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Characterization of a bacterial tyrosine ammonia lyase, a biosynthetic enzyme for the photoactive yellow protein

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Abstract During genome sequence analysis of *Rhodobacter capsulatus*, nearby open reading frames were found that encode a photoactive yellow protein (PYP) and a hypothetical biosynthetic enzyme for its chromophore, a tyrosine ammonia lyase (TAL). We isolated the TAL gene, overproduced the recombinant protein in *Escherichia coli*, and after purification analyzed the enzyme for its activity. The catalytic efficiency for tyrosine was shown to be approximately 150 times larger than for phenylalanine, suggesting that the enzyme could in fact be involved in biosynthesis of the PYP chromophore. To our knowledge it is the first time this type of enzyme has been found in bacteria.

Key words: Tyrosine ammonia lyase; Photoactive yellow protein; *p*-Hydroxycinnamic acid; Phenylalanine ammonia lyase; *Rhodobacter capsulatus*

1. Introduction

Phenylalanine ammonia lyase (PAL) catalyzes the elimination of ammonia from L-phenylalanine to yield *trans*-cinnamic acid (Fig. 1). This reaction is the first step in the phenylpropanoid pathway in plants [1]. PAL activity has been found in some fungi and in all higher plants analyzed to date, but not in animals or bacteria. Because of the importance of PAL in the formation of lignin, isoflavonoids, and other secondary metabolites, this enzyme is one of the best studied in plants (for reviews see [2–4]).

Tyrosine ammonia lyase (TAL) catalyzes the formation of *trans*-*p*-hydroxycinnamic acid from L-tyrosine (Fig. 1). As pointed out by Rösler et al. [5], in monocotylic plants, PAL and TAL activities reside on the same polypeptide. In these plants, both activities have very similar catalytic efficiencies, in spite of large differences in K_m and turnover number. The enzyme from dicotylic plants, on the other hand, only utilizes L-phenylalanine efficiently. In contrast to PAL and TAL, histidine ammonia lyase (HAL) is a degradative enzyme that is commonly found in bacteria.

p-Hydroxycinnamic acid is the chromophore of the so-called photoactive yellow protein (PYP), a protein found in a small number of purple phototrophic bacteria where it is thought to act as a light sensor [6–8]. The chromophore of PYP is thought to be synthesized from tyrosine via a TAL and then to be activated by a specific ligase (pCL) for binding to the PYP apo-protein. Genes encoding the possible PYP biosynthetic enzyme pCL are located downstream of the gene for PYP in *Ectothiorhodospira halophila* and *Rhodobacter sphaeroides* [8,9]. The *Rhodobacter capsulatus* genome project (www.integratedgenomics.com) has reported an open reading frame (ORF) whose sequence is homologous to the *pal* sequences of plants (1626 bp, contig 58–60, ORF 1844) but which is located upstream of the PYP gene. The *R. capsulatus* PAL/TAL protein has e.g. 32% identity with the PAL sequence of *Pinus taeda*, but the *R. capsulatus* sequence shows a lower homology to the PAL sequences of yeasts. PAL from plants is thought to be post-translationally modified by the formation of a dehydroalanine residue in the catalytic center [10]. The Ser residue, which is the precursor of the autocatalytically formed dehydroalanine, is conserved in the present bacterial sequence, implying a similar reaction mechanism.

To our knowledge, this is the first time that a *pal*-homologous gene has been found in bacteria. Because we consider the enzyme to be an important step in the synthesis of holo-PYP, we have cloned and overexpressed the gene from *R. capsulatus* (ORF 1844) to produce the recombinant protein in *Escherichia coli* and to characterize the enzymatic activity of the gene product. Both TAL and PAL activity were investigated and were found to follow Michaëlis–Menten kinetics. The kinetic studies established that L-tyrosine, and not L-phenylalanine, is the natural substrate of the enzyme under physiological conditions.

