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Tryptophan decarboxylase from *Catharanthus roseus* is a pyridoxo-quinoprotein

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Tryptophan decarboxylase (EC 4.1.1.28) from *Catharanthus roseus* was purified to homogeneity. The native enzyme has an M_r of about 96000 as estimated from native PAGE. After SDS-PAGE, three protein bands were visible corresponding with M_r 49000, 33000 and 17000. The N-termini of the 49 kDa protein and the 33 kDa protein were identical. Antibodies against the 49 kDA protein also reacted strongly with the two smaller proteins. It is concluded that the native enzyme consists of two subunits of M_r 49000. Tryptophan decarboxylase appears to be a pyridoxo-quinoprotein, since two molecules of pyridoxal phosphate and two molecules of covalently-bound pyrroloquinoline quinone were found per enzyme molecule.

Tryptophan decarboxylase; Hydrophobic interaction chromatography; Pyrroloquinoline quinone; Pyridoxal Phosphate; Pyridoxo-quinoprotein; (*Catharanthus roseus*)

1. INTRODUCTION

Tryptophan decarboxylase (TDC) catalyzes the formation of tryptamine from L-tryptophan. The decarboxylation of L-tryptophan is the first step in the formation of terpenoid-derived indole alkaloids in the plant family of the Apocynaceae. There is evidence that the level of TDC activity may be one of the bottlenecks for the production of these compounds in plant cell suspension cultures [1,2]. As we are interested in the mechanisms that govern the biosynthesis of these alkaloids, we decided to clone the TDC gene from *Catharanthus roseus* and to study its expression at the protein level. For this purpose, we have purified the decarboxylase from a cell suspension culture of *C. roseus* [3] and we have raised TDC-specific antibodies (Goddijn et al., in preparation).

Recently, it was found that pyrroloquinoline quinone (PQQ) is present as the second organic cofactor in 3,4-dihydroxyphenylalanine (dopa) decarboxylase (EC 4.1.1.28) from pig kidney [4]. Glutamic acid decarboxylase (EC 4.1.1.15) from *Escherichia coli* is another example of a decarboxylase which is a pyridoxo-quinoprotein [5]. In both cases, PQQ is covalently bound to the protein and the amount of PQQ can be assessed after purification of the enzyme to homogeneity. Covalently-bound PQQ has also been found as a cofactor in enzymes of plant origin. Examples are lipoxygenase-1 (EC 1.13.11.12) from soybean [6] and diamine oxidase (EC 1.4.3.6) from pea (*Pisum sativum*) [7].

The biosynthesis of PQQ has not yet been clarified. Recent results could indicate that covalently-bound PQQ is synthesized in situ, i.e. in the quinoprotein itself, via a route that is different

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Abbreviations: HIC, hydrophobic interaction chromatography; PLP, pyridoxal-5'-phosphate, PQQ, pyrroloquinoline quinone; PAGE, polyacrylamide gel electrophoresis; TDC, tryptophan decarboxylase

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from that of free PQQ [5]. The enzymes involved are unknown.

In view of these results, we became interested to see if TDC from *C. roseus* is also a pyridoxoquinoprotein. If so, this may have implications for our goals concerning the cloning of the TDC gene. In this paper, the presence of covalently-bound PQQ in TDC from *C. roseus* is demonstrated, after purification of the enzyme to apparent homogeneity. Some implications of this finding are discussed.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

Cell suspension cultures of *C. roseus* (L.) G. Don were routinely grown as described [8]. Cells were subcultured every 10–12 days by a 5-fold dilution. For induction of TDC, a 12-day-old suspension culture was transferred to the induction medium described in [9], also by a 5-fold dilution. After 5 days, the cells were harvested.

2.2 *Purification of TDC*

TDC was extracted from *C. roseus* cells and purified as described in [10]. This yielded a TDC preparation (3 mg protein) with a specific activity of 2.9 nkat/mg. SDS-PAGE was used to assess the purity of this preparation.

High-performance hydrophobic interaction chromatography (HIC), originally intended as an extra purification step, appeared to give additional information on the purity. Moreover, this step appeared to be suitable for concentration of the enzyme, needed for further characterization, and for removal of free pyridoxal-5'-phosphate (PLP), allowing the subsequent determination of PLP bound to the enzyme. HIC was carried out on a TSK Phenyl-5PW column (7.5 × 75 mm) (Pharmacia LKB Biotechnology) at a flow rate of 0.5 ml/min. The column was equilibrated with 1 M $(NH_4)_2SO_4$ in 50 mM Na_2HPO_4 buffer, pH 7.4 (eluent B). A sample (0.7 mg protein) of the preparation obtained as described in [10], in 50 mM Tris-HCl (pH 7.5) containing 0.02 mM PLP, was brought to 1 M $(NH_4)_2SO_4$ and applied to the column by 8 repetitive 1-ml injections. After elution of the unretained components, a linear gradient (volume 7.5 ml) was started from 100% B to 100% H_2O (eluent A).

