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Serine-202 is the putative precursor of the active site dehydroalanine of phenylalanine ammonia lyase

Site-directed mutagenesis studies on the enzyme from parsley (*Petroselinum crispum* L.)

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Abstract

To investigate the possible role of serine as a precursor of dehydroalanine at the active site of phenylalanine ammonia lyase, two serines, conserved in all known PAL and histidase sequences, were changed to alanine by site-directed mutagenesis. The resulting mutant genes were subcloned into the expression vector pT7.7 and the gene products were assayed for PAL activity. Mutant PALMutS209A showed the same catalytic property as wild-type PAL, whereas mutant PALMutS202A was devoid of catalytic activity, indicating that serine-202 is the most likely precursor of the active site dehydroalanine.

Key words: Phenylalanine ammonia lyase; PAL; Dehydroalanine; Site-directed mutagenesis

1. Introduction

Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) is one of the most extensively studied enzymes in higher plants and plays an important role in their metabolism. It catalyses the elimination of ammonia from L-phenylalanine to give *trans*-cinnamic acid which is an intermediate in the biosynthesis of various phenylpropanoids, such as lignin, flavonoids and coumarins [8,11].

Since its discovery by Koukol and Conn [20] PAL from various plants and fungi have been studied. Hanson and Havir reported that PAL from potato [9] and maize [10,13], when inactivated and specifically tritiated with NaB^3H_4 at the active site, yielded DL-[3-^{3}H]alanine and tritiated water after hydrolysis. They also reported that ^{14}CN-inactivated PAL upon hydrolysis gave DL-[4-^{14}C]aspartic acid [14]. Treatment with $^{14}CH_3NO_2$ led also to inactivation. After hydrolysis with HCl, $^{14}CO_2$, $H^{14}CO_2H$ and DL-[^{14}C]aspartic acid were formed. Hanson and Havir concluded that PAL contains dehydroalanine at the active site and postulated cysteine or serine as a possible precursor [15].

Histidase (histidine ammonia-lyase, HAL; EC 4.3.1.3), which catalyses a similar elimination of ammonia by converting histidine into urocanic acid, has also been found to contain dehydroalanine [7,38].

Histidase and PAL are very similar enzymes, showing high sequence homology, catalyzing the same reaction type and possessing the same prosthetic group. The biosynthesis of this group is thus likely to be analogous. Here we report on the exchange of serine-202 and -209 for alanine by site-directed mutagenesis and its effect on the catalytic activity of PAL.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

E. coli TG1 cells were used for the isolation of single-stranded DNA from M13 phages to carry out site-directed mutagenesis. Cells were grown and infected as described in the lab manual [29]. *E. coli* BL21(DE3) cells served for the expression of either wild-type or mutant phenylalanine ammonia-lyase. For overexpression cells were grown in 1l of Luria-Bertani medium supplemented with ampicillin (85 μg/ml) at 37°C. At an OD_{600} of 1.0, 400 mM isopropyl thio-β-D-galactoside (IPTG) was added [32]. Cells were harvested 4 h after induction. The expression vector pT7.7 was generously provided by Dr. Stanley Tabor [34]. pT7.7PAL was produced by subcloning the PAL gene into pT7.7 by using the restriction sites *NdeI* at the 5′ end of the gene (created by site-directed mutagenesis) and *SalI* at the 3′ end.

The phage M13BM21 was from Boehringer Mannheim. pBS(+) was purchased from Stratagene.

2.2. Subcloning the PAL gene

The PAL1 gene was isolated as described by Schulz et al. [31] from a cDNA library from elicitor-treated parsley (*Petroselinum crispum* L.) cells [26]. An *Eco*RI digest of pBS(+)PAL led to a 720 bp fragment including the start codon of the PAL gene. This fragment was cloned into the phage M13BM21 for the introduction of a 5-terminal *NdeI* site. Digestion with *NdeI* and *Eco*RI of the recombinant M13BM21 led to several fragments. The 660 bp fragment, coding for the first 219 amino acids, was separated by electrophoresis using a 1% agarose gel and recovered by excising it from the gel. This fragment was subcloned into pT7.7 containing the 3′ end an *Eco*RI–*SalI* fragment from recombinant pBS, coding for the missing 474 amino acids.