2. Materials and methods

2.1. Cloning of the tal gene

Cloning of the *tal* gene of *R. capsulatus* (DSMZ 1710, type strain) in the overexpression vector pKK223-3 (Pharmacia, Uppsala, Sweden) was performed using PCR-based techniques. The oligonucleotides ECOTAL (CGGAATTCATGCTCGATGCAACC) and TALHIND (GCCCAAGCTTTCATGCCGGGGGATC) were complementary to the DNA encoding the TAL N- and C-termini. ECOTAL and TALHIND contained an *Eco*RI and a *Hin*dIII restriction site, respectively. As template for the PCR we used *R. capsulatus* genomic DNA. High fidelity polymerase (Roche Diagnostics, Brussels, Belgium) was used in the following reaction: 30 cycles, 1 min denaturation at 95°C, 1 min annealing at 50°C, and 2 min elongation at 72°C. After purification out of a 1% agarose gel (with Qiaquick Gel extraction kit, Qiagen) the 1.6 kb fragment was digested with *Eco*RI

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Abbreviations: ORF, open reading frame; TAL, tyrosine ammonia lyase; PAL, phenylalanine ammonia lyase; HAL, histidine ammonia lyase; PYP, photoactive yellow protein; K_m, Michaëlis–Menten constant; k_{cat}, turnover number; pCL, *p*-coumaryl:coenzyme A ligase; TFA, trifluoroacetic acid

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and *Hin*dIII and inserted into the pKK223-3 plasmid, yielding pKK223-3(TAL).

2.2. Overproduction and purification of TAL

pKK223-3(TAL), with expression of the *tal* gene under control of a strong *tac* promotor, was transformed into *E. coli* XL1-Blue competent cells. The overexpression culture, induced with 1 mM isopropyl-β-D-1-thiogalactoside at an OD_{600} of 0.5, was grown overnight at 37°C. Expression samples were taken after several induction times and analyzed by SDS–PAGE. After 2 h of induction, recombinant protein could already be detected, but maximum production was achieved after overnight induction. The cells were pelleted by centrifugation, resuspended in Tris–HCl buffer (20 mM, pH 8.0), and fractionated by sonication. After centrifugation to remove the cell debris, the TAL protein was precipitated by ammonium sulfate at a saturation of 25–40%. Following dialysis, the TAL-containing fraction was loaded onto a 1 ml Resource Q column (Pharmacia, Uppsala, Sweden) using an ÄKTA Explorer (Pharmacia) high-performance liquid chromatography (HPLC) system. Buffer A contained 20 mM Tris–HCl, pH 8.0; buffer B was the same as A supplemented with NaCl to a final concentration of 1 M. The TAL-containing fractions were pooled and concentrated on Ultrafree 4 centrifugal filters (Millipore, Bedford, MA, USA). The purification was continued by size exclusion chromatography on a Superdex 75 column (Hiload 16/60, Pharmacia) with 100 mM Tris–HCl pH 8.0, supplemented with 150 mM NaCl. After this step, the TAL was approximately 90% pure. An additional anion exchange 'polishing' step (Resource Q) removed practically all remaining contaminating proteins.

2.3. Enzymatic assay

PAL activity was assayed by following cinnamic acid formation at 280 nm using a double beam spectrophotometer (Uvikon, Kontron, Herts, UK) in 10 mM Tris buffer at 35°C. The pH optimum was determined between the pH values 6 and 10. The substrate concentrations were varied between 5 mM and 0.2 mM. TAL activity was assayed by monitoring *p*-hydroxycinnamic acid formation at 310 nm and 35°C. The substrate concentration was varied between 2 mM and 0.01 mM. All reactions were performed in a total volume of 1 ml.

2.4. Purification of cinnamic acid and p-hydroxycinnamic acid

For the determination of PAL and TAL specificities, 1 mM L-Phe or 1 mM L-Tyr was used. The reaction was left at 25°C for 20 min in Tris–HCl buffer (10 mM, pH 8.0) and followed spectrophotometrically. The reaction was stopped by adding 30 μl 2 N HCl. After filtration on a 0.45 μM Millipore filter, samples were analyzed on a C18 column (220×2.1 mm, Applied Biosystems, Foster City, CA, USA) connected to the SMART HPLC system (Pharmacia). Solvent A consisted of 0.1% trifluoroacetic acid (TFA) and solvent B of 0.08% TFA containing 80% acetonitrile. The flow rate was 100 μl/min. Peaks were detected by the UV absorbance at 313 nm.

