aspartate aminotransferase, rat serine–pyruvate aminotransferase and human alanine:glyoxylate aminotransferase, all of which exhibit high sequence identity with *M. loti* PPAT and phosphoserine aminotransferases from *E. coli* and *Bacillus alcalophilus*, which are the only type I class V aminotransferases whose three-dimensional structures are known so far. The multiple sequence alignment carried out using ClustalX (version 1.83) is shown in Figure 5(A). A molecular phylogenetic tree was drawn using the neighbour-joining method (Figure 5B). The tree clearly shows that the probable serine–pyruvate aminotransferase/archaeal aspartate aminotransferase of *Rhodospirillum rubrum* is closest to, the *Methanobacterium thermoformicicum* aspartate aminotransferase, rat serine–pyruvate aminotransferase and human alanine:glyoxylate aminotransferase are next closest to, and the two phosphoserine aminotransferases are farthest from *M. loti* PPAT. The sequence alignment (Figure 6A) also reflects the above relationship; the two phosphoserine aminotransferases show significant insertions

in the active-site regions. However, several residues are strongly conserved among the six enzymes (see below).

DISCUSSION

Comparison of *M. loti* and *Pseudomonas* enzymes

The properties of PPAT from *M. loti* and *Pseudomonas* MA-1 are compared in Table 2. Both are homotetrameric enzymes and share the same optimum pH. The *M. loti* enzyme showed larger K_m values for PM, PL and L-alanine, and a similar one for pyruvate. Both enzymes are specific for the L-enantiomer. Although the *Pseudomonas* enzyme has been reported to show activity towards PMP and PLP [3], the *M. loti* enzyme did not show any activity towards them. The *M. loti* enzyme showed activity towards 2-oxobutyrate as well as pyruvate, like the *Pseudomonas* enzyme [3]. The *M. loti* enzyme showed activity towards (S)-2-aminobutyrate, the corresponding amino acid of 2-oxobutyrate.

Sequence identity with PLP-dependent aminotransferases and spectrophotometric properties of PPAT–substrate complexes

Although PPAT is a PLP-independent enzyme, its primary structure is identical with those of enzymes of fold type I, aminotransferases class V [11] or α family, AT IV [10] in the superfamily of PLP-dependent enzymes. This is the first known case of a PLP-independent enzyme showing high sequence identity with a PLP-dependent one with the same reaction specificity. Glutamate racemase shows significant sequence identity with mammalian myoglobins [16], but not with PLP-dependent racemases. L-Serine dehydratase from *Peptostreptococcus asaccharolyticus* was characterized as an iron–sulphur protein and showed no consensus sequence with PLP-dependent enzymes [15]. Two types of histidine decarboxylases are known. The PLP-dependent one belongs to α family DCII (decarboxylase II) [33]. However, the PLP-independent pyruvoyl-dependent one shows no similarity to any PLP-dependent enzyme [13]. When substrate PL is bound to PPAT, the enzyme–substrate complex gave an absorption band at 412 nm, which is ascribed to the protonated Schiff base between PL and the active-site Lys¹⁹⁷. That the Schiff base is protonated at pH 8.0 indicates that the PL–Lys¹⁹⁷ Schiff base has a high pK_a value. This is in accordance with the finding described above that PPAT is a subclass V

Figure 5 Multiple sequence alignment and phylogenetic tree of class V aminotransferases

