Mutation of cysteine 111 in Dopa decarboxylase leads to active site perturbation

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Abstract

Cysteine 111 in Dopa decarboxylase (DDC) has been replaced by alanine or serine by site-directed mutagenesis. Compared to the wild-type enzyme, the resultant C111A and C111S mutant enzymes exhibit k_{cat} values of about 50% and 15%, respectively, at pH 6.8, while the K_m values remain relatively unaltered for L-3,4-dihydroxyphenylalanine (L-Dopa) and L-5-hydroxytryptophan (L-5-HTP). While a significant decrease of the 280 nm optically active band present in the wild type is observed in mutant DDCs, their visible co-enzyme absorption and CD spectra are similar to those of the wild type. With respect to the wild type, the Cys-111→Ala mutant displays a reduced affinity for pyridoxal 5'-phosphate (PLP), slower kinetics of reconstitution to holoenzyme, a decreased ability to anchor the external aldimine formed between D-Dopa and the bound co-enzyme, and a decreased efficiency of energy transfer between tryptophan residue(s) and reduced PLP. Values of pK_a and pK_b for the groups involved in catalysis were determined for the wild-type and the C111A mutant enzymes. The mutant showed a decrease in both pK values by about 1 pH unit, resulting in a shift of the pH of the maximum velocity from 7.2 (wild-type) to 6.2 (mutant). This change in maximum velocity is mirrored by a similar shift in the spectrophotometrically determined pK value of the 420 → 390 nm transition of the external aldimine. These results demonstrate that the sulfhydryl group of Cys-111 is catalytically nonessential and provide strong support for previous suggestion that this residue is located at or near the PLP binding site (Dominici P, Maras B, Mei G, Borri Voltattorni C. 1991. *Eur J Biochem* 201:393–397). Moreover, our findings provide evidence that Cys-111 has a structural role in PLP binding and suggest that this residue is required for maintenance of proper active-site conformation.

Keywords: active site; decarboxylase; pyridoxal 5'-phosphate; site-directed mutagenesis

The versatility of pyridoxal 5'-phosphate (PLP) as a co-factor is demonstrated by the wide variety of reactions that enzymes dependent upon it catalyze. Although detailed catalytic mechanisms and information correlating structure and function have been reported for many PLP dependent enzymes, such information is conspicuously lacking for PLP dependent decarboxylases.

Even though aromatic amino acid decarboxylases have been cloned from a number of mammalian (Ichinose et al., 1989; Tanaka et al., 1989; Kang & Joh, 1990; Taketoshi et al., 1990), insect (Eveleth et al., 1986), and plant sources (DeLuca et al., 1989; Kawalleck et al., 1993; Facchini & DeLuca, 1994), little or no

information is available about the protein from sources other than pig kidney. Dopa decarboxylase (DDC; 4.1.1.28) is a stereospecific aromatic amino acid α decarboxylase (Borri Voltattorni et al., 1983). The enzyme possesses a broad substrate specificity although the physiologically recognized substrates are L-3,4-dihydroxyphenylalanine (L-Dopa) and L-5-hydroxytryptophan (L-5-HTP), which are decarboxylated to form the important neurotransmitters dopamine and serotonin, respectively. PLP is bound as an internal aldimine to K303 in the pig kidney enzyme (Maras et al., 1991), which, when expressed in *Escherichia coli* contains 2 mol of co-factor per enzyme homodimer (Moore et al., 1996). Since no crystallographic data are yet available for the enzyme, the identification of residues involved in catalysis and substrate/co-enzyme binding must rely upon chemical modification studies or homology to other better characterized enzymes. While an arginine (Tancini et al., 1985), a histidine (Dominici et al., 1985), and a cysteine (Dominici et al., 1991) residue have been implicated in enzymatic function, only the latter has been localized in the primary structure. By use of the co-enzyme affinity analog N-(bromoacetyl)pyridoxamine 5'-phosphate (BAPMP), it was

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Abbreviations: PLP, pyridoxal 5'-phosphate; DDC, Dopa decarboxylase; Dopa, 3,4-dihydroxyphenylalanine; 5-HTP, 5-hydroxytryptophan; BAPMP, N-(bromoacetyl) pyridoxamine 5'-phosphate; DTT, dithiothreitol; C111A, the mutant enzyme wherein cysteine 111 is altered to alanine; C111S, the mutant enzyme wherein cysteine 111 is altered to serine.

