Cloning and expression of pig kidney dopa decarboxylase: comparison of the naturally occurring and recombinant enzymes

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L-Aromatic amino acid decarboxylase (dopa decarboxylase; DDC) is a pyridoxal 5′-phosphate (PLP)-dependent homodimeric enzyme that catalyses the decarboxylation of L-dopa and other L-aromatic amino acids. To advance structure–function studies with the enzyme, a cDNA that codes for the protein from pig kidney has been cloned by joining a partial cDNA obtained by library screening with a synthetic portion constructed by the annealing and extension of long oligonucleotides. The hybrid cDNA was then expressed in *Escherichia coli* to produce recombinant protein. During characterization of the recombinant enzyme it was unexpectedly observed that it possesses certain differences from the enzyme purified from pig kidney. Whereas the latter protein binds 1 molecule of PLP per dimer, the recombinant enzyme was found to bind two molecules of coenzyme per dimer. Moreover, the V_{max} was twice that of the protein purified from tissue. On addition of substrate, the absorbance changes accompanying transaldimination were likewise 2-fold greater in the recombinant enzyme. Examination of the respective apoenzymes by absorbance, CD and fluorescence spectroscopy revealed distinct differences. The recombinant apoprotein has no significant absorbance at 335 nm, unlike the pig kidney apoenzyme; in the latter case this residual absorbance is associated with a positive dichroic signal. When excited at 335 nm the pig kidney apoenzyme has a pronounced emission maximum at 385 nm, in contrast with its recombinant counterpart, which shows a weak broad emission at about 400 nm. However, the holoenzyme–apoenzyme transition did not markedly alter the respective fluorescence properties of either recombinant or pig kidney DDC when excited at 335 nm. Taken together, these findings indicate that recombinant pig kidney DDC has two active-site PLP molecules and therefore displays structural characteristics typical of PLP-dependent homodimeric enzymes. The natural enzyme contains one active-site PLP molecule whereas the remaining PLP binding site is most probably occupied by an inactive covalently bound coenzyme derivative; some speculations are made about its origin. The coenzyme absorbing bands of recombinant DDC show a modest pH dependence at 335 and 425 nm. A putative working model is presented to explain this behaviour.

INTRODUCTION

3,4-Dihydroxyphenylalanine (dopa) decarboxylase (DDC; EC 4.1.1.28) is a homodimeric pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyses the conversion of L-dopa and L-5-hydroxytryptophan to their corresponding amines. The enzyme will also accept other catechol- or indole-related L-amino acids and has therefore also been described as L-aromatic amino acid decarboxylase [1–4]. In addition to the former reaction, the enzyme also catalyses a minor side reaction, referred to as decarboxylation-dependent transamination, which occurs to varying extents depending on the substrate [5,6]. Most of the information about the enzyme has been obtained from pig kidney, although DDCs from several sources have been examined. DDC from pig kidney has a molecular mass of about 54 kDa and contains 1 molecule of PLP per enzyme dimer [7]. In addition to the usual absorbance at 280 nm, the enzyme displays absorbance bands at 335 and 425 nm due to bound coenzyme. Atypically for PLP-dependent enzymes, the apoenzyme retains an unexplained catalytically inactive absorbance at 335 nm [5,7]. Several amino acids have been identified by chemical modification that seem to be critical for enzymic catalysis. In particular, a cysteine, an arginine and a histidine residue have been implicated in the reaction mechanism [8–10]. The complete primary structure has been determined by Edman degradation [11] and crystallographic studies are in progress [12]. Although DDC has been cloned from human, rat, guinea pig, *Drosophila* and bovine sources [13–17], only rat liver DDC has been expressed to produce recombinant protein for structural/mechanistic studies [18].

Because the enzyme from other sources has remained relatively uncharacterized, progress in understanding the structure/function relationships operating in this enzyme has been hampered by the lack of a cDNA clone for pig kidney (pk)DDC with the ultimate goal of producing recombinant protein and site-directed mutants. Toward this end, a full-length pig kidney cDNA coding for DDC has been constructed by joining a partial clone isolated from a cDNA library with a synthetic portion of about 700 bp obtained by the annealing and extension of long oligonucleotides. The resulting cDNA was then cloned into an expression vector to produce recombinant enzyme in *Escherichia coli*. The recombinant protein has been purified to homogeneity and compared with the enzyme purified from tissue. Interestingly, recombinant DDC possesses certain differences from the enzyme purified from a natural source. These distinctions raise the possibility of clarifying several peculiarities present in the nat-

Abbreviations used: DDC, L-aromatic amino acid decarboxylase (EC 4.1.1.28); DTT, dithiothreitol; L-5-HTP, L-5-hydroxytryptophan; PLP, pyridoxal 5′-phosphate; pk, pig kidney.

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urally occurring protein. The significance of these variances is discussed below.

MATERIALS AND METHODS

Materials

Restriction and DNA-modifying enzymes were purchased from Promega, Pharmacia or New England Biolabs. Radionucleotides were from Amersham. Sequenase was from U.S. Biochemicals. Protease inhibitors were from Sigma. A pig kidney λgt11 cDNA library was kindly provided by Dr. Claudia Evans and Dr. Paul Srere (V.A. Medical Center, University of Texas, Dallas, TX, U.S.A.). A λgt10 pig liver cDNA library was purchased from Clontech. A near full-length cDNA clone for DDC from rat phaeochromocytoma was a gift from Dr. J. Thibault (Biochimie Cellulaire, Collège de France, Paris, France). Oligonucleotides were synthesized by Professor A. Galizzi (Dipartimento di Genetica, Università di Pavia, Pavia, Italy). pkDDC was purified to homogeneity as previously described [19]. All other reagents were of the highest purity available from commercial sources.

Screening of cDNA libraries and isolation of a partial cDNA for pkDDC

A λgt11 cDNA library was plated on 150 mm plates in *E. coli* Y1090 at a density of about 30000 plaque-forming units per plate. The plaques were then immobilized on nylon membranes and processed by standard procedures [20]. After prehybridization at 42 °C, a rat DDC probe (bp 13–1404) labelled by random priming was added to the mixture and hybridized overnight at the same temperature. After the final wash at 50 °C in 0.1 × SSC containing 0.1 % SDS, the filters were then autoradiographed. Positive signals were isolated and rescreened until all plaques were positive. DNA was then prepared from plate lysates, analysed by restriction digestion and subcloned into plasmid vectors. After over 1.5×10^6 plaques had been screened, seven positive clones were obtained, the largest of which, designated 3L, had an insert of 1.3 kb.