(A) Multiple sequence alignment of the class V aminotransferases using ClustalX (version 1.83). The parameters used were as follows: gap opening, 5.00; gap extension, 1.00; delay divergent sequences, 30%; no negative matrix, and Gonnet series protein mass matrix. MloPPAT, *Mesorhizobium loti* PPAT; RruSPT, *Rhodospirillum rubrum* probable serine-pyruvate aminotransferase; RatSPT, rat serine-pyruvate aminotransferase; HumAGT, human alanine:glyoxylate aminotransferase; BalPSAT, *Bacillus alcalophilus* phosphoserine aminotransferase; EcoPSAT, *Escherichia coli* phosphoserine aminotransferase; MthAAT, *Methanobacterium thermoformicum* aspartate aminotransferase. Asterisks (*) indicate the positions of single fully conserved residues; : and . indicate that one of the 'strong' groups (NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY and FYW) and 'weaker' groups (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM and HFY) respectively is fully conserved. The bars under the amino acid sequences indicate the strength of consensus at the corresponding positions. The residues important for catalysis and substrate recognition are: phenylalanine, tyrosine or tryptophan at position 109 on the ruler (Trp¹⁰⁸ of human alanine:glyoxylate aminotransferase), aspartate at position 186 (Asp¹⁸³ of human alanine:glyoxylate aminotransferase), lysine at position 212 (Lys²⁰⁹ of human alanine:glyoxylate aminotransferase) and arginine at position 365 (Arg³⁶⁰ of human alanine:glyoxylate aminotransferase). (B) Phylogenetic tree of the class V aminotransferases drawn using the bootstrap neighbour-joining method of ClustalX (version 1.83). The numbers indicate the branch length. The abbreviations of the enzyme names are the same as in (A).

aminotransferase, which has a high Schiff base pK_a value (>9) compared with a subclass I aminotransferase having a relatively low Schiff base pK_a value (6–7).

The molar CD value, $16 \text{ M}^{-1} \cdot \text{cm}^{-1}$, of the PPAT–PL complex is similar to the molar CD value at 430 nm ($19 \text{ M}^{-1} \cdot \text{cm}^{-1}$) of the acidic form of *E. coli* aspartate aminotransferase, a fold type I aminotransferase subclass Ia enzyme. This suggests that the environment of PL at the active site of PPAT is similar to that around the PLP of fold type I aminotransferase, as discussed below.

Insights into the active-site structure

Sequence alignment of the class V aminotransferases (Figure 5A) showed that the residues that have been shown to

interact with PLP, based on the crystallography of *E. coli* phosphoserine aminotransferase [35] and human alanine:glyoxylate aminotransferase [36], are conserved among the seven aminotransferases. Besides Lys²⁰⁹ (numbering of human alanine:glyoxylate aminotransferase), which forms a Schiff base with the aldehyde group of PLP, Asp¹⁸³ forms a hydrogen bond/salt bridge with the pyridine nitrogen of PLP (Figure 6). These residues are also conserved in other aminotransferases shown in Figure 5(A). The aromatic ring is on the *re* face of the pyridine ring of PLP.

In spite of these similarities, there should be clear differences between the active-site structures of *M. loti* PPAT and other 'amino acid' aminotransferases with respect to the recognition of the phosphate group of PLP; PPAT binds to PL, but not to PLP. In human alanine:glyoxylate aminotransferase, the phosphate group of PLP forms hydrogen bonds with the main-chain amide nitrogen

Figure 6 Active-site structure of human alanine:glyoxylate aminotransferase

The structure was drawn using the crystal structure of human alanine:glyoxylate aminotransferase in the unliganded form (PDB code 1H0C), with specific reference to the residues corresponding to those involved in substrate recognition by *M. luti* PPAT. Broken lines indicate the hydrogen-bond interactions of the phosphate group of PLP with the main-chain nitrogen atoms of Gly⁸² and His⁸³, and the side chain of Ser⁸¹.

Table 2 Comparison of the properties of PPATs from *M. luti* and *Pseudomonas* MA-1

Data for *Pseudomonas* MA-1 are taken from the indicated references.

Property	PPAT	
	<i>M. luti</i>	<i>Pseudomonas</i> MA-1
Molecular mass (Da)		
Native	154 000	148 000 [7]
Subunit	41 000	38 000 [7]
Number of subunits	4	4 [7]
Optimum pH	9.5	9.1 [3]
Substrate [K_m (mM) or activity]		
PM	0.044	0.013 [4]
PL	0.059	0.012 [4]
Pyruvate	0.34	0.35 [4]
L-Alanine	11	1.6 [4]
PMP	No	Yes [3]
PLP	No	Yes [3]

atoms of Gly⁸² and His⁸³, and the side-chain hydroxy group of Ser⁸¹. The side chain at position 82 is not bulky in the aminotransferases, except in *M. luti* PPAT, and thus does not interfere with the binding of the phosphate group. The corresponding residue in *M. luti* is proline. Therefore the lone pair electrons of the main-chain amide nitrogen of the position 82 residue are occupied by the side-chain carbon atom of proline, and cannot be used for the recognition of the phosphate group. This can explain why PLP does not bind to *M. luti* PPAT.