2.3 *Determinations*

Decarboxylase activity was determined by the HPLC method described previously [8].

During the purification, protein was measured by the dye-binding method of [11] with bovine γ-globulin as a reference. The protein contents of the enzyme preparations, used for cofactor determinations, were calculated from the 280 nm absorbance. The $A_{280\,nm}^{1\,mg/ml}$ was determined according to [12] in the final enzyme preparation after the high-performance HIC step.

After basification with NaOH, the PLP content of the final enzyme preparation was calculated from the absorbance at 388 nm, using a molar absorption coefficient of 6600 $M^{-1} \cdot cm^{-1}$ [13]. This method was also used to assay PLP in the first fractions eluted from the TSK Phenyl-5PW column.

PQQ was determined according to [5], in a sample purified as described in [10], except that the high-performance size-exclusion chromatographic step was carried out in 50 mM phosphate buffer, pH 7.0.

2.4 *Gel electrophoresis*

Native PAGE and SDS-PAGE were performed with the PhastSystem electrophoresis equipment (Pharmacia LKB Biotechnology) in Phastgel gradient media 8-25 and 10-15, respectively, according to the manufacturers' instructions. For SDS-PAGE, samples were prepared in 2.5% (w/v) SDS, 5.0% (v/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue and heated at 100°C for 5 min. Protein staining occurred with Phastgel Blue R. Immunological detection of TDC was carried out after SDS-PAGE and Western blotting, with antibodies raised against the 49 kDa protein obtained with SDS-PAGE (Goddijn et al., in preparation).

3. RESULTS AND DISCUSSION

Since the enzyme preparation obtained with the previously described procedure [10] appeared to consist of three bands after analysis by SDS-PAGE, a step consisting of high-performance HIC was devised to detect any impurities present. TDC was concentrated on the column by 8 consecutive injections, at 100% B as solvent. Each injection resulted in two peaks eluting with solvent B (fig.1). The corresponding fractions did not stain with the Coomassie reagent [11]. The fraction correspon-

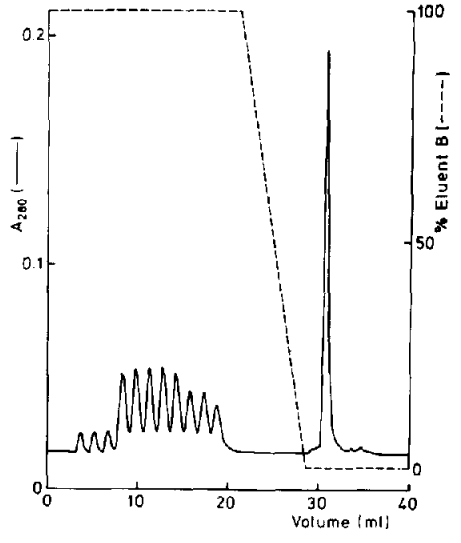

Fig.1. HIC of TDC on a TSK Phenyl-5PW column. The sample was applied by 8 repetitive 1-ml injections. Flow rate was 0.5 ml/min. The linear gradient (delay volume 3.5 ml) was from 100% eluent B (1 M $(NH_4)_2SO_4$ in 50 mM Na_2HPO_4 buffer, pH 7.4) to 100% eluent A (H_2O) during 15 min.

ding with the first peak contained PLP. After elution of all the peaks with solvent B, the gradient was started and TDC was eluted as a sharp peak which was collected for further analysis. The elution pattern (fig.1) indicated that native TDC already had an adequate purity (estimated higher than 95%) before injection. SDS-PAGE of TDC after this step in a 10–15% gradient gel again showed the presence of the above-mentioned three bands. These bands corresponded with M_r values 49 000, 33 000 and 17 000. Native PAGE in an 8–25% gradient gel showed the presence of only one band corresponding to M_r 96 000. Antibodies directed against the 49 kDa band appeared to react strongly with all three bands. These results suggested that the 49 kDa protein is the real subunit of TDC and that the two other proteins are degradation fragments. This view is supported by the results of N-terminal sequence analysis showing that the N-termini of the 49 kDa band and the 33 kDa band are identical (Goddijn et al., in preparation). It is concluded that TDC from *C. roseus* is a dimeric protein consisting of two subunits of *M*r 49 000 and that the smaller proteins of M_r 49 000 and that the smaller proteins of M_r 33 000, constituting the N-terminal portion, and of M_r 17 000 result from degradation of TDC.