Synthesis of a partial cDNA coding for the N-terminus of pkDDC

A synthetic fragment coding for residues 1–222 was obtained by the extension of mutually priming long oligonucleotides as four subfragments covering nucleotides −9 to 667. Codon usage from *E. coli* was used to design the synthetic portion. Each subfragment was made from pairs of mutually priming oligonucleotides. The four fragments, the nucleotides covered, and the restriction sites used for cloning are as follows: EF, −9 to 173, *Eco*RI–*Pst*I; GH, 173–343, *Pst*I–*Xho*I; AB, 343–496, *Xho*I–*Aat*II; CD, 496–667, *Aat*II–*Pst*I. At least four nucleotides were included beyond restriction sites to allow for cleavage; overlaps for annealing ranged from 18 to 20 nucleotides. Eight oligonucleotides varying in length from 98 to 110 bp were synthesized and gel-purified. Reaction mixtures were set up in a final volume of 50 μl containing 0.5 μg each of the appropriate oligonucleotides, 4 mM dNTP, and 1 × reaction buffer (New England Biolabs). The mixtures were then incubated at 75 °C for 5 min followed by slow cooling to allow for annealing. Vent DNA polymerase (1–2 units) was added and the elongation was allowed to proceed for 4 min at 75 °C. The DNA was then extracted with phenol/chloroform, precipitated with ethanol, digested with the appropriate restriction enzymes and ligated into plasmid vectors. The products of annealing, extension and digestion were analysed by PAGE [5 % (w/v) gel]. As fragment GH did not elongate efficiently, PCR was performed with 18 bp primers to amplify the fragment. All the resulting nucleotide sequences were verified.

Assembly of a full-length cDNA coding for pkDDC

By using the restriction sites indicated above, fragment AB was inserted into a plasmid containing fragment CD to give pDDCPX340(SK). The resulting *Xho*I–*Pst*I fragment was then cloned into the same sites in pUC18X to create pDDCPX340. (pUC18X was created by digestion of pUC18 with *Sma*I followed by the addition of an *Xho*I linker). The wild-type pkDDC cDNA (p3L) was digested with *Hin*dIII, and partly digested with *Pst*I to obtain a 950 bp fragment, which was then inserted into the *Pst*I–*Hin*dIII sites of pDDCPX340, creating pDDCXH1250. Using the above indicated sites, fragments EF and GH were joined in a three-fragment ligation. The resulting plasmid was then digested with *Eco*RI and *Xho*I and the insert was transferred into the same sites of pDDCXH1250 to create pDDC1600, which contains the full-length cDNA.

DNA sequence analysis

Single-stranded DNA was prepared from clone 3L and various subframents, and subjected to dideoxy sequencing by using modified T7 DNA polymerase and [^{35}S]dATP. All restriction junctions were sequenced. DNA sequencing of the oligonucleotide clones was performed by denaturation of double-stranded plasmid DNA.

Expression of pkDDC in *E. coli*

The *Eco*RI–*Hin*dIII fragment from pDDC1600 was excised and cloned in the same sites of pKK223-3 (Pharmacia) to yield pKKDDC. There was no detectable expression with this plasmid. To optimize the distance between the ribosomal binding site and the initiator ATG, pKKDDC was digested with *Eco*RI and treated with mung bean nuclease, removing 4 bp of the *Eco*RI site to create pKKDDCΔ4. In JM109 this plasmid expressed low levels of DDC that were not inducible with isopropyl β-D-thiogalactoside. The cDNA was also inserted into pET-3a (Novagen) using the *Nde*I site at −1; when transformed into BL21(DE3), expression levels were less than one-tenth of those obtained with pKKDDCΔ4 in JM109, and were uninducible. The latter system was therefore used to obtain recombinant enzyme. Routinely, approximately 5 litres of LB broth containing 150 mg/ml ampicillin was inoculated 1:20 with an overnight culture and allowed to grow for 6–8 h at 37 °C. The cells were then processed as described below.

Purification of recombinant pkDDC

Extract preparation

From the above-mentioned culture the cells were pelleted and resuspended in 10 mM potassium phosphate buffer, pH 7.4, containing 10 μM PLP, 100 μM dithiothreitol (DTT), 1 mM EDTA and 1 mM PMSF. Lysozyme was then added to a concentration of 100 μg/ml followed by a 15 min incubation at room temperature. After a freeze–thaw, leupeptin (0.5 μg/ml) and pepstatin (0.7 μg/ml) were added and the suspension was then centrifuged at 30000 ***g*** for 30 min at 4 °C.

DEAE-Biogel chromatography

The cleared lysate was diluted to approximately 20 mg/ml and loaded on a DEAE-Biogel column (3.5 cm × 10 cm) previously

equilibrated with 10 mM potassium phosphate buffer, pH 7.4, containing 100 μM DTT and 10 μM EDTA at 4 °C. The column was then washed with equilibration buffer at a flow rate of 1.5 ml/min until the A_{280} of the eluent was about 0.1. The enzyme was eluted with 250 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 100 μM DTT and 10 μM EDTA. The active fractions were pooled and loaded directly on FPLC.

Phenyl-Sepharose chromatography

FPLC was performed at room temperature. The combined eluent from the DEAE-Biogel column was adjusted to 400 mM with potassium phosphate buffer, and loaded on a HiLoad 26/10 Phenyl-Sepharose HP column (Pharmacia) previously equilibrated with 400 mM potassium phosphate buffer, pH 7.0, containing 100 μM DTT and 10 μM EDTA, at a flow rate of 4 ml/min. After the elution of the void volume, a linear gradient (120 min; 100–0 % B) was inserted at a flow rate of 6 ml/min with B as 50 mM potassium phosphate buffer, pH 7.0, containing DTT and EDTA. Absorbance was monitored at 280 nm; the peak eluting at about 60 % B was collected and concentrated by ultrafiltration.

Enzyme assays

DDC activity was measured as described by Sherald et al. [21] as modified by Charteris and John [22]. Reactions contained 100 μM PLP except when kinetic measurements were made. One unit of enzymic activity is defined as the amount of protein that catalyses the production of 1 nmol of amine/min. The enzyme concentration was determined by using an ϵ_m of 1.3×10^5 $M^{-1} \cdot cm^{-1}$ [23].

