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Identification of a plant gene encoding glutamate/aspartate-prephenate aminotransferase: The last homeless enzyme of aromatic amino acids biosynthesis

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ABSTRACT

In all organisms synthesising phenylalanine and/or tyrosine via arogenate, a prephenate aminotransferase is required for the transamination of prephenate into arogenate. The identity of the gene encoding this enzyme in the organisms where this activity occurs is still unknown. Glutamate/aspartate-prephenate aminotransferase (PAT) is thus the last homeless enzyme in the aromatic amino acids pathway. We report on the purification, mass spectrometry identification and biochemical characterization of *Arabidopsis thaliana* prephenate aminotransferase. Our data revealed that this activity is housed by the prokaryotic-type plastidic aspartate aminotransferase (At2g22250). This represents the first identification of a gene encoding PAT.

1. Introduction

The aromatic amino acids biosynthesis pathway is only found in microorganisms and in plants, and never occurs in animals. This pathway links the metabolism of carbohydrates to the biosynthesis of aromatic amino acids involved in protein synthesis, and also to the biosynthesis of a large diversity of aromatic secondary metabolites [1–3]. The aromatic amino acid pathway is thus of central importance for the growth of these organisms and for their interaction with the environment. There is also a strong economic interest in this pathway as a source of natural molecules with high nutritional or pharmaceutical values and as a potential target of new antibiotics or herbicides [4].

Depending on the organisms, there are two different routes for the post-chorismate branch of the pathway leading to phenylalanine and tyrosine, the phenylpyruvate/*p*-hydroxyphenylpyruvate route, or the arogenate route. The existence of an arogenate route for the biosynthesis of phenylalanine and/or tyrosine was first reported by Stenmark et al. [5]. It was then shown mainly by the group of Jensen that depending on the organisms, dehydrogenases and dehydratases dedicated to tyrosine and phenylalanine biosyntheses, respectively, present different specificities for the cyclohexadienyl substrates arogenate or prephenate. Some are exclusively arogenate dependent [6–9], others exclusively prephenate dependent [10,11], and some are able to use both substrates [11–14]. This alternative explains the observed great diversity both in terms of organization and regulation in the post-chorismate branch (for reviews, see [4,15–17]). Until now, higher plants are the sole documented organisms where both tyrosine and phenylalanine are synthesized via arogenate [18–20]. Whatever the situation, in all organisms synthesising phenylalanine and/or tyrosine via arogenate, a prephenate aminotransferase is required for the transamination of the keto acid prephenate into arogenate (Fig. 1A). Little is known about this prephenate aminotransferase activity. The enzyme was partially purified and characterized from *Nicotiana sylvestris* [21] and from *Sorghum bicolore* [19] and to apparent homogeneity from *Anchusa officinalis* [22], but the identity of the gene encoding this prephenate aminotransferase in all the organisms where this activity occurs is still

Abbreviation: PAT, glutamate/aspartate-prephenate aminotransferase

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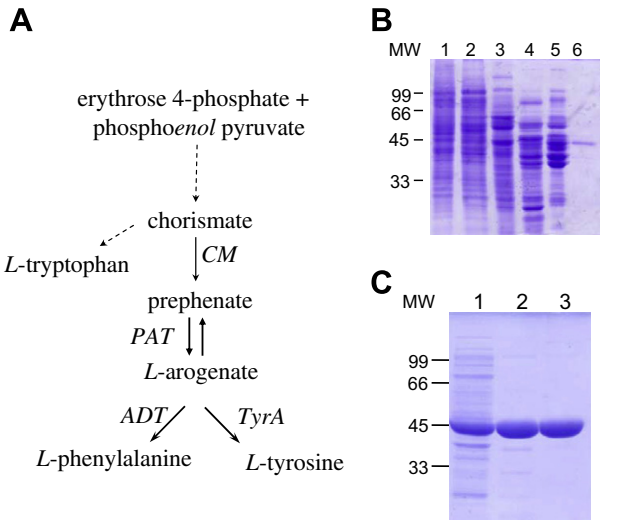