Table 1. Kinetic parameters for wild-type and mutant DDCs^a

Enzyme	L-Dopa			L-5-HTP			PLP (μM)
	K_m ($\text{M} \times 10^{-4}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1} \times 10^4$)	K_m ($\text{M} \times 10^{-5}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1} \times 10^4$)	
wild-type	0.91	9.1	10	2.74	1.3	4.7	0.044
C111A	1.45	4.2	2.9	4.53	0.6	1.3	3.2
C111S	4.40	1.2	0.3	4.10	0.2	0.5	n.d.

^aKinetic parameters were determined at pH 6.8 and 25 °C as described in Materials and methods. n.d., not determined.

shown that in apoDDC Cys-111 is specifically labeled, suggesting that this residue lies at or near the PLP-binding site of the enzyme (Dominici et al., 1991).

To directly test this possibility and to assess its role in enzymatic function, Cys-111 in pig kidney DDC has been changed to an alanine or a serine residue by site-directed mutagenesis. The resulting mutant proteins have substantial enzymatic activity, indicating that Cys-111 is not a catalytically essential residue. However, mutation of this amino acid results in a protein that displays aberrant behavior with respect to co-enzyme affinity, reactivation of apoprotein by PLP, and interaction with the active-site-directed inhibitor D-Dopa. Moreover, mutation of Cys-111 affects the ionization state of the external aldimine and/or catalytic active-site residues. As described herein, it is postulated that Cys-111 plays an important role in maintaining local conformational integrity as its substitution leads to effects which are felt throughout the active site. These are the first reported results involving characterization of mutant forms of a PLP-dependent aromatic amino acid decarboxylase.

Results

Purification and kinetic parameters of wild-type, C111A, and C111S DDC

Purification of wild-type DDC is performed in three steps: homogenization and clarification of the extract, DEAE-FF chromatography, and Phenyl Sepharose chromatography. When subjected to the same procedure, the mutant proteins irreversibly lost about 75% of their activity during the final chromatographic step. It was found that inclusion of 10^{-5} M PLP in the buffers used during this stage increased the recovery to about 95%, similar to that routinely seen for the wild-type protein. However, during concentration by ultrafiltration, the purified mutant enzymes lost about 40% of their activity and were further subject to slow inactivation during storage at -20 °C. Since this loss of activity was even more drastic for the C111S mutant, the bulk of the studies were carried out with the more stable C111A mutant.

The steady-state turnover and apparent K_m values of the physiological substrates, L-Dopa and L-5-HTP, are compared in Table I for the wild-type and C111A and C111S mutant enzymes. At pH 6.8 and 25 °C, the k_{cat} for both substrates drops by a factor of about two and seven when C111 is mutated to alanine or serine, respectively. Extremely modest increases were observed in the K_m for both substrates relative to the wild type, indicating that binding is not significantly impaired.

The binding constants, K_D , for PLP were measured using L-Dopa. It was found that the K_D value for C111A mutant was significantly higher than that of the wild-type protein (Table 1).

pH dependence of kinetic parameters of wild-type and C111A mutant DDCs

The pH-dependence of the steady-state values for K_m and k_{cat} for wild-type and C111A-catalyzed reaction of L-Dopa has been examined in a limited range of pH values from 5.9 to 8.5. This was due to inactivation of the enzyme below pH 5.9 and to oxidation of the substrate above pH 8.5. For the wild-type enzyme, a plot of $\log k_{cat}$ versus pH yielded a bell-shaped curve with limiting slopes of +1 on the acid side and -1 on the alkaline side. The C111A mutant shows apparent limiting slopes of $<\pm 1$ (Fig. 1). When fit to Equation 1, the data yielded $\text{p}K_a$ and $\text{p}K_b$ values of 6.6 ± 0.1 and 7.9 ± 0.1 , respectively, for the wild type, and of 5.5 ± 0.2 and 7.0 ± 0.1 , respectively, for the C111A mutant.