Apoenzyme preparation

Apoenzyme was prepared by incubation of about 1–2 mg of protein with 5 mM hydroxylamine in 0.5 M potassium phosphate buffer, pH 6.8, containing 100 μM DTT; this was followed by gel-filtration (0.9 cm × 40 cm column) in the same buffer without hydroxylamine. The enzyme was then concentrated and washed in microconcentrators with 0.1 M potassium phosphate buffer, pH 6.8, containing 100 μM DTT. For reconstitution of the apoenzyme, a 5-fold molar excess of PLP was added, followed by gel-filtration in 0.1 M potassium phosphate buffer, pH 6.8, containing 100 μM DTT, to remove unbound PLP.

Spectroscopy

Absorbance measurements were made with a Jasco V-550 spectrophotometer. Fluorescence spectra were taken with a Kontron SFM 25 spectrofluorimeter, using 5 nm bandwidths on both sides. CD spectra were obtained with a Jasco 710 spectropolarimeter with a thermostat-controlled cell compartment at 20 °C. For near-UV and visible wavelengths, protein concentrations were from 0.6 to 1 mg/ml in a cuvette with a 1 cm path length. Routinely, five spectra were recorded at a scan speed of 50 nm/min with a bandwidth of 2 nm, and averaged automatically. For the far UV, the protein concentration was from 0.05 to 0.1 mg/ml in a cuvette with a 0.1 cm path length.

RESULTS

Isolation of a cDNA clone for pkDDC

To obtain a cDNA coding for pkDDC, a λgt11 pig kidney library was screened by using a rat DDC cDNA, which yielded seven positive clones. The longest of these clones (3L) had an insert of 1.3 kb. DNA sequence analysis confirmed that hypothetical translation of clone 3L codes for pkDDC starting at amino acid 207 giving a product identical to the protein sequence determined by Edman degradation [11] with the exception of an additional serine at the C-terminus (Figure 1). It seems most likely that this residue escaped detection during protein sequence analysis. Screening of a λgt10 pig liver cDNA library gave no positive signals. Construction of a pig kidney cDNA library from

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-10                                        GAATTCACATATGAATGCCAGCGATTTC
                                                       M  N  A  S  D  F     6

19   CGTCGACGCGGCAAAGAAATGGTGGATTACATGGCGGATTACCTGGAAGGCATCGAAGGT
      R  R  R  G  K  E  M  V  D  Y  M  A  D  Y  L  E  G  I  E  G     26

79   CGTCAGGTGTACCCGGATGTGCAGCCGGGGTACCTGCGTCCGCTGATCCCGGCGACCGCC
      R  Q  V  Y  P  D  V  Q  P  G  Y  L  R  P  L  I  P  A  T  A     46

139  CCGCAGGAACCGGATACCTTCGAAGATATCCTGCAGGATGTGGAAAAAATCATCATGCCG
      P  Q  E  P  D  T  F  E  D  I  L  Q  D  V  E  K  I  I  M  P     66

199  GGGGTGACCCACTGGCACAGCCCGTACTTCTTCGCGTACTTCCCGACCGCCAGCAGCTAC
      G  V  T  H  W  H  S  P  Y  F  F  A  Y  F  P  T  A  S  S  Y     86

259  CCGGCGATGCTGGCGGATATGCTGTGCGGTGCGATCGGATGCATCGGTTTCAGCTGGGCG
      P  A  M  L  A  D  M  L  C  G  A  I  G  C  I  G  F  S  W  A    106

319  GCTAGCCCGGCGTGCACCGAACTCGAGACCGTGATGATGGATTGGCTGGGCAAAATGCTC
      A  S  P  A  C  T  E  L  E  T  V  M  M  D  W  L  G  K  M  L    126

379  CAGCTTCCGGAAGCGTTCCTGGCGGGCGAAGCCGGTGAAGGCGGCGGCGTGATCCAGGGT
      Q  L  P  E  A  F  L  A  G  E  A  G  E  G  G  G  V  I  Q  G    146

439  AGCGCCAGCGAAGCCACCCTGGTGGCGCTGCTGGCGGCGCGTACCAAAGTGGTGCGACGT
      S  A  S  E  A  T  L  V  A  L  L  A  A  R  T  K  V  V  R  R    166

499  CTGCAAGCGGCGAGCCCGGGCCTGACCCAGGGCGCGGTGCTGGAAAAACTAGTGGCGTAC
      L  Q  A  A  S  P  G  L  T  Q  G  A  V  L  E  K  L  V  A  Y    186

559  GCGAGTGATCAGGCGCACAGCAGCGTGGAACGTGCCGGCCTGATCGGCGGCGTGAAACTG
      A  S  D  Q  A  H  S  S  V  E  R  A  G  L  I  G  G  V  K  L    206

619  AAAGCGATCCCGAGCGATGGCAAATTCGCGATGCGTGCGAGCGCGCTGCAGGAGGCCCTG
      K  A  I  P  S  D  G  K  F  A  M  R  A  S  A  L  Q  E  A  L    226

679  GAGAGAGACAAGGCTGCCGGCCTGATTCCTTTCTTCGTGGTGGCTACGCTGGGGACCACA
      E  R  D  K  A  A  G  L  I  P  F  F  V  V  A  T  L  G  T  T    246

739  TCGTGCTGCTCCTTTGACAATCTCTTAGAAGTGGGACCCATCTGTCACGAAGAGGACATA
      S  C  C  S  F  D  N  L  L  E  V  G  P  I  C  H  E  E  D  I    266

799  TGGCTGCACGTGGATGCTGCCTACGCAGGCAGTGCCTTCATCTGCCCTGAGTTCCGGCAC
      W  L  H  V  D  A  A  Y  A  G  S  A  F  I  C  P  E  F  R  H    286

859  CTGCTGAATGGAGTGGAGTTTGCAGATTCATTTAACTTTAATCCCCACAAATGGCTCTTG
      L  L  N  G  V  E  F  A  D  S  F  N  F  N  P  H  K  W  L  L    306

919  GTGAATTTTGACTGCTCGGCTATGTGGGTGAAAAGGAGAACGGACCTGACTGGAGCCTTC
      V  N  F  D  C  S  A  M  W  V  K  R  R  T  D  L  T  G  A  F    326