The resemblance with dopa decarboxylase from pig kidney is striking [14]. This enzyme could be nicked by trypsin with complete loss of activity yielding fragments of M_r 38 000 and 14 000. The nicked protein retained its native molecular weight and its capacity to bind PLP. The fragments could only be dissociated and separated under denaturing conditions. The 38 kDa fragment was found to constitute the N-terminal portion of the protein. It seems that TDC, too, has a site particularly susceptible to proteolysis and located about two-thirds from the N-terminus. By comparing the amino acid sequence for the N-terminus of the 14 kDa fragment [14] with the sequences for TDC from *C. roseus* [15] and for dopa decarboxylase from *Drosophila melanogaster* [16,17], it can be deduced that the site of proteolytic cleavage might be lysine-350 of TDC. Cleavage at this site would yield fragments with calculated M_r values of 38 735 and 17 489. Similar to dopa decarboxylase [14], these fragments seem to be tightly bound in the enzyme since they cannot be isolated by the HIC step described in this paper.

Although the HIC step turned out to give only a slight additional purification (fig.1; and results of SDS-PAGE combined with silver staining, not shown), it is attractive for concentration of the enzyme and removal of exogenous PLP. The observed decrease in specific activity from 2.9 nkat/mg before injection to 2.1 nkat/mg in the collected fraction might be explained by the inactivation due to proteolysis, as discussed above.

An $A\ ^{1\ mg/ml}_{280\ nm}$ of 1.45 ± 0.02 was calculated for TDC from the UV absorbancies. With this value the specific activity of the final TDC preparation was calculated to be 3.4 nkat/mg protein, 1.6 times higher than the value based on the protein content determined by the method of [11]. The absorption spectrum in the 300–400 nm region (fig.2) is typical of a PLP-containing enzyme and is similar to that of dopa decarboxylase [18] and glutamic acid decarboxylase [19].

From the cofactor determinations it was calculated that TDC contains 2.4 molecules of PLP and 1.8 molecules of covalently-bound PQQ per enzyme molecule, assuming homogeneity of the enzyme and taking a molecular mass of 96 kDa. This suggests that each subunit contains one molecule of each of the cofactors. A content of 1 PLP per enzyme molecule has been reported by others [20]. An explanation for the difference might be the omission of PLP from the buffers [20], leading to extraction of the cofactor from the enzyme during the purification procedure. This view is supported by the finding [20] that PLP enhanced the activity

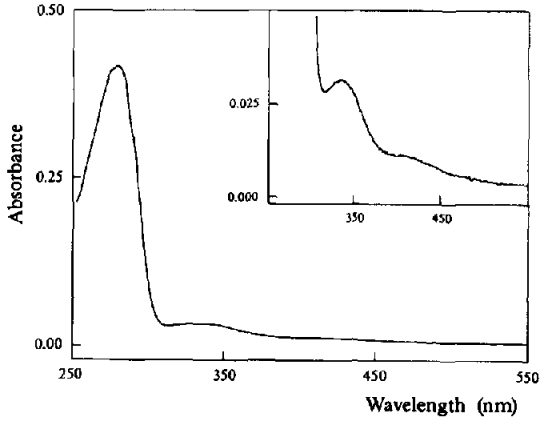

Fig.2. Absorption spectrum of TDC. The spectrum was measured in the preparation eluted from the TSK Phenyl-5PW column (pH 7.4).

of TDC between 2- and 3-fold, depending upon the degree of purity of the enzyme.

TDC from *C. roseus* is the third example of a decarboxylase which is a pyridoxo-quinoprotein. Although L-tyrosine and L-glutamic acid are the precursors of bacterial (free) PQQ [21], it is still unknown whether the same building blocks are being used for the biosynthesis of the covalently-bound form. As was recently demonstrated for glutamic acid decarboxylase from *E. coli* [5], the presence of free PQQ is not required for assemblage of the active enzyme. As suggested in [22], covalently-bound PQQ might be synthesized in situ, either from amino acids present in a pro-form of the enzyme or from building blocks supplied by the organism. If this view is correct and if the presence of covalently-bound PQQ is essential for functionality [23], it could have important implications for production of active *C. roseus* TDC in a different organism. In case the machinery for synthesis of covalently-bound PQQ works in a specific way, synthesis of functional TDC in e.g. *E. coli* would require additional genes from *C. roseus* for synthesis and/or insertion of the cofactor. Recent findings [15] in which TDC activity could be detected in *E. coli*, transformed with a plasmid containing the TDC cDNA insert from *C. roseus*, might point to rather unspecific reactions for the insertion of PQQ. Another possibility is that the enzyme has some basal activity in the absence of the cofactor. Further studies are required to clarify these points.

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