Fig. 1. pH dependence of k_{cat} in the steady-state reaction catalyzed by wild-type and C111A mutant DDCs at 25 °C at $I_c = 0.1$. Assay conditions are described in the text. Closed circles, wild-type; the points represent experimental values, while the curve is theoretical from a fit of the data to Equation 1 with a pH-independent value of k_{cat} of $15.2 \pm 1 \text{ s}^{-1}$, $\text{p}K_a = 6.6 \pm 0.1$ and $\text{p}K_b = 7.9 \pm 0.1$. Open circles, C111A mutant; the points represent experimental values, while the curve is theoretical from a fit of the data to Equation 1 with a pH-independent value of k_{cat} of $7.4 \pm 1 \text{ s}^{-1}$, $\text{p}K_a = 5.5 \pm 0.2$ and $\text{p}K_b = 7.0 \pm 0.1$.

respectively, for the C111A mutant. It is possible that the reaction catalyzed by the mutant enzyme is not absolutely dependent on the groups with the above pK values, although they could influence the chemistry of the reaction and/or product release. Nonetheless, replacement of Cys-111 with alanine gives rise to a pH profile for k_{cat} markedly different from that of the wild-type enzyme: The mutant shows an acidic shift of about 1 pH unit in both pK values. The maximum velocity is therefore achieved at pH 7.2 and 6.2 for wild-type and C111A mutant enzymes, respectively.

Spectroscopic Properties of the C111A and C111S Mutants

As for the wild-type enzyme, the mutant proteins bind 2 mol of PLP per dimer and display co-enzyme absorbance bands centered at 425 nm and 335 nm in potassium phosphate buffer, pH 6.8, $I_c = 0.1$ (Fig. 2A). Although it should be noted that the ratio of these two absorbances ($A_{335\text{ nm}}/A_{425\text{ nm}}$) was somewhat variant between

different enzymatic preparations, it was consistently lower than that of the wild-type protein. In wild-type DDC, the co-enzyme absorbances at 335 nm and 425 nm show pH dependency: At basic pH the former is predominant, while the latter predominates at acidic pH (Moore et al., 1996). The C111A and C111S mutants exhibit the same, but less pronounced, pH-dependent changes in the PLP absorption spectrum between pH 6 and 8.5 (data not shown).

The CD spectrum of wild-type DDC in the visible region has two positive bands at wavelengths corresponding to the two absorption bands (at 335 nm and 420 nm) at pH 6.8. Likewise, the mutant proteins showed similar dichroic signals (Fig. 2B). The optical activity (mdeg/absorbance unit) was measured to determine if these forms of the enzyme exhibit similar properties with respect to the asymmetric environment of the bound PLP. The wild-type, C111A, and C111S enzymes display optical activity values of 54, 66, and 51 mdeg/ A_{425} and 53, 43, and 33 mdeg/ A_{335} , respectively, suggesting that the chiral environment of the internal aldimine is only slightly different among the three enzymes. Dramatic differences were observed in the aromatic region and would seem to indicate that the tyrosine and/or tryptophan residue(s) giving rise to this positive Cotton effect are in a different microenvironment in the C111A and C111S mutants (Fig. 2B). CD spectra taken in the far-UV region were practically superimposable for the wild-type and mutant proteins, indicating no gross alterations in secondary structure (data not shown).

While intrinsic fluorescence emission spectra of C111A showed no consistent changes in either emission maximum or intensity with respect to the wild-type protein, intrinsic and co-enzyme fluorescence emission spectra of NaBH_4 -reduced C111A reveal differences when compared with those of reduced wild-type. In fact, when excited at 335 nm the reduced C111A mutant exhibits a decreased emission fluorescence intensity at 385 nm compared to wild-type DDC (Fig. 3B); moreover, as shown in Figure 3A, there is a decreased efficiency of the energy transfer process occurring between tryptophan residue(s) and the reduced co-enzyme. These data suggest that mutation of C111 causes conformational changes at or near the microenvironment of the co-enzyme which affects the distance and/or orientation of the donor-acceptor pair.