3. Results

3.1. Production and purification of the TAL

The 1.6 kb fragment was expressed in *E. coli* using the pKK223-3(TAL) vector. After DNA sequencing, it was found that there were two differences in the gene sequence compared to what was found in the on-going genome sequence project. Both differences were found in non-conserved regions, namely the mutation His521→Asp and the deletion of Ala534. The PCR and sequencing reactions were repeated three times, but gave the same results each time. We attribute these differences to either strain differences or genome sequencing errors. After production and purification of the protein, as described above, we were able to reach a purity of 99%, based on a silver stain procedure (Fig. 2). The final yield of the recombinant protein after purification was approximately 5 mg/l culture.

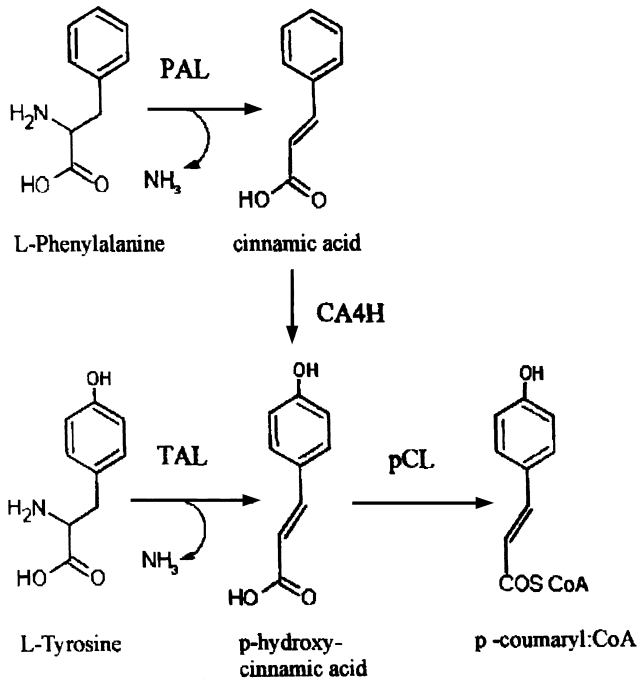

Fig. 1. Some crucial steps in the phenylpropanoid pathway in plants. CA4H: cinnamic acid 4-hydroxylase.

3.2. Enzymatic characterization of the TAL

The purified protein was investigated with respect to its enzymatic activity and specificity by determining the K_m and k_{cat} values for the conversion of L-tyrosine to *p*-hydroxycinnamic acid and L-phenylalanine to cinnamic acid. Both activities were followed by a spectrophotometric assay [5].

The pH optima of both activities were found to be slightly different as shown in Fig. 3; the optimum was pH 8.5 for TAL and pH 9.4 for PAL. Fig. 4 presents Lineweaver–Burk

Table 1
Comparison of the enzymatic properties for PAL and TAL

Organism	Enzymatic properties	L-Tyrosine	L-Phenylalanine
R. capsulatus	K_m (μM)	15.6	1277
	k_{cat} (s^{-1})	27.7	15.1
	k_{cat}/K_m	1.77	0.0118
Z. mays	K_m (μM)	19	270
	k_{cat} (s^{-1})	0.9	10
	k_{cat}/K_m	0.0473	0.037
P. crispum	K_m (μM)	2500	17.2
	k_{cat} (s^{-1})	0.3	22
	k_{cat}/K_m	0.00012	1.28

The K_m and k_{cat} values for the *R. capsulatus* enzyme are the mean values of six independent measurements. The values for the maize enzyme are taken from [5], those from parsley are from [11].