979  AAATTGGACCCCGTGTACTTAAAGCACAGCCACCAGGGCTCGGGGCTTATCACGGACTAC
      K  L  D  P  V  Y  L  K  H  S  H  Q  G  S  G  L  I  T  D  Y    346

1039 AGGCACTGGCAGCTGCCACTGGGTCGGCGATTCCGGTCCCTGAAAATGTGGTTTGTTTTT
      R  H  W  Q  L  P  L  G  R  R  F  R  S  L  K  M  W  F  V  F    366

1099 AGGATGTACGGAGTCAAGGGACTGCAGGCCTATATCCGCAAGCACGTGCAGCTGTCTCAT
      R  M  Y  G  V  K  G  L  Q  A  Y  I  R  K  H  V  Q  L  S  H    386

1159 GAGTTTGAGGCATTTGTGCTTCAGGATCCACGCTTTGAAGTCTGTGCCGAAGTCACCCTG
      E  F  E  A  F  V  L  Q  D  P  R  F  E  V  C  A  E  V  T  L    406

1219 GGGCTGGTGTGTTTCCGGCTGAAGGGCTCCGACGGACTGAATGAAGCGCTTCTGGAAAGG
      G  L  V  C  F  R  L  K  G  S  D  G  L  N  E  A  L  L  E  R    426

1279 ATAAACAGCGCCAGGAAAATCCACTTGGTTCCCTGTCGCCTGAGGGGCCAGTTCGTGCTG
      I  N  S  A  R  K  I  H  L  V  P  C  R  L  R  G  Q  F  V  L    446

1339 CGGTTCGCCATCTGCTCGCGCAAGGTGGAGTCGGGCCACGTGCGGCTGGCCTGGGAGCAC
      R  F  A  I  C  S  R  K  V  E  S  G  H  V  R  L  A  W  E  H    466

1399 ATCCGAGGGCTGGCGGCCGAGCTGCTGGCCGCGGAGGAGGGAAAGGCAGAGATCAAAAGT
      I  R  G  L  A  A  E  L  L  A  A  E  E  G  K  A  E  I  K  S    486

1459 TGAAGTGCCCTGAAGAGCAGAATCGGAGGAGACGCGTCGTCCCCGCTCCGAGGCGTAGAG
      *
1519 CCTGCAATGGTCCCCCCAGTTCTTTAGCCCACGTTCTCCAGAAAGAAGCTTGTGCCTACG
1579 TCTGACCAGCCTCTCAGCAATGAAGAAGTATTATTTGCTCTTTGAAAAGTTAATCCCAGT
1639 GGAGACAGCTTTTACTCTTTATTTGGCTGTGATTGTTTGTTGATTAAAACATCATAGGTT
1699 TCTGCATCCTTGAAGTTGTCAGCGGTGGTCCACTTTCCGGGGCAACCTATGCTGATGGGA
1759 TTTGAGATGATACCCGTGGTCTTTAAATTACTCTGTCCTGTGGCTTATGCTTAATAAATG
1819 ATGTGAAGTGTAAAAAAAAAAAAAAAAAAA
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Figure 1 DNA sequence and hypothetical translation of pDDC1600

The sequence shown from nucleotide 667 to the end of the cDNA is derived from clone p3L and is the wild-type coding sequence. The sequence preceding nucleotide 667 was obtained by synthetic means as described in the text and in Figure 2. The nucleotide sequence is numbered on the left-hand side; protein sequence is numbered on the right-hand side. The stop codon is indicated with an asterisk. The sequence downstream from the *Hin*dIII site at 1566 has also been included. A potential polyadenylation site is underlined.

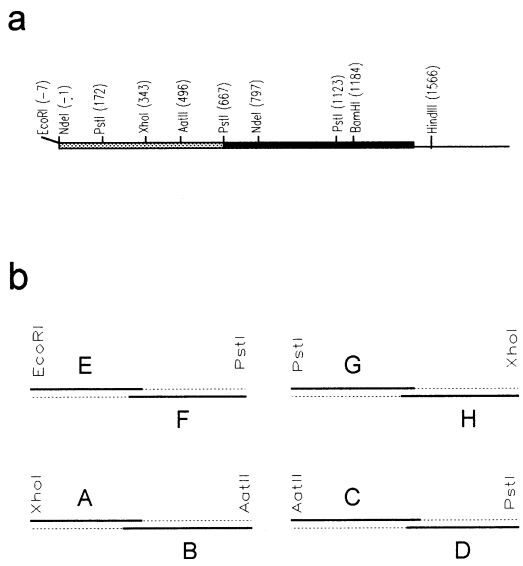


Figure 2 Restriction map of the full-length cDNA for pkDDC and synthesis strategy for the N-terminus of the cDNA

(**a**) Restriction map of cloned DDC. The wild-type coding sequence is represented by a thick black line, the synthetic coding sequence by a thick grey line and the non-coding portion by a thin line. Relevant restriction sites are as indicated. (**b**) Strategy for the synthesis of a cDNA coding for the N-terminus of pkDDC. The indicated oligonucleotides (indicated by a thick line) were synthesized and processed as described in the text. The fill-in reaction is shown by dotted lines. The extended oligonucleotide pairs were then digested with the appropriate restriction enzymes and cloned. The fragments were then assembled as outlined and joined to the wild-type partial cDNA.

poly(A)$^+$ RNA in λgt10 unfortunately resulted in positive clones that were shorter than clone 3L (P. S. Moore, unpublished work). As library screening seemed to be ineffectual, an alternative strategy was used to obtain a full-length cDNA for pkDDC.

Because the primary structure of the protein has been determined, a coding region based on the codon bias from *E. coli* was designed, to obtain a complete cDNA by synthetic means (Figures 1 and 2). In this strategy, pairs of mutually priming long oligonucleotides are annealed, extended and then cloned (Figure 2). This protocol differs slightly from previous protocols (see, for example, [24,25]) in that Vent DNA polymerase is used to extend the primed oligonucleotides, giving two advantages. First, problems with secondary structure are minimized because the extension reactions are carried out at high temperature. Secondly, Vent DNA polymerase apparently has a lower error rate than other commonly used polymerases such as Sequenase, Klenow or Taq. Even though the fragments are relatively small, this problem can be significant; with the other three polymerases errors were invariably incorporated (P. S. Moore, unpublished work). The synthesis was performed as four separate fragments, each approx. 175 bp, covering a final length of 670 bp (Figure 2). These synthetic fragments were then assembled along with the isolated partial cDNA to create a hybrid synthetic–wild-type full-length cDNA coding for pkDDC.