Kinetics of reconstitution of wild-type and C111A apoenzymes with PLP

When the wild-type apoenzyme is mixed with PLP under pseudo-first-order conditions, the phases involved in the formation of an enzymatically holoenzyme are too rapid to be studied by conventional techniques. In fact, the regain of enzymatic activity and spectral characteristics occurs within the time required for manual mixing of PLP and apoenzyme. Under the same experimental conditions, the C111A mutant shows widely different kinetics. The regain of activity upon the addition of PLP to apoC111A DDC as a function of time reaches a near saturation value within 1 h (Fig. 4A). The calculated rate constant, k_{obsd} , for this process was 0.071 min^{-1} . This slow reactivation was also followed spectrophotometrically. Immediately after mixing, an absorbing band at 400 nm was seen, which was gradually converted to an absorbance at 425 nm (Fig. 4B). Following the spectral shift with time (inset of Fig. 4B), the rate constant for this process was calculated to be 0.051 min^{-1} . This spectral shift from 400 to 425 nm parallels the observed increase in activity, as reflected in the near identity of the

Fig. 2. Absorption and CD spectra of wild-type, C111A and C111S mutants of DDC. **A:** Absorption spectra of (—) wild-type, (---) C111A, and (·····) C111S in potassium phosphate buffer, $I_c = 0.1$, pH 6.8 at a concentration of $4\text{ }\mu\text{M}$. **B:** CD spectra, same as absorption.

Fig. 3. Intrinsic and co-enzyme fluorescence emission spectra of NaBH_4 -reduced enzymes. **A:** Excitation at 295 nm. **B:** Excitation at 335 nm. (—) wild-type, (---) C111A mutant. For all spectra, the protein concentration was 0.8 μM .

two rate constants. In order to determine if the 400 nm absorbing species is a non-covalent or covalent complex, rapid chemical quenching studies were carried out by NaBH_4 reduction as a function of time after the addition of equimolar amounts of PLP to apoC111A. As equivalent amounts of reduced co-enzyme were present in samples at all the time points examined (i.e., from 30 sec to 1 h), it can be deduced that the 400 nm absorbing species is bound as an internal aldimine, although catalytically inactive. Thus, the reconstitution of apoC111A can be separated in at least two distinct steps: Formation of an enzymatically inactive internal aldimine, followed by its conversion to an enzymatically active internal aldimine.

Fig. 4. Kinetics of reconstitution of apoC111A with PLP as monitored by enzyme activity and absorption spectral measurements. Apoenzyme (8 μM) was mixed with PLP (50 μM) in 100 mM potassium phosphate buffer, pH 6.8, at 25 $^{\circ}\text{C}$. **A:** Aliquots were removed at the periods indicated on the figure and assayed for decarboxylase activity. **B:** Spectra were taken at 1, 3, 5, 15, 40, 50, 60 min (from top to bottom). **Inset:** Plot of λ_{max} as a function of time.

Spectral changes of wild-type and C111A mutant DDC with L-Dopa

As for the enzyme purified from pig kidney, under steady-state conditions in the presence of L-Dopa, two forms of the co-enzyme-substrate complex absorbing at 420 and 390 nm are also observed for recombinant DDC. It has been postulated that this 420 $\rightarrow$ 390 nm transition is caused by deprotonation of the 4'-nitrogen of the enzyme-substrate complex and that this ionization influences the equilibrium between the two tautomers that absorb at 420 and 390 nm (Borri Voltattorni et al., 1983). For the recombinant enzyme, the apparent pK of this spectral change was 7.2 (data not shown), identical to that previously determined for the pig kidney enzyme (Borri Voltattorni et al., 1983). Similar high and low pH forms of the enzyme-substrate intermediate complexes were also observed for the C111A mutant DDC (Fig. 5), with the exception that the E_{M} of these intermediate complexes is significantly reduced. However, plots of absorbance values at 420 and 390 nm for C111A mutant as a function of pH fit titration curves with an apparent pK value of 6.3 (inset of Fig. 5). Thus, replacement of Cys-

Fig. 5. pH dependence of C111A spectra in the presence of L-Dopa. Spectra of 4.2 μ M C111A plus 2 mM L-Dopa run in potassium phosphate buffer, $I_c = 0.1$, at pH 5.9 (—), 6.3 (---), and 7.4 (----). **Inset:** Absorbance at 420 nm (○) and 390 (●) nm as function of pH. The points represent the experimental values, while the curves are theoretical from a fit of the data to Equation 2, which in both cases gave a pK_a of 6.3.

111 by alanine shifts the ionization which controls the tautomeric equilibrium of the external aldimine spectrum by about 1 pH unit.