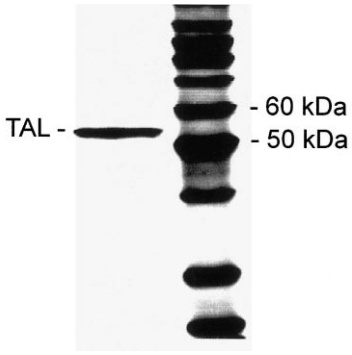

Fig. 2. SDS–PAGE of the purified TAL (loaded onto a 10% gel).

plots for the two substrates at their respective pH optimum. The kinetic parameters calculated for TAL and PAL activities are summarized in Table 1. As a consequence of a smaller K_m and a slightly larger k_{cat}, we can conclude that the enzyme shows a strong preference for L-tyrosine over L-phenylalanine. For comparison, Table 1 also includes the kinetic parameters for the PAL/TAL enzyme from the monocot *Zea mays*, for both substrates at pH 8.7 [5]. It can be seen that there is a 15-fold higher K_m value for L-Phe compared to L-Tyr. With the *R. capsulatus* enzyme, we found an even more pronounced specificity for L-Tyr, with an 80-fold higher K_m for L-Phe than for L-Tyr. In maize, the turnover numbers (k_{cat}) for TAL activity are about one tenth those for the PAL activity, resulting in a comparable catalytic efficiency (k_{cat}/K_m). In *R. capsulatus*, on the other hand, the k_{cat} for TAL activity is almost two times higher than for PAL activity. This results in a 150-fold greater catalytic efficiency for L-Tyr than for L-Phe.

Table 1 also shows that the K_m value and turnover number of the *R. capsulatus* enzyme, with L-tyrosine as substrate, are very comparable to the kinetic parameters from the PAL-1 isozyme from parsley (*Petroselinum crispum*), with L-phenylalanine as a substrate. The latter is a typical PAL from dicots [11].

To verify that both the PAL and TAL activities resulted in the expected products, the enzyme was incubated with the respective substrates for 20 min, as described above, and the products were analyzed by reversed phase HPLC. As shown in Fig. 5, the products had retention times as expected for cinnamic acid and *p*-hydroxycinnamic acid, which is in agreement with PAL and TAL activities.

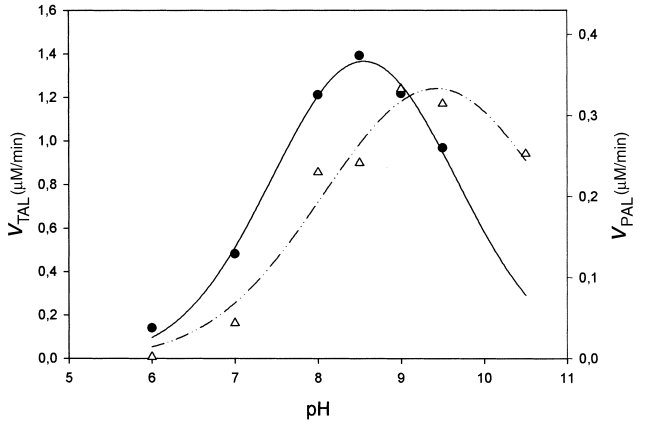

Fig. 3. pH optima for PAL/TAL activity. The solid line is a theoretical curve for the pH dependence of the rate of TAL reaction, the dotted line is for the PAL reaction.