Expression of pkDDC in *E. coli*

The cDNA was initially cloned into pET-3a by using the *Nde*I site at the initiator ATG and transformed in BL21(DE3). In this system, extremely low levels of DDC activity were observed that were uninducible by isopropyl β-D-thiogalactoside (results not shown). The cDNA was then cloned into pKK223-3 and transformed into JM109; no detectable activity was observed. To optimize the distance between the initiator ATG and the ribosome binding site, 4 bp of the *Eco*RI site was removed to create pKKDDCΔ4. This plasmid showed activity levels that were about 10-fold higher than those observed in BL21(DE3) under the control of a T7 promoter but were, however, uninducible. None the less, modest quantities of enzyme can be purified from bacteria harbouring pKKDDCΔ4.

Purification of recombinant pkDDC

The purification scheme used is presented in Table 1. After an initial clearing of the extract, a DEAE-Biogel chromatography step was performed and the enzyme was eluted with a step gradient. This eluate was then passed over a hydrophobic column in FPLC. The peak eluting at about 60% B contained homogeneous enzyme with a specific activity of about 5000 nmol/min per mg. The recombinant enzyme has an electrophoretic mobility identical with that of the enzyme purified from pig kidney. As for the latter protein, the recombinant enzyme shows a single band after SDS/PAGE analysis (results not shown). Direct N-terminal sequence analysis of this material revealed the expected MNASD, with no underlying sequences, as further confirmation of its identity and purity.

Spectral properties of recombinant pkDDC

The absorbance spectra of the recombinant enzyme is shown in Figure 3(A). In addition to the protein absorption at 280 nm, two peaks are observed at 335 and 425 nm characteristic of the bound coenzyme. The ratio of the two bands consistently falls between 2.2 and 2.4; on addition of NaOH to 0.1 M, 1.8–2.2 mol of PLP are released per mol of enzyme dimer, depending on the preparation. For comparison, the spectrum of the enzyme purified from tissue is shown in Figure 3(B). The ratio of the 335

Table 1 Summary of purification of pkDDC expressed in *E. coli*

Step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cleared lysate	210	4620	35280	7.6	100	1.0
DEAE-Biogel	78	146	30708	209	87	28
Phenyl-Sepharose	118	4.4	22435	5135	64	675

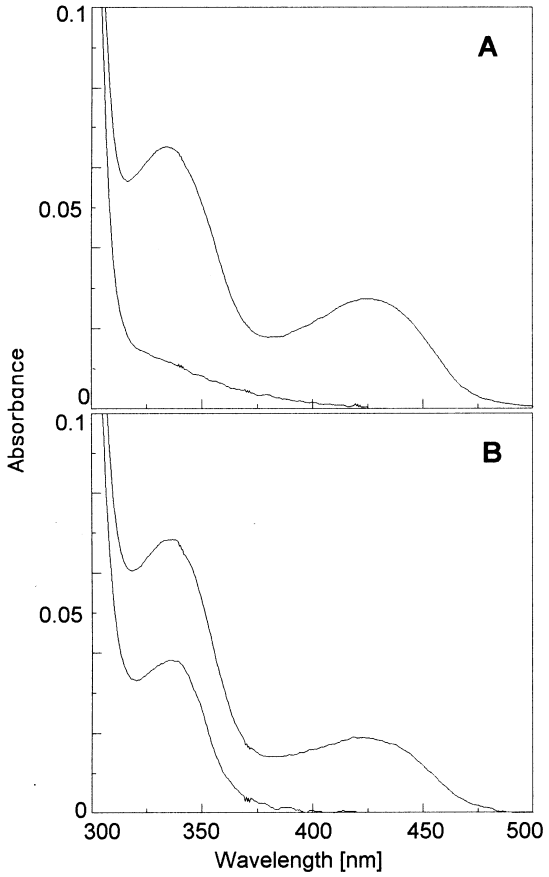


Figure 3 Absorbance spectra of recombinant and naturally occurring pkDDC holoenzymes and apoenzymes

Spectra were taken in 0.1 M potassium phosphate buffer, pH 6.8, containing 100 μM DTT. (**A**) Recombinant DDC holoenzyme is shown in the upper spectrum. The apoenzyme is seen in the lower spectrum. (**B**) Tissue-purified pkDDC. The holoenzyme is represented by the upper spectrum. The spectrum of the apoenzyme is the lower tracing. The enzyme concentration was 10 μM.

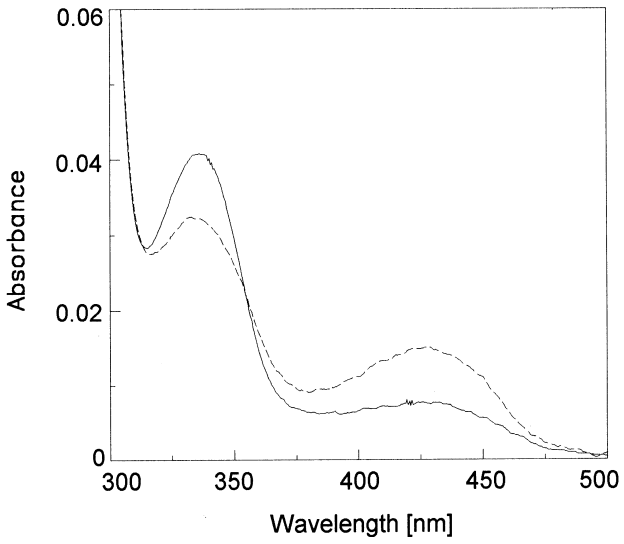


Figure 4 Absorbance spectra of recombinant DDC at pH 6.5 and 8.4

Spectra were taken in 0.1 M potassium phosphate buffer at pH 6.5 (broken line) and pH 8.4 (solid line) at an enzyme concentration of 4 μM.

and 425 nm coenzyme-associated bands is somewhat higher, ranging from about 3.4 to 3.8. In the presence of 0.1 M NaOH, 0.9–1.1 mol of PLP are generally released per mol of enzyme dimer [1,7].