Fig. 1. Plant aromatic amino acid pathway and purification of the native and recombinant *A. thaliana* prephenate aminotransferase (PAT) (A) Biosynthesis pathway leading to tryptophan, tyrosine and phenylalanine in plant CM: chorismate mutase, PAT: prephenate aminotransferase, TyrA: arogenate dehydrogenase, ADT: arogenate dehydratase. (B) Purification of native *A. thaliana* PAT. Proteins were separated on a 12% SDS–PAGE stained with Coomassie brilliant blue R-250. (Lane 1) crude soluble extract (35 μg). (Lane 2) 45–75% ammonium sulfate pellet (35 μg). (Lane 3) heat shock supernatant (35 μg). (Lane 4) pool of the fractions containing PAT eluted from the Q-Sepharose column (35 μg). (Lane 5) pool of the fractions containing PAT eluted from the S200 column (35 μg). (Lane 6) fraction containing PAT eluted from the Q-Sepharose column (1 μg). (C) Purification of recombinant *A. thaliana* PAT. (Lane 1) crude soluble protein extract (15 μg). (Lane 2) pool of active fractions eluted from the EMD DEAE column (10 μg). (Lane 3) pool of active fractions eluted from the S200 gel filtration (6 μg). MW: Molecular weight markers (kDa).

unknown. PAT is thus the last enzyme in the aromatic amino acids pathway that is not associated with a gene.

In this study we report on the purification, mass spectrometry identification and biochemical characterization of *Arabidopsis thaliana* prephenate aminotransferase. Our data revealed that *A. thaliana* prephenate aminotransferase activity is housed by the prokaryotic-type plastidic aspartate aminotransferase (At2g22250). This represents the first identification of a gene encoding a PAT activity.

2. Materials and methods

2.1. Chemicals

Amino acids, prephenate, oxaloacetate, α-ketoglutarate, NADP, NADH and malate dehydrogenase were obtained from Sigma–Aldrich (St. Quentin Fallavier, France).

2.2. Protein quantification

Proteins were quantified either by the method of Bradford [23] for crude extracts with bovine γ-globulin as standard or, for pure proteins, by measuring A_{205nm} [24]. Pure enzyme concentration is expressed on a monomer basis (45 kDa).

2.3. Purification of native prephenate aminotransferase from A. thaliana cultured cells

A. thaliana (ecotype Columbia) cell suspension cultures were grown as previously described [25]. 167 g (wet weight) of cells were powdered in liquid nitrogen and dispersed in a Potter grinder in 450 ml of 50 mM Potassium-Phosphate buffer (pH 7.5) containing 0.1 mM pyridoxal-5′-phosphate (PLP), 1 mM EDTA, 1 mM DTT, 20% (v/v) glycerol, 1 mM benzamidine and 5 mM ε-aminocaproic acid. The soluble protein extract (550 ml, 4.2 g) was fractionated using ammonium sulfate. The 45–75% ammonium sulfate pellet (2.2 g protein) containing PAT activity was re-suspended in 200 ml of 50 mM KPi buffer (pH 8.0), supplemented as described above and subsequently heated at 65 °C for 10 min, then placed on ice for 1 h before centrifugation (30 000×*g*, 45 min, 4 °C). The desalted proteins (210 mg) were applied to a Q-Sepharose (Amersham Pharmacia) column (16 × 150 mm^2), equilibrated with 25 mM KPi buffer (pH 8.0), 20% (v/v) glycerol and 1 mM EDTA. The proteins were eluted with a linear gradient of KCl (0–0.75 M, 300 ml). The fractions containing PAT activity (15.3 mg protein) were pooled and concentrated on macrosep 10 K concentrator (Pall Filtron) prior to loading on a 26/60 superdex S200 column (Pharmacia), equilibrated with 25 mM KPi buffer (pH 7.3), 20% (v/v) glycerol. PAT activity was eluted in the fractions corresponding to an apparent molecular weight of 90 kDa. The active fractions were concentrated and loaded (3.4 mg) on a Q-Sepharose column equilibrated with 10 mM KPi buffer (pH 7.2), 20% (v/v) glycerol. Proteins were eluted with a linear gradient of KPi (pH 7.2), (10–75 mM, 150 ml). SDS–Page analysis of the more active fraction revealed the presence of a single major band migrating with an apparent molecular weight of 43 kDa.

2.4. Identification of prephenate aminotransferase

The 43 kDa band was cut from the gel and proteins and peptides were prepared as described in [26]. Nanoliquid chromatography, LTQ-Orbitrap and bioinformatics analyses were performed as described in [27].

2.5. Determination of enzyme activities

Prephenate aminotransferase activity was assayed by coupling the reaction with purified Tyr-insensitive arogenate-specific dehydrogenase from *Synechocystis* [28] and following the reduction of NADP at 340 nm. The reaction was carried out at 30 °C in 50 mM Hepes buffer (pH 8.0) in the presence of 40 nM coupling enzyme, 100 μM NADP and variable amounts of prephenate, aspartate or glutamate. Activities were calculated using an epsilon for NADPH of 6250 M^{-1} cm^{-1} at 340 nm. Glutamate–oxaloacetate aminotransferase activity was measured in Hepes 50 mM pH 8.0 at 30 °C by the spectrophotometric assay of oxaloacetate at 280 nm (ε_{280nm} = 550 M^{-1} cm^{-1}) [29]. Aspartate–α-ketoglutarate aminotransferase activity was measured using malate dehydrogenase (6 units) and followed by the decrease of NADH at 340 nm. This assay was also used to test the potential aspartate–*p*-hydroxyphenylpyruvate and aspartate–phenylpyruvate aminotransferase activity of PAT. In all the assays the reaction was initiated by the addition of PAT.