Interaction of wild-type and mutants DDCs with D-Dopa

As already seen for the pig kidney enzyme (Borri Voltattorni et al., 1983; Moore et al., 1995), the addition of D-Dopa to wild-type DDC causes an increase in absorbance at 430 nm with a concomitant decrease at 335 nm (Fig. 6A). This is accompanied by a decrease of the positive dichroic band at 420 nm and the near disappearance of the signal at 335 (Fig. 6B). These spectral features remain unchanged with time. The addition of D-Dopa to the mutant enzymes causes the immediate increase of an absorption band at 430 nm, with an E_M about half that of the wild-type enzyme and a modest blue shift of the 335 nm absorbance band (Fig. 6A). CD spectra were also altered showing a reduction in the intensity of the dichroic band at 420 nm and a shift of the positive dichroic signal at 335 nm to 325 nm (Fig. 6B). These absorption (Fig. 6C) and dichroic bands (data not shown) undergo slow time-dependent changes. After a 2-h incubation of the mutant enzymes with D-Dopa and passage over a PD-10 column, the proteins were devoid of enzymatic activity and exhibited absorption and CD spectra typical of apoenzyme (data not shown). Original activity, co-enzyme-associated absorption, and dichroic bands were restored by the addition of PLP. While these time-dependent spectral changes might be indicative of an enzymatic reaction, no dopamine, 3,4-dihydroxyphenylacetaldehyde, or pyridoxamine 5'-phosphate could be detected either in a decarboxylase assay or by HPLC.

Discussion

Although distant from the co-factor-binding lysine (K303) in the primary structure, Cys-111 was found to bind a PLP analog, BAPMP, thus suggesting its localization at or near the PLP binding site (Dominici et al., 1991). To probe the role of Cys-111, we have changed this residue to alanine or serine using site-directed muta-

Fig. 6. Absorption and CD spectra of wild-type and C111A mutant enzymes in the presence of D-Dopa. **A:** Absorption spectra of wild-type DDC (—) and in the presence of 2 mM D-Dopa (---); absorption spectra of C111A (----) and immediately after the addition of 2 mM D-Dopa (----). The enzyme concentration was in each case 5 μ M. **B:** CD spectra, same as absorption. **C:** Absorption spectra of 8.5 μ M mutant C111A (----) and of the mutant enzyme plus 2 mM D-Dopa at 1, 5, 15, 60 min (from top to bottom). An identical behavior was observed for C111S mutant.

genesis. The initial observation that the inclusion of exogenous PLP is necessary in the final purification step in order to recover full enzymatic activity for both mutants, unlike for the wild-type

DDC, immediately suggested that co-enzyme binding is altered. It may also be that the irreversible loss of activity in the absence of exogenous co-enzyme is due in part to the greater inherent instability of the mutants. It is interesting to note that this loss of decarboxylase activity is more pronounced for the more conservative hydroxyl (C111S) replacement.

Both the C111A and C111S mutants have substantial, although lower, steady-state catalytic activity than does the wild-type enzyme. Moreover, these mutations do not markedly affect the affinity of the substrates L-Dopa and L-5-HTP (Table 1). Thus, the sulfhydryl moiety of Cys-111 cannot be considered either a catalytic or a substrate binding group. When compared with other type II decarboxylases (Sandmeier et al., 1994), Cys-111 is highly, although not absolutely, conserved; interestingly, alanine is found among the plant aromatic amino acid decarboxylases (DeLuca et al., 1989; Kawalleck et al., 1993; Facchini & DeLuca, 1994). Thus, it is perhaps not surprising that this residue has no essential enzymatic role.

As for the wild-type enzyme, both these mutants bind 2 mol of PLP per dimer. Their CD spectra in the visible region have two positive bands at positions corresponding to the two absorption bands (at 335 nm and 425 nm) at pH 6.8, very similar to those of the wild-type enzyme. However, the ratio of ellipticity to absorbance of the two spectral bands of the mutants would suggest a slightly altered chiral environment of the internal aldimine. The possibility that the state of the bound co-enzyme in the active site of mutant proteins has been perturbed is also demonstrated by the fact that, when excited at 335 nm, NaBH₄-reduced C111A displays a decreased fluorescence emission intensity and, when excited at 295 nm, it exhibits a decreased efficiency of the energy transfer between tryptophan residue(s) and the reduced co-enzyme.