Spectra of the recombinant enzyme were also taken in the pH range 6.0–9.0; at these pH extremes both absorbing species are still clearly visible. Although no differences were observed from pH 6.0 to 7.0, further increasing the pH caused a decrease in the 425 nm species accompanied by a corresponding increase in the band at 335 nm. This transition seems to take place in the pH range from 7.5 to 8.0. The spectra of the recombinant enzyme at pH 6.5 and 8.4 are shown in Figure 4. Although a more detailed analysis is required for an accurate determination of a pK for this transition, a precise evaluation is complicated by the fact that the differences between the two absorbance bands are rather small, and therefore is prone to error unless large amounts of enzyme are used. This pH dependence is similar to what has been previously seen for the tissue-purified enzyme [7,26].

Characteristics of the recombinant apoenzyme

As attempts to prepare recombinant apoenzyme by using 0.1 M hydroxylamine [5] resulted in extremely modest levels of re-activation (about 15–20%), a more desirable protocol was sought. After subsequent modification of the procedure, the resulting apoenzyme had a residual activity that did not exceed 0.5%, whereas the reconstituted enzyme completely recovered its original activity after incubation with excess PLP. As shown in Figure 3(A), the absorbance spectrum of the recombinant apoenzyme has almost no remaining absorbance in the visible region. In contrast, the apoprotein prepared from enzyme purified from tissue displays different characteristics. With the modified method to remove the coenzyme, the protein has a similar amount of residual activity and likewise all of its original activity is restored after the addition of PLP. However, as is shown in Figure 3(B), a significant proportion of the 335 nm peak remains associated with the protein. In both cases reconstitution of holoenzyme (as described in the Materials and methods section) restores the original absorbance spectral properties (results not shown). Although the re-activation percentages are greatly improved with respect to the previous protocol (which gave re-activations of about 40%), the apoprotein consistently retains the 335 nm band. Denaturation of this apoenzyme in 5 M guanidinium chloride and passage over a gel-filtration column equilibrated with denaturant does not remove the 335 nm absorbing band (results not shown).

CD spectra of holoproteins and apoproteins

It has been shown that the protein from pig kidney displays two positive CD bands at 335 and 425 nm, reflecting the asymmetry of the coenzyme bound at the active site (Figure 5B) [7,23]. Similar positive Cotton effects are seen for the recombinant enzyme (Figure 5A). It is also shown here that both enzymes possess positive dichroic bands in the aromatic region at 275–285, 288 and 296 nm (Figures 5A and 5B), which would indicate the asymmetry of certain aromatic amino acids, most probably associated with the active site. Whereas the 288 and 296 nm bands can most probably be attributed to tryptophan residue(s), the broad overlapping region from 275 to 285 nm may be due to the contribution of both tryptophan and tyrosine residues. The CD spectrum of recombinant apoDDC is completely devoid of

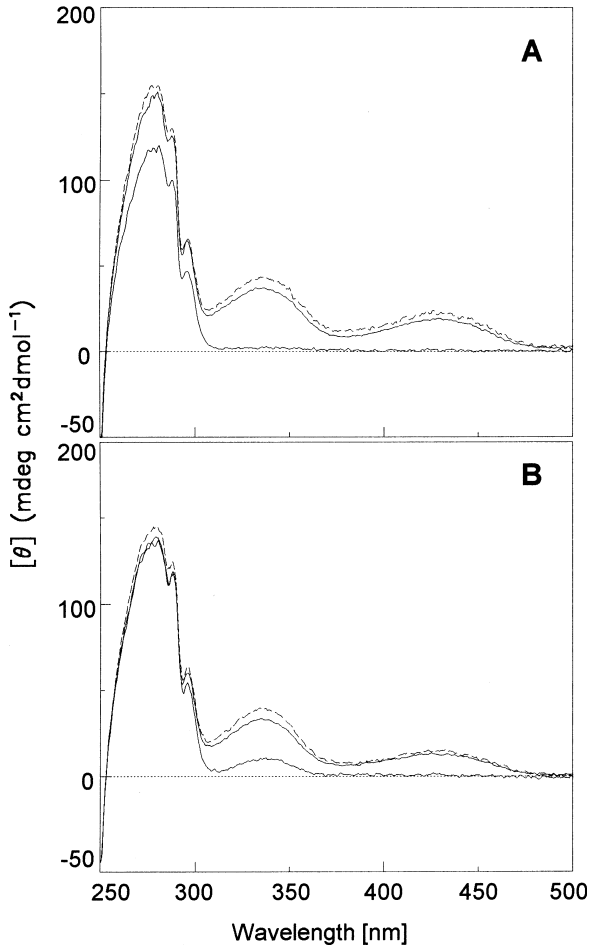


Figure 5 Near-UV and Visible CD spectra of recombinant and naturally occurring pkDDC holoenzyme, apoenzyme and reconstituted enzyme

Spectra were recorded in 0.1 M potassium phosphate buffer, pH 6.8, containing 100 μM DTT. (**A**) Recombinant holoenzyme is represented by the solid line in the upper spectrum, the corresponding apoenzyme by the bottom spectrum and the reconstituted apoenzyme by the broken line. (**B**) The tissue-purified enzyme is represented by the solid line in the upper tracing, the corresponding apoenzyme by the lower spectrum and the reconstituted protein by the broken line.

the coenzyme-associated dichroic bands, accompanied by a slight decrease in the aromatic region (Figure 5A). In contrast, the naturally occurring apoenzyme is devoid of the 425 nm band, but still retains a significant fraction of the 335 nm band (Figure 5B); the positive signal around 280 nm remains unaltered. On reconstitution of recombinant holoenzyme, the 335 and 425 nm coenzyme-associated bands are restored to an intensity comparable to that of the starting material. Similarly, in the aromatic region the positive signal returns to its original intensity (Figure 5A). The reconstitution of the naturally occurring enzyme also restores all the positive coenzyme-associated dichroic bands (Figure 5B); the aromatic region remains unaltered throughout this process. For both proteins, the far-UV CD spectra remain unmodified between the holoproteins and apoproteins (results not shown).