2.6. Construction of recombinant vector

The predicted positions of the transit peptide cleavage site for At2g22250 and At4g31990 proteins were identified using ChloroP program. The cDNA sequences corresponding to the predicted mature proteins were amplified by PCR using cDNAs prepared from *A. thaliana* leaves. The oligonucleotides used contained NcoI and XhoI restriction sites for cloning of the PCR fragments into pET23 d(+) vector. Oligonucleotides used for PCR amplification of At2g22250 were At2g22250 5′: GAATATGTCATCTAGAACCATGGCTATGGCCA AACCAAATG and At2g22250 3′: CACATTTCTGTGCTCGAGCTGTTAA ACGGAGAC. An initiating Met is introduced in place of C53. Oligonucleotides used for PCR amplification of At4g31990 were At4g31990 5′: CTAAAAGCAAAGTCTTTTACCATGGTGACTATGACGG TTGCAG and At4g31990 3′: GTCATCTACTGAGACTCGAGCTCA GCT TACGTTATGG. An initiating Met was inserted at position 41 in

place of R41. The recombinant proteins are devoid of their predicted transit peptides and are produced without any tags.

2.7. Overproduction conditions and recombinant enzyme purification strategy

Fresh colonies of transformed BL21 (DE3) Rosetta2 bacteria (Novagen, Darmstadt, Germany) were transferred into 15 ml of LB media supplemented with the antibiotics and grown at 37 °C. Saturated culture was transferred into 800 ml LB medium supplemented with the antibiotics and growth was continued for 3 h at 37 °C. IPTG was added (0.4 mM) at A_{600nm} = 0.6. Growth was continued for 18 h at 20 °C. Pelleted bacteria were re-suspended in 50 mM KPi pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol (v/v), 5 mM ε-aminocaproïc acid and 1 mM benzamidine, 100 μM PLP, 10 ml per litre culture and sonicated for 10 min at 4 °C on a Vibracell disruptor. Streptomycin sulfate (0.1% (p/v)) was added to precipitate DNA and the solution was centrifuged for 35 min at 30 000×*g* at 4 °C. Purification steps were carried out at room temperature. The first step consisted in an anion exchange chromatography [DEAE EMD 650(M) column 26 × 260 mm^2, (Merk, Darmstadt, Germany)], equilibrated in 50 mM KPi, pH 7.5, 1 mM DTT, 1 mM EDTA, 10% glycerol (v/v) and 100 μM PLP. Proteins were eluted by a linear gradient of KCl in this buffer. Active fractions were pooled and loaded on HiPrep 16/60 Sephacryl S-200 column (Amersham Pharmacia) equilibrated with 20 mM KPi, pH 7.5, 100 mM KCl and 10% glycerol (v/v). Pure proteins were concentrated using centricon (Pall Filtron), quickly frozen in liquid nitrogen and stored at −80 °C.

2.8. Kinetic data analysis

Kinetic data were fitted with the appropriate theoretical equations by using the Kaleidagraph program (Synergy Software, Reading, PA, USA).

3. Results and discussion

3.1. Prephenate aminotransferase activity is housed by the A. thaliana prokaryotic-type aspartate aminotransferase (At2g22250)

Purification of prephenate aminotransferase from cultured *A. thaliana* cells was performed by a set of five steps (Supplementary Table 1). The specific activity in the more purified fraction was 470 nmol s^{-1} mg^{-1} with a purification factor of at least 1500. SDS–PAGE analyses of the active fractions eluted from the last Q-Sepharose column revealed the presence of a major polypeptide with an apparent molecular mass of 43 kDa (Fig. 1B). The corresponding polypeptide band was extracted from the SDS–PAGE and identified by mass spectrometry, which revealed the presence of three proteins (see Supplementary Table 2). The more abundant one (At2g22250, 15 spectral counts) corresponded to an aspartate aminotransferase (SwissProt accession number, Q9SIE1). The two other proteins corresponded to a glutamate-1-semialdehyde aminomutase (At3g48730, 8 spectral counts), and an isopropylmalate dehydrogenase (At1g80560, 3 counts). Only aspartate aminotransferase (At2g22250) was considered as a putative prephenate aminotransferase (PAT) candidate since PAT activity is known to utilize either aspartate or glutamate as amino donors [19,21,22]. Neither the glutamate-1-semialdehyde aminomutase, which is known to be involved in heme and chlorophyll synthesis [30] and does not display aminotransferase activity, nor the isopropylmalate dehydrogenase were further considered.