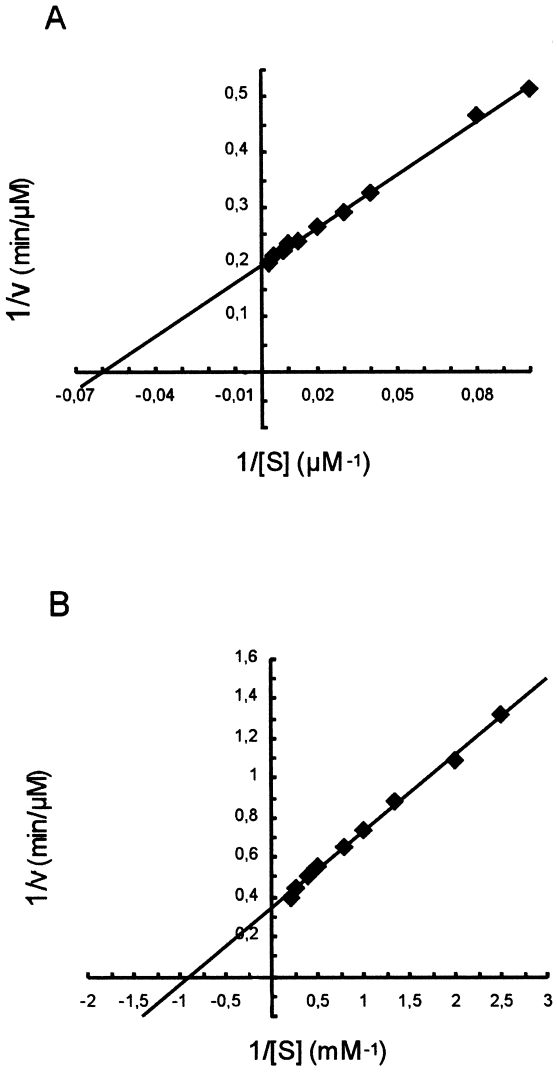

Fig. 4. Lineweaver–Burk plots of the TAL (A) and PAL (B) activities of the *R. capsulatus* enzyme.

Since PAL and TAL sequences show significant homology to HALs (see Capsulapedia for a sequence alignment), which are common enzymes in bacteria, we also tested L-histidine as a substrate for the recombinant enzyme. However, using the spectrophotometric assay described by Schwede et al. [12], we could not detect any activity. Also L-tryptophan is not a substrate, since no activity was detected even up to a 5 mM concentration.

4. Discussion

Our findings clearly show that the *pal*-homologous gene, which was found in *R. capsulatus*, encodes a TAL. The activity with tyrosine as substrate has a K_m of approximately 15.6 µM and a k_{cat} of 27.7 s^{-1}, values that are comparable to those of typical PALs of plants. Since the protein showed high homology to known PAL and HAL sequences, we also tested the conversion of L-phenylalanine and L-histidine. The cata-