Alanine or serine at position 111 might perturb the active site directly through unfavorable electrostatic or steric interactions with PLP or indirectly by inducing conformational changes in the vicinity of the active site. This latter possibility seems to be favored by the consistent diminution of the 280-nm optically active band in the mutant enzymes. This indicates that the conformation of tyrosine and/or tryptophan residue(s), possibly at the active site, has been altered without major structural changes, since the secondary structure remains unchanged. Furthermore, the effects of mutation of Cys-111 on the ionization state of the external aldimine and of the catalytic active-site residues seem to be consistent with structural perturbation of the active center. Although Cys-111 is not a catalytically essential group, replacement of this residue with ala (C111A) results in an acidic shift of the pK values found in the k_{cat} versus pH profile. Therefore, while the maximum velocity for wild-type DDC is achieved at pH 7.2, the V_{max} for the mutant enzyme is reached at pH 6.2. Similarly, the pK of the 420 → 390 nm transition of the external aldimine reflects the same acidic shift. From this data, it can be forwarded that the maximal catalytic efficiency is achieved when the acidic group is unprotonated, the basic group is protonated, and both the 420- and 390-nm absorbing forms are equally present. While the lack of information concerning the topography of the active site of DDC makes the interpretation of these results difficult, it can nonetheless be suggested that the effect of this mutation would be to induce a structural change that either alters the ionization of residues involved in catalysis or leads to the involvement of different catalytic residues.

It is nonetheless evident that A → C replacement weakens non-covalent interactions between co-enzyme and protein that are nec-

essary for correct and tight anchorage of PLP to the active site. In fact, the affinity of the mutant enzyme for PLP is greatly reduced, leading to a $\Delta\Delta G$ of 2.5 kcal/mol. The rate constant of reconstitution to the holo form for the apomutant is also significantly lower than that for the wild-type apoprotein. Indeed, upon addition of PLP to apoenzymes, wild-type DDC completely recovers its native activity and spectral properties within the time required for manual mixing, while almost 1 h is required for the mutant enzyme. A more detailed analysis of the reconstitution of apoC111A performed by measuring the recovery of enzyme activity and spectral characteristics revealed that at least two steps are involved in this process. The initial fast phase, for which no rate constant is given due to the rapid formation of the 400-nm absorbing band, leads to an inactive internal aldimine absorbing at 400 nm. This is followed by a relatively slow phase with a pseudo-first order rate constant of 0.051 min^{-1} leading to a catalytically active internal aldimine absorbing at 420 nm. Therefore the simplest mechanism which explains this reconstitution is $A + \text{PLP} \leftrightarrow E^*\text{-PLP} \leftrightarrow E\text{-PLP}$, where A is the apoenzyme, $E^*\text{-PLP}$ is the inactive PLP-K303 internal aldimine, and E-PLP is the catalytically active enzyme. Although we have not investigated the kinetics of PLP association for wild-type DDC by stopped-flow techniques, it is likely that the same mechanism can be applied. If this is the case, it would appear that an isomerization event, probably reflecting conformational changes involving rearrangement of the enzyme into an active form, is required for the complete reconstitution of apoenzyme. Similar rearrangements have been observed for other PLP-dependent enzymes (Churchich & Farrelly, 1969; Reed & Schnackerz, 1979; Bartholmes et al., 1980).

The peculiar time-dependent spectral changes observed in the presence of D-Dopa for both mutant proteins merit comment. While these spectral modifications might be indicative of an enzymatic reaction, the absence of detectable products, together with the finding that incubation of the C111A mutant with D-Dopa leads to the formation of apoenzyme, would seem to exclude this possibility. The simplest explanation is that C111A has a decreased ability to anchor the external aldimine formed between D-Dopa and the bound co-factor. The formation of an external aldimine is not impaired but, unlike in wild-type DDC, this enzyme-bound intermediate dissociates to yield apoenzyme and PLP-Dopa. This event would lead to an inactivation of C111A that is reversible upon addition of PLP, which is what is seen.