With regard to the recognition of amino/keto acids, it is noteworthy that *M. luti* PPAT has Arg³⁴⁵ corresponding to Arg³⁶⁰ of alanine:glyoxylate aminotransferase, which is conserved among all class V aminotransferases and is known to bind to the α -carboxy group of the substrate amino/keto acids. Therefore we can expect that alanine/pyruvate bind to the active site through their α -carboxy groups, forming a hydrogen bond/salt bridge to

Arg³⁴⁵ and the α -amino/keto groups forming Schiff bases with PL/PM, and the transamination proceeds in the same way as for other aminotransferases with the catalytic assistance of Lys¹⁹⁷ and Asp¹⁷¹, and the electron-withdrawing ability of the pyridine ring of PL/PM.

Catalytic reaction of PPAT in comparison with those of PLP-dependent aminotransferases

The fact that PL and PM are not only catalysts but also substrates for PPAT makes the catalytic reaction different from those of PLP-dependent aminotransferases. In PLP-dependent aminotransferases, PLP forms a Schiff base with the active-site lysine. The protonated imine bond of the Schiff base is reactive, and thus PLP can react with the incoming substrate amino acid. The catalytic reaction of PPAT, on the other hand, must include the step of Schiff base formation from the aldehyde and the amino group, which is slower than the Schiff base exchange reaction (transimination). This is reflected in the K_m and K_d values for PL and PM. For PM, the K_m and K_d values are essentially identical. This indicates that the PPAT–PM complex, as observed by means of CD, is the principal species (i.e. the Michaelis complex in the Michaelis–Menten reaction) that exists before the rate-determining catalytic reaction. On the other hand, the K_d value for PL is approx. 100-fold lower than the K_m value for PL. This suggests that the formation of the PL–Lys¹⁹⁷ Schiff base is partially rate-determining in the catalytic cycle. That is, PL first associates weakly with PPAT, the aldehyde group of PL being in either the free aldehyde or hemiacetal form and does not form a covalent bond with Lys¹⁹⁷. Then PL forms a Schiff base with the ϵ -amino group of Lys¹⁹⁷ and is used in the subsequent reactions. The ‘weak’ association species is the one that gives the observed K_m value for PL. The similar K_m values for PL and PM are considered to reflect that both Michaelis complexes have no covalent bond with PPAT, and are bound to the enzyme via hydrogen bonds and stacking interactions. This in turn suggests that the Schiff base formation of PL–Lys¹⁹⁷ is an important step in the catalysis, and PPAT may have been designed to promote this step.

Evolutionary aspects

PPAT uses PM and PL as substrates, which are dephosphorylated forms of the coenzymes of PLP-dependent aminotransferases, but shows no activity towards L-aspartate, L-serine or the co-enzymes. Aspartate aminotransferase, a representative PLP-dependent aminotransferase, showed a transamination reaction between PM and 2-oxo acid when the coenzyme, PLP or PMP, was removed from its active site [22,34]. Thus apo forms of aspartate aminotransferases from pig heart cytosol [22] and mitochondria, *E. coli* and yeast showed low but measurable pyridoxamine–oxalacetate aminotransferase activity: the ratio of specific activity to that of aspartate–2-oxoglutarate transamination catalysed by the corresponding holo forms of enzymes was 0.03–0.94% [35]. The apoenzymes from the cytosol and yeast also showed low (0.05 and 0.04 unit/mg respectively) PM–2-oxoglutarate transamination reaction rates. Considering the properties of aspartate apoaminotransferase, there is one main possible explanation for the relationship between the molecular evolution of PLP-dependent and -independent aminotransferases, although several other explanations are possible. Both types of aminotransferase could be descendants of a prototypic aminotransferase, which had the active-site lysine residue, but not PLP. One type of prototypic aminotransferase acquired PLP, and then evolved into many PLP-dependent aminotransferases with different substrate specificities. Another one increased the efficiency of the catalysis

without acquiring PLP. Further studies are required to elucidate the relationships between the molecular evolution of PLP-independent and -dependent aminotransferases.

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