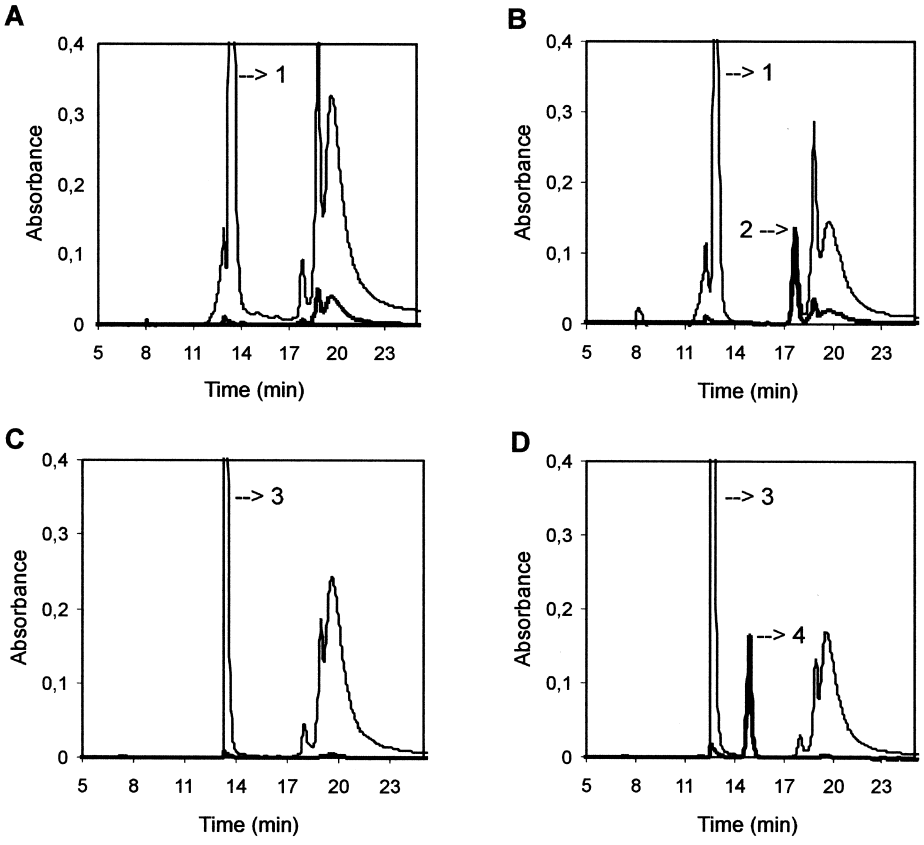

Fig. 5. HPLCs for PAL and TAL assays, showing the reaction mixture compounds at zero time (A and C, peaks 1 and 3 are L-Phe and L-Tyr, respectively) and the formation of cinnamic acid (B, peak 2) and *p*-hydroxycinnamic acid (D, peak 4) after 20 min of incubation. Thin lines are absorbances at 220 nm in A and B, and at 280 nm in C and D. Thick lines are at 280 nm in A and B, and at 310 nm in C and D.

lytic efficiency for L-phenylalanine is 150 times less than that with L-tyrosine as the substrate, suggesting that the principal physiological role of this enzyme is the production of *p*-hydroxycinnamic acid. To our knowledge, this is the first enzyme of the PAL/TAL family that has a higher enzymatic efficiency with L-tyrosine than with L-phenylalanine. In addition, it is also the first bacterial TAL that has been identified. The most commonly encountered enzyme of this family in bacteria is HAL, but our enzyme had no activity with histidine at all.

There is only a single crystal structure for the family of the ammonia lyases, that of the HAL from *Pseudomonas putida* [12]. The 12 amino acid residues at the active site are conserved in HAL enzymes, but there are two amino acid substitutions in the PAL enzymes, His83→Leu and Glu414→Gln. The *R. capsulatus* enzyme has the PAL substitutions of Leu and Gln, which is consistent with the absence of activity with histidine. However, it is not obvious what determines the specificities of the monocot, dicot, and bacterial PAL/TAL enzymes.

The *tal* sequence was found on the genome in proximity to the gene for the PYP (approximately 2 kb upstream of the *pyp* gene, contig 58–60, ORF 1849). The cofactor of PYP was identified as *p*-hydroxycinnamic acid [13,14], which is covalently bound by a thioester bond to a cysteine [15]. Since TAL catalyzes the elimination of ammonia from L-Tyr to yield *p*-hydroxycinnamic acid, we believe this enzyme to be one of the biosynthetic enzymes of holo-PYP because of its specificity, of the proximity of its gene, and of the scarcity of TAL in bacteria. The *p*-hydroxycinnamic acid is thought to be activated for binding to the apo-protein through formation of a thioester with coenzyme A (CoA) [8]. In this regard it is notable that approximately 1 kb downstream of *pyp*, a gene occurs encoding a presumed *p*-hydroxycinnamyl:CoA ligase (pCL, 1266 bp, contig 58–60, ORF 1852). More generally, *pcl*-homologous genes have been found in two other photosynthetic bacteria [8,9], and in both cases the gene was in close proximity of the *pyp* gene. *R. capsulatus* is the first *pyp*-containing organism where, in addition to the *pcl* gene, a *tal* gene has been found.

Although many ligases are involved in the intermediary metabolism of bacteria, the gene adjacent to that for PYP may not necessarily be involved in its biosynthesis. The fact, however, that the TAL is likely to be involved in the biosynthesis of the chromophore encourages the idea that the nearby pCL is also involved. Therefore the presumed pCL is currently being purified and characterized, with the goal to determine whether TAL and pCL are necessary and sufficient for the biosynthesis of PYP from apo-protein and L-tyrosine.

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