With regards to the co-factor-binding pocket in the active site of PLP-dependent enzymes, details of how the different proteins interact with the co-enzyme have been well-defined only in the cases for which high-resolution crystal structures have been determined (Kirsch et al., 1984; Hyde et al., 1988; McPhalen et al., 1992; Antson et al., 1993; Momany et al., 1995b; Toney et al., 1995). In particular, a glycine-rich loop has been recognized at the co-factor binding site in those enzymes for which crystal structures are available and has also been identified through site-directed mutagenesis in threonine and D-serine dehydratases (Datta et al., 1987; Marceau et al., 1990) and more recently in aminolevulinate synthase (Gong et al., 1995, 1996). Interestingly, a similar glycine-rich region is present in DDC (138-GEGGG; Maras et al., 1991), although its function has not been directly probed. By homology, DDC has been identified as a group II decarboxylase (Sandmeier et al., 1994) and as such is a member of the fold-type I structural superfamily as defined by Grishin et al. (1995) and Momany et al. (1995a). Ornithine decarboxylase from *Lactobacillus* 30a, whose structure has been recently solved (Momany et al., 1995b), is also

a member of the aforementioned superfamily. In the latter enzyme, the glycine-rich loop is immediately preceded by a short linker domain, which provides residues that contribute to dimer stabilization and fills in portion of the PLP binding pocket of the other subunit. Cys-111 in DDC also precedes its glycine-rich region in the primary structure. If the folding pattern is indeed conserved between DDC and the *Lactobacillus* decarboxylase, it is thus possible that Cys-111 is at the PLP binding site. This consideration is also made in the light of the fact that this cysteine residue was identified by means of a co-enzyme-affinity probe (Dominici et al., 1991).

Although some of the structural and physicochemical properties of DDC have been determined, there is no direct structural information concerning catalysis and co-factor/substrate binding. Recently, Ishii et al. (1996) have performed systematic mutations of rat liver DDC on residues conserved among group II decarboxylases and determined the kinetic parameters of the proteins that showed activity. In the absence of a more detailed analysis, however, little correlation regarding enzymatic structure and function could be obtained. The possibility of purifying sufficient quantities of enzyme has allowed for an in-depth characterization of Cys-111 mutants.

Cysteine residues have been found to be associated with the active site in other PLP-dependent enzymes, even though their exact roles have not been elucidated. In particular, Cys-360 in mouse ornithine decarboxylase, originally identified as the binding site of the inhibitor α -difluoromethylornithine (Poulin et al., 1992), was found to be located at the active site (Coleman et al., 1993) and Cys-298 in tryptophan indole-lyase is at the active site, possibly in contact with co-factor (Phillips & Gollnick, 1989). Additionally, two cysteine residues in β -cystathionase were found to be conformationally important and facilitate internal Schiff base formation (Gentry-Weeks et al., 1995). In the absence of a crystal structure, it remains difficult to define exactly what structural changes introduced by mutation of Cys-111 lead to catalytic and co-factor binding alterations. In summary, these studies provide evidence that this residue has a structural role in PLP binding and suggest that it is necessary for proper active site conformation.

Materials and methods

Materials

L-Dopa, L-5-HTP, and substrate analogs were purchased from Sigma. Restriction and DNA-modifying enzymes were from either Pharmacia or New England Biolabs. Sequenase version 2.0 was from Amersham. All other chemicals and reagents were of the highest quality available and used without further purification.

Site-directed mutagenesis

Alterations of Cys-111 in the pig kidney DDC cDNA were performed by cassette mutagenesis. The following oligonucleotides were synthesized: For the C111A mutant, 5'-CTAGCC CGGCGGCCACCGAAC-3' and 5'-TCGAGTTCGGTGGCCGC CGGG-3pr, and for the C111S mutant, 5'-CTAGCCCCGGCGT CCACCGAAC-3' and 5'-TCGAGTTCGGTGGACGCCGGG, with the changed bases underlined. The oligonucleotides were phosphorylated, annealed, and ligated into pDDCKX229 cut with Nhe I and Xho I. (pDDCKX229 is the Kpn I (111)–Xho I (340) fragment from the pig kidney DDC cDNA (Moore et al., 1996)). The

resulting Kpn I–Xho I fragment was then cloned into pKKDDC Δ 4 (Moore et al., 1996) (see below) for expression of the mutant proteins. All final constructs were sequenced directly using an internal primer to verify the presence of the desired mutation.

Expression and purification of wild-type and mutant DDCs

The conditions used for expression of the mutant proteins in *E. coli* (JM109 or JM105) were as described for the wild-type enzyme (Moore et al., 1996). Purification of the wild-type and mutant proteins was also carried out in an identical manner (Bertoldi et al., 1996; Moore et al., 1996), except for the Phenyl Sepharose chromatography step (see text), in which 10^{-5} M PLP was included in all buffers. Exogenous co-enzyme was also included in the buffer used for concentration by ultrafiltration (0.1 M potassium phosphate, pH 6.8, 10^{-4} M DTT, and 10^{-5} M PLP). The enzyme concentration was determined using an E_M of 1.3×10^5 M $^{-1}$ cm $^{-1}$ (Dominici et al., 1993). PLP content of holoDDC enzymes was determined by releasing the co-enzyme in 0.1 N NaOH and by using $E_M = 6600$ M $^{-1}$ cm $^{-1}$ at 388 nm.