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2.3. Site-directed mutagenesis

To introduce a *Nde*I site as well as for constructing the two Ser/Ala mutants, the *Eco*RI flanking 720 bp fragment was subcloned from recombinant pBS into M13BM21. Site-directed mutagenesis was performed following the protocol of the Amersham mutagenesis kit (Skulptor). The oligonucleotides were used in the mutagenesis reactions:

NDEPRIMER: 5′-GTACGT*CATA*TGGAGAACGG-3′

S202A: 5′-CACTGCT*G*CTGGTGATC-3′

S209A: 5′-GCCATTG*G*CTTACATTGCTGG-3′

The introduction of the *Nde*I-site was confirmed by restriction analysis of double-stranded DNA of the M13BM21 clones. The Ser/Ala exchanges were checked by sequence analysis using the dideoxynucleotide chain-termination method of Sanger et al. [30]. Either Sequenase (USB) at 37°C or *Taq* polymerase (Boehringer-Mannheim) at 70°C were used for the sequencing reactions [16].

2.4. Transformation

Either *E. coli* TG1 or *E. coli* BL21(DE3) cells were grown in 0.5 l of LB medium to an OD_{600} of 0.3–0.4. The cells were sedimented at 4500 × *g*. The cell pellet was resuspended in 0.5 l ice-cold 10% glycerol solution. In five steps the cells were made competent by reduction of the resuspension volume of 10% glycerol to a final volume of 1 ml. All steps were performed at 4 °C. 140-μl aliquots of the concentrated competent cells were stored at −70°C for two months without loss of competence. Transformation was performed by electroporation using a gene pulser from Bio-Rad. 70 μl of competent cells was transformed with 20 ng of vector DNA in a 0.4 mm cuvette at 2.5 kV and 0.2 kΩ (6.25 kV/cm) for 4.4 ms. Recombinant phages were selected by using blue/white screening. Recombinant bacteria were detected either by blue/white screening or by restriction analysis.

2.5. Purification

Transformed *E. coli* BL21 cells were grown in 1 l LB medium containing 85 μg/ml to an OD_{600} of 1,0. Then 0.4 mM IPTG was added. Cells were harvested 4 h after induction by centrifugation at 4,500 × *g*. The cell pellet was resuspended in 10 ml of 50 mM Tris-HCl buffer, pH 7.2, supplemented with 40 units of Benzonase (Merck, Darmstadt) 5 mM benzamidine, and 0.5 mM phenylmethanesulfonyl fluoride. Sonication (Branson Model 450, 70% power setting, 10 min ice bath) was followed by centrifugation at 30,000 × *g* for 30 min. The clear supernatant was applied to a TSK DEAE-3SW Ultrapac column using 50mM Tris-HCl buffer, pH 7.2, containing 10 mM phenylalanine as buffer A, and as eluent buffer A supplemented with 0.5 M KCl by a flow rate of 3 ml/min. The PAL-active fractions were collected, concentrated to 4 ml (centricon 30, Amicon) applied to a HiLoad 26/60 Superdex 200 preparative-grade column and isocratically fractionated at a flow rate of 1.75 ml/min. 50 mM Tris-HCl buffer, pH 7.2, containing 0.1 M KCl and 10 mM phenylalanine, was used as eluent. PAL was eluted after 110 min. The active fractions were pooled and stored at +4°C.

2.6. SDS/PAGE, Western-blot, protein determination and enzyme assay

SDS/PAGE using a 10% polyacrylamide gel was performed according to Laemmli [21] with Coomassie brillant blue R 250 staining. Western blotting was performed using the protocol of Symingteon et al. [33]. Protein determinations were carried out according to Warburg and Christian [23]. PAL activity was determined spectrophotometrically as described in [40].

3. Results and discussion

It has been previously reported that the use of *E. coli* BL21(DE3) cells combined with the expression system pT7.7 leads to high expression of recombinant urocanase and histidase [19,22,25]. Schulz et al. have shown, that the expression of PAL from parsley in *E. coli* leads to catalytically active enzyme [31]. In an attempt to achieve higher expression rates we now cloned PAL from parsley into the expression system pT7.7 and used this construct to transform *E. coli* BL21(DE3) cells. Maximum expression was reached after 4 h (Fig. 1). On longer incubation PAL is eventually degraded, leading to weaker signals on the Western blot. Isolation from 1 l of Luria-Bertani medium yielded a total activity of 12 U. After purification by anion-exchange and gel-filtration chromatography the enzyme had a specific activity of 0.42 U/mg, comparable with PAL preparations from soybean [15] or *Rhizoctonia solani* [18]. SDS/PAGE revealed that the purification procedure is still incomplete. The K_m value was estimated to be 0.17 mM very similar to the K_m of PAL from *Rhodotorula glutinis* [1] and *Helianthus annuus L* [17], which show values of 0.29 and 0.27 mM, respectively. The purified enzyme was not very stable. Addition of protease inhibitors and phenylalanine [2] to the buffer solutions prevented the degradation of PAL during the isolation procedure. The enzyme was stored in 50% glycerol at −20 °C without loss of activity for several weeks.