In order to definitely confirm that this aspartate aminotransferase carried the prephenate aminotransferase activity, the recombinant protein was expressed in *Escherichia coli* and purified to apparent homogeneity by a two step standard chromatography procedure (Fig. 1C). Biochemical analyses revealed that, when tested in a coupled assay with a Tyr-insensitive, arogenate-specific arogenate dehydrogenase (TyrA from *Synechocystis* [28]), prephenate in the presence of glutamate or aspartate was effectively transaminated into arogenate by At2g22250 gene product. Production of arogenate was confirmed by HPLC according to [21]. In a direct assay containing only PAT, arogenate could be clearly detected. In addition, in a coupled assay using TyrA, arogenate was transformed into tyrosine (Supplementary Fig. 1). No activity could be detected using *p*-hydroxyphenylpyruvate or phenylpyruvate as ketoacid or using tyrosine or phenylalanine as amino donors. The At2g22250 gene product was previously identified as a prokaryotic-type plastid localized aspartate–α-ketoglutarate aminotransferase [31]. The plastidic localization of this protein is entirely consistent with a chloroplastic localization of aromatic amino acids biosynthesis [16,32]. Kinetic characterizations (Table 1 and Supplementary Figs. 2–4) were then carried out to determine the efficiency of At2g22250 protein for the transamination of the keto acid prephenate with glutamate or aspartate as amino donor and to examine how this additional kinetic competence compares with the originally identified aspartate–α-ketoglutarate aminotransferase activity.

Table 1
Kinetics parameters of recombinant *A. thaliana* glutamate/aspartate-prephenate aminotransferase (PAT) (At2g22250). Values given are the average of at least three independent determinations. The differences in each set of data were <10%.

Aspartate aminotransferase kinetic parameters	
$K_{m\ (2ketoglutarate\ saturating)}^{Asp}$	12 000 μM
$K_{m\ (oxaloacetate\ saturating)}^{Glu}$	5600 μM
$K_{m\ (Glutamate\ saturating)}^{oxaloacetate}$	25 μM
$K_{m\ (Aspartate\ saturating)}^{2ketoglutarate}$	200 μM
$k_{cat}^{Glu,\ oxaloacetate}$	200 s^{-1}
$k_{cat}^{Asp,\ ketoglutarate}$	65 s^{-1}
Prephenate aminotransferase kinetic parameters	
$K_{m\ (Aspartate\ saturating)}^{Prephenate}$	13 μM
$K_{m\ (Glutamate\ saturating)}^{Prephenate}$	14 μM
$K_{m\ (prephenate\ saturating)}^{Glutamate}$	1500 μM
$K_{m\ (prephenate\ saturating)}^{Aspartate}$	2200 μM
$k_{cat}^{Glu,\ prephenate}$	95 s^{-1}
$k_{cat}^{Asp,\ prephenate}$	28 s^{-1}

3.2. Biochemical characterization of A. thaliana PAT activity

Specificity constants (k_{cat}/K_m) for the keto acids calculated from the kinetic parameters displayed in Table 1 indicate that in the presence of glutamate as amino donor PAT is nearly as efficient to transaminate prephenate (k_{cat}/K_m = 7 μM^{-1} s^{-1}) or oxaloacetate (k_{cat}/K_m = 8 μM^{-1} s^{-1}). These results indicate that PAT can operate both as a glutamate/aspartate-prephenate aminotransferase (PAT) and as a classical aspartate aminotransferase. Aspartate aminotransferase activity in *A. thaliana* chloroplast is represented by two isoforms. The eukaryotic-type (At4g31990, [33]) and the prokaryotic-type (At2g22250, [31]). In order to see whether PAT activity is a fortuitous activity of these aspartate aminotransferases the eukaryotic-type chloroplastic aspartate aminotransferase (At4g31990) was produced as a recombinant protein and purified. This protein displayed an aspartate aminotransferase activity, as previously reported by [33] but did not display any PAT activity even in the presence of a high concentration of prephenate (1 mM). Thus PAT activity is a specific property of the prokaryotic-type enzyme (At2g22250) in *Arabidopsis*.

As PAT is not only competent to function as aspartate aminotransferase – but also as prephenate aminotransferase, it will be interesting to compare its structure with that of crystallized specific aspartate aminotransferases to understand at the molecular level how the neo-functionalization of PAT occurred during evolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.09.037.

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