Enzyme activity

Decarboxylase activity was measured as described by Sherald et al. (1973), as modified by Charteris and John (1975). Determination of the kinetic parameters for L-Dopa and L-5-HTP were performed at 25 °C in potassium phosphate buffer at $I_c = 0.1$, with varying amounts of substrate (0.025–0.5 mM), and appropriate dilutions of enzyme. For determination of the binding constants for PLP, K_D , the Dopa concentration was maintained at 0.5 mM and the wild-type and C111A mutant apoenzyme concentrations were 0.1 μ M and 10 μ M, respectively. The PLP concentrations were varied from 0.01 to 1 μ M and 0.1 to 50 μ M, respectively.

Preparation and reconstitution of apoenzymes

Apoenzymes were prepared as described previously (Moore et al., 1996) and concentrated by ultrafiltration. Reconstitution of apoenzymes with PLP was monitored by enzymatic activity and conventional spectrophotometry measurements. Eight μ M apoenzyme was incubated with 50 μ M PLP in 100 mM potassium phosphate buffer, pH 6.8, at 25 °C. At various times, aliquots were removed and diluted (usually 30-fold) into a normal assay mixture containing 0.5 mM L-Dopa, and enzymatic activity was determined as described above. For spectrophotometric measurements, PLP was added to a final concentration of 50 μ M to both the apoenzyme (8 μ M) and reference cuvette and spectra were taken at various times. Rapid quenching of the internal aldimine was performed as described (Bartholmes et al., 1980).

Spectroscopy

Absorption spectra were carried out using a Jasco V-550 spectrophotometer at 25 °C. For measurements of the spectra of DDC and mutants in the absence and in the presence of L-Dopa at different pH values, potassium phosphate buffer was used, at $I_c = 0.1$. Fluorescence spectra were taken with a Kontron SFM 25 spectrofluorometer using 5-nm bandwidths on both sites at a protein concentration of 0.8 μ M. CD spectra were obtained with a Jasco 710 spectropolarimeter with a thermostatically controlled cell at 25 °C. For near-UV and visible wavelengths, protein concentrations were

from 0.8 to 1 mg/mL using a 1-cm path length. Routinely, four spectra were recorded at a scan speed of 50 nm/min with a bandwidth of 2 nm and averaged automatically. For far-UV measurements, the protein concentration was 0.1 mg/mL with a 0.1-cm path length.

Data analysis

Kinetic parameters were obtained using the non-linear regression program, ENZFITTER (Elsevier), to fit the data to hyperbolic form of the Michaelis-Menten equation. pH profiles for k_{cat} were fit using the same program to the logarithmic form of Equation 1.

$$k = k_{lim} / (1 + 10^{pK_a - pH} + 10^{pH - pK_b}) \quad (1)$$

where $k = k_{cat}$, k_{lim} is the corresponding maximum value of k_{cat} , and $pK_b > pK_a$.

For spectrophotometric determination of pK values, spectra of wild-type and C111A DDCs in the presence of saturating concentrations of L-Dopa were monitored at various pH values. The optical density at 420 nm and 390 nm were collected and fitted to Equations 2a and 2b, respectively.

$$A = \frac{A_1 - A_2}{1 + 10^{pH - pK_a}} + A_2 \quad (2a)$$

$$A = \frac{A_1 - A_2}{1 + 10^{pK_a - pH}} + A_2 \quad (2b)$$

where A_1 and A_2 are the higher and lower absorbance limits at a particular wavelength, respectively.

K_D values of the enzyme-co-enzyme complex were obtained using the Eadie plot (Fersht, 1985).

$$\Delta A = \Delta A_{max} - K_D(\Delta A/[PLP]) \quad (3)$$

where ΔA refers to enzymatic activity change at the PLP concentration, $[PLP]$, and ΔA_{max} to the enzymatic activity change when all enzyme molecules are complexed with co-enzyme.

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