Fluorescence properties

As for the naturally occurring protein [23], on excitation of the recombinant holo- and apoenzymes at 280 nm a characteristic

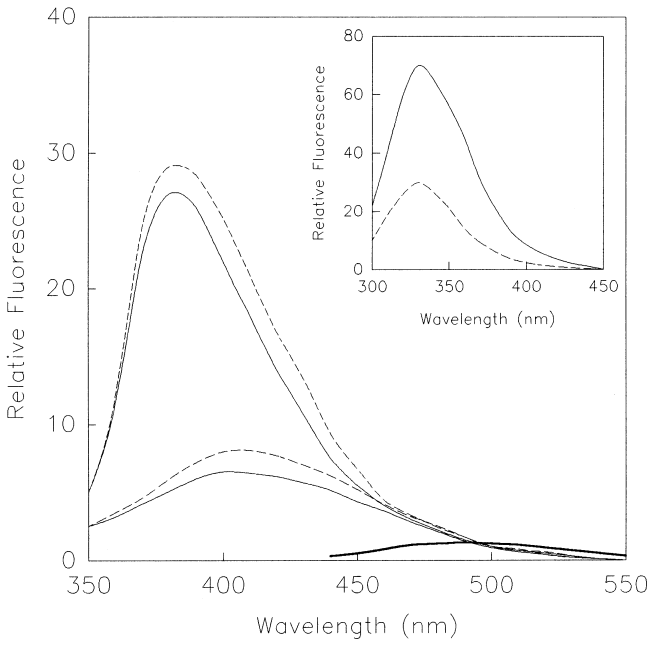


Figure 6 Fluorescence spectra of recombinant and naturally occurring pkDDC holoenzyme and apoenzyme

Excitation was at 335 nm. The upper two tracings were obtained from the tissue-purified enzyme. The lower two tracings are from the recombinant enzyme. Holoenzymes are indicated by the solid lines, and the corresponding apoproteins by broken lines. The thick line at the bottom right indicates the fluorescence when excited at 420 nm (only one tracing is indicated because for both the tissue-purified and recombinant holoenzymes the emission at around 490 nm is practically superimposable). This faint signal apparently disappears in the respective apoproteins. Inset: intrinsic fluorescence of recombinant DDC: broken line, holoenzyme; solid line, apoprotein. Excitation was at 280 nm. For all spectra, the protein concentration was 0.5 μM in 50 mM potassium phosphate, pH 6.8.

emission maximum at about 326 nm is seen (Figure 6, inset). However, the quantum yield of the holoenzyme fluorescence is considerably less than that of the respective apoenzyme owing to quenching by bound PLP. This phenomenon is more pronounced for the recombinant enzyme than for the tissue-purified protein (Figure 6, inset) [23]. When the recombinant enzyme is excited at 335 nm, a weak broad emission is observed centred at about 400 nm (Figure 6). For the naturally occurring protein, a well defined emission maximum is seen at 385 nm with a much higher fluorescence intensity (Figure 6) [7]. Excitation spectra verified that these emissions are due to the 335 nm absorbing peaks, although as expected the excitation peak is better perceived in the natural enzyme (results not shown). Fluorescence emission spectra obtained from the apoproteins revealed that this emission is slightly higher with respect to the intensity seen in the respective holoenzymes (Figure 6). As the intensity of this emission is not decreased in the corresponding apoenzymes, it can be deduced that this fluorescence is not due to active PLP. As previously observed for the enzyme purified from tissue [7], excitation of the recombinant enzyme at 425 nm results in an extremely faint emission at about 490 nm, which apparently does not remain in the apoenzyme (Figure 6).

Reaction of recombinant DDC with substrates

For the recombinant enzyme, the K_m for L-dopa is close to 200 μM, which is in agreement with that previously found for the naturally occurring protein [4]. The V_{max}, however, is sub-

stantially higher, being 5959 nmol/min per mg compared with 2881 nmol/min per mg [4]. It should be noted that the latter value of V_{max} has been corrected for the difference in ϵ_m, which was originally underestimated and later determined more accurately [23]. As for the natural protein, the addition of saturating concentrations of L-5-hydroxytryptophan (L-5-HTP) to the recombinant enzyme leads to the conversion of the internal aldimine at 425 nm to an external aldimine that absorbs at 430 nm [4,27]. Recombinant DDC also shows a doubling of the absorbance change at 430 nm after saturation with L-5-HTP when compared with the natural enzyme (results not shown). Taken together these results suggest that the recombinant enzyme has two catalytically active PLP binding sites. The enzyme is also capable of transamination, as evidenced by the observation that in the presence of α-methyldopa the 425 nm absorbing band gradually disappears while a new absorbance at 325 nm appears owing to the formation of pyridoxamine 5′-phosphate [5]. Thus the recombinant enzyme is capable of performing decarboxylation-dependent transamination, as is the protein purified from tissue.

DISCUSSION

The availability of a full-length clone for pkDDC obtained by a modified protocol for gene synthesis has permitted the expression of the enzyme in *E. coli*. The finding that the enzyme has an additional serine residue at the C-terminus results in a calculated subunit molecular mass of 53936 kDa. It is unfortunate that conditions for overexpression of the protein have not yet been found. It is possible that the protein is not well tolerated; however, systems that should nevertheless allow the production of proteins that are detrimental to the cell do not increase the expression of the cDNA. The fact that the cDNA has about one-half of the coding region optimized for *E. coli* does not have any apparent benefit in the case of DDC. In spite of its uninducibility the enzyme can be easily purified at levels sufficient for characterization.

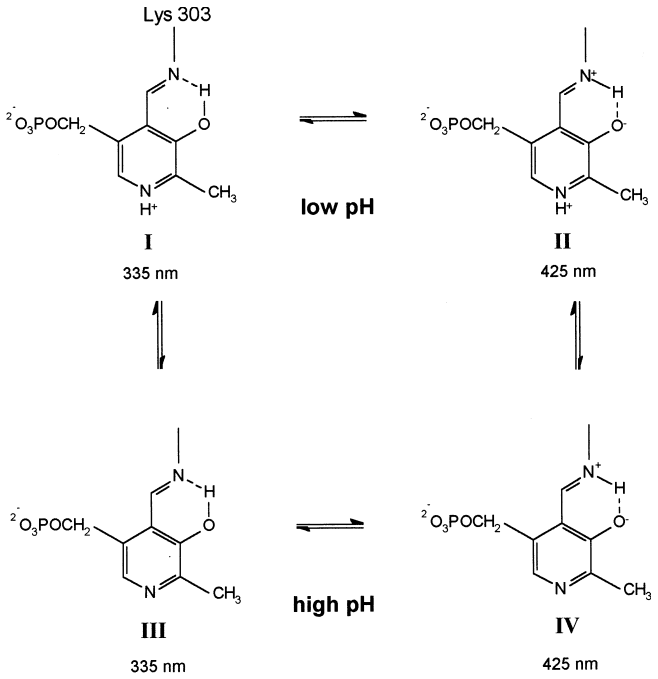
Recombinant DDC binds two molecules of PLP per enzyme dimer, in contrast with the enzyme purified from pig kidney, which normally releases about 1 molecule of PLP per enzyme dimer. Moreover, the recombinant enzyme shows a doubling in V_{max} and in the absorbance change occurring on external aldimine formation. This indicates that the recombinant enzyme has two catalytic binding sites whereas the tissue-purified enzyme has one functional active site per dimer.