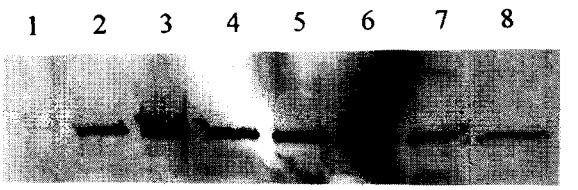

Fig. 1. Western blot of recombinant PAL from *E. coli* BL21 (DE3). Induction was performed with IPTG. Lane 1, time of addition of IPTG; lane 2, after 2 h; lane 3, after 4 h; lane 4, after 6 h; lane 5, after 8 h; lane 6, after 10 h; lane 7, after 16 h; lane 8, after 24 h.

In the deduced amino acid sequence of PAL from *Petroselinum crispum* [31], *Rhodotorula toruloides* [3], *Rhodotorula rubra* [6], *Oryza sativa* [35], *Lycopersicon esculentum* [24] and *Ipomea batatas* [35] and the amino acid sequence of histidase from *Pseudomonas putida* [5], *Bacillus subtilis* [28], *Streptomyces griseus* [39], from rat liver [36] and mouse liver [37] four conserved serines can be identified. Langer et al. [22] reported that an exchange of serine 143 to alanine by site-directed mutagenesis of histidase from *P. putida* led to an inactive enzyme. Comparison of the two sequences from *P.crispum* PAL and

PAL *P.crispum*	197-208	GTITA**S**GDLVPL**S**YIA
PAL *R.toruloides*	205-216	GTISA**S**GDLSPL**S**YIA
PAL *R.rubra*	211-222	GTISA**S**GDLSPL**S**YIA
PAL *O.sativa*	184-195	GTITA**S**GDLVPL**S**YIA
PAL *L.esculentum*	204-215	GTITA**S**GDLVPL**S**YIA
PAL *I.batatas*	187-198	GTITA**S**GDLVPL**S**YIA
HAL *P.putida*	138-149	GSVGA**S**GDLAPLATMS
HAL *B.subtilis*	137-148	GSLGA**S**GDLAPL**S**HLA
HAL *Str.griseus*	142-153	GSLGC**S**GDLAPL**S**HCA
HAL *R.norvegicus*	250-261	GTVGA**S**GDLAPL**S**HLA
HAL *M.musculus*	250-261	GTVGA**S**GDLAPL**S**HLA

Fig. 2. Comparison of the amino acid composition of six PAL and five histidases around serine-202 and -209 in PAL from *P. crispum*. Comparison was carried out using the MultAlign program in HUSAR (Heidelberg Unix Sequence Analysis Resources).

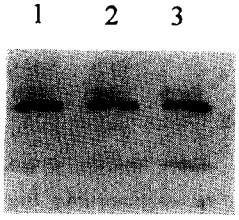

Fig. 3. Western blot of overexpressed wilde-type PAL (lane1), PALMutS202A (lane 2) and PALMutS209A (lane 3).

P. putida histidase suggested that serine-202 of PAL corresponds to serine 143 of histidase. In the amino acid sequence of PAL close to serine-202 a second conserved serine (serine-209) is found which is part of a highly conserved sequence (Fig. 2). Both serines were converted into alanine by site-directed mutagenesis. PALMutS202A and PALMutS209A were isolated as described for wild-type PAL. PALMutS209A exhibited the same K_m value as the wild-type enzyme. In contrast, PALMutS202A showed no activity (<0.01% of that of the wild-type). Western blot analysis revealed that PALMutS202A was expressed in the same quantity as wild-type PAL or PALMutS209A and, importantly, the inactive mutant had the same molecular mass as wild-type PAL and PALMutS209A (Fig. 3). These results suggest that serine-202 plays a very important role in producing catalytically active PAL. Hanson and Havir [12] proposed that dehydroalanine is formed by desulfurization of cysteine or by dehydratisation of serine. Böttge [4] labelled PAL with [^{14}C]serine, and isolated [^{14}C]alanine, after inactivation with $NaBH_4$ under denaturing conditions. Langer et al. [22] identified serine-143 as the precursor of dehydroalanine in histidase from *P. putida*. They showed that the circular dichroism spectra of wild-type histidase and MutS143A are identical. This indicates that the lack of catalytic activity is due to the change of serine into an alanine and not to a change in the secondary structure. We conclude that serine-202 plays the same important role in the biosynthesis of catalytically activite PAL by being the most likely precursor of dehydroalanine at the active site.

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