For both the naturally occurring and recombinant enzymes, completely resolved apoenzymes that regain all of their original activity upon addition of PLP were obtained. An important difference in the spectroscopic properties of the respective apoenzymes was observed. Although the recombinant apoprotein does not have a significant absorbance at 335 nm, the apoprotein prepared from the tissue-purified enzyme retains a notable catalytically incompetent 335 nm absorbance, which is associated with a positive dichroic band at the same wavelength. It has been speculated that the 335 nm species in apoDDC is a substituted aldamine or is related to one of the adducts found in the various subforms of aspartate aminotransferase [5,18]. When excited at 335 nm, the tissue-purified enzyme in the apo form shows a significant emission at 385 nm, whereas under the same conditions the recombinant apoprotein has a much smaller fluorescence intensity. Rat liver apoDDC similarly displays a prominent 335 nm absorption band [28], which is not distinctly present in the same enzyme obtained by recombinant techniques [18]. It was suggested that the difference in the remaining absorbances at 335 nm in the tissue-purified and recombinant enzymes was due to the reagent used to resolve the coenzyme from the protein [18]. The possibility of directly comparing apoproteins prepared from the recombinant and tissue-purified enzymes, both obtained by hydroxylamine treatment, has permitted the exclusion of the above possibility and clarifies that the 335 nm absorbing band is present in both enzymes but to widely varying extents.

The presence of this absorbing species is not related to the extent of the resolution or to its reactivation by coenzyme. Instead it appears to be related to the amount of PLP that is released by NaOH. In the natural homodimer, which releases one molecule of PLP, a significant amount of this adduct remains in the apoenzyme, whereas in the recombinant enzyme, which releases about 2 molecules of coenzyme per dimer, an extremely modest fraction of this adduct is present in the corresponding apoprotein. It can therefore be surmised that the natural enzyme indeed contains two molecules of chromophore, only about one-half of which is released by NaOH and reacts with hydroxylamine. Thus the tissue-purified enzyme, unlike the recombinant protein, has a large portion of coenzyme covalently bound in an inactive form, which most probably does not participate in the equilibrium between the forms absorbing at 335 and 425 nm. It is thus not surprising that the ratio A_{335}/A_{425} is different for the recombinant and naturally occurring enzymes. In fact, if the remaining absorbances at 335 nm in the apoenzymes are subtracted from those of their respective holoenzymes, the ratio A_{335}/A_{425} becomes nearly identical for the two proteins, indicating that the equilibrium ratio of active-site PLP is the same in the two enzymes. The unexpected finding that this species is present to widely varying extents in the recombinant and tissue-purified enzymes is difficult to explain as its structural nature remains elusive. Although the possibility cannot be excluded that the inactive chromophore is an artificial modification produced during purification, this possibility seems unlikely because the two procedures are extremely similar.

It is noteworthy that the spectroscopic differences between the two pig kidney holo/apoproteins are not limited to the visible region as they also show differences in near-UV CD. Whereas the recombinant apoprotein displays noticeable conformational changes in the aromatic region compared with the holoenzyme, no such changes are apparent for the tissue-purified enzyme, seeming to indicate that the microenvironment of these aromatic residues remains detectably unchanged in the latter case. Such a conformational change is reversible because the addition of cofactor restores the original near-UV CD signal. It seems reasonable to hypothesize that the adduct that remains in the natural apoenzyme keeps the protein (or certain active-site aromatic residues) in the same conformation as the holoenzyme.

The absorbing species at 425 nm in recombinant DDC can be attributed to a protonated aldimine, as for natural pkDDC. However, as many different species absorb around 335 nm, its assignment is more problematic. The observed behaviour of the coenzyme bands absorbing at 335 and 425 nm rules out a pH-dependent interconversion of the two species, which would be expected if the 335 nm form were an unprotonated aldimine. A pK in the range 7.5–8.0 would, however, be appropriate for ionization of the nitrogen in the PLP ring. These results are consistent with the attribution of the 335 nm species to an enolimine and are explainable by a simple tautomerism. As shown in Scheme 1, such a model involves protonated (II) and unprotonated (IV) ketoenamine forms absorbing at 425 nm and enolimine tautomers [protonated (I) and unprotonated (III)] absorbing maximally at 335 nm. At pH values much less than pK, I and II will be present, whereas III and IV represent the forms at pH values much greater than the pK. Accordingly, ring nitrogen ionization would not be directly responsible for the



Scheme 1

spectral transition but would instead influence the equilibrium between the two tautomers that absorb at 335 and 425 nm. Although the absence of a fluorescence emission at 500 nm on excitation at 335 nm is in apparent contrast with the above attribution of the 335 nm species to an enolimine, it is possible that its fluorescence is quenched. In fact, pkDDC is devoid of any fluorescence emission due to active PLP, in contrast with rat liver DDC, which shows an emission band at 520 nm when excited at either 335 or 425 nm. Although the difference in fluorescence between the recombinant rat liver and naturally occurring pig kidney enzymes was attributed to the difference in PLP content [18], this idea can no longer be substantiated as both the respective recombinant enzymes have the same amount of active coenzyme.

The characterization of recombinant pkDDC has permitted the revision of the long-standing idea that the enzyme has its subunits asymmetrically arranged to form a single active site per dimer. As the enzyme has two independent catalytically active PLP binding sites, it has in fact structural attributes more characteristic of other PLP-dependent homodimeric proteins.

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