Reaction Specificity of Native and Nicked 3,4-Dihydroxyphenylalanine Decarboxylase*

3,4-Dihydroxyphenylalanine (Dopa) decarboxylase is a stereospecific pyridoxal 5'-phosphate (PLP)-dependent α-decarboxylase that converts L-aromatic amino acids into their corresponding amines. We now report that reaction of the enzyme with D-5-hydroxytryptophan or D-Dopa results in a time-dependent inactivation and conversion of the PLP coenzyme to pyridoxamine 5'-phosphate and PLP-α-amino acid Pictet-Spengler adducts, which have been identified by high performance liquid chromatography. We also show that the reaction specificity of Dopa decarboxylase toward aromatic amines depends on the experimental conditions. Whereas oxidative deamination occurs under aerobic conditions (Bertoldi, M., Moore, P. S., Maras, B., Dominici, P., and Borri Voltattorni, C. (1998) J. Biol. Chem. 271, 23954–23958; Bertoldi, M., Dominici, P., Moore, P. S., Maras, B., and Borri Voltattorni, C. (1998) Biochemistry 37, 6552–6561), half-transamination and Pictet-Spengler reactions take place under anaerobic conditions. Moreover, we examined the reaction specificity of nicked Dopa decarboxylase, obtained by selective tryptic cleavage of the native enzyme by multiple sequence alignment with other group II decarboxylases combined with site-directed mutagenesis (4). The enzyme is a stereospecific α-decarboxylase that catalyzes the conversion of L-aromatic amino acids into their corresponding amines (5). D-Aromatic amino acids have also been shown to bind to the active site of the enzyme. Whereas aromatic amino acids with a catechol-related structure in the D form have been shown to inhibit enzymatic activity and to form stable intermediate complexes with the enzyme absorbing at 430 nm, the indole-related D forms exert a time-dependent inactivation and form intermediate complexes absorbing at 430 nm which undergo time-dependent modification (5). The behavior of the latter amino acids has been ascribed to a half-transamination reaction, although the stoichiometry of this reaction appeared to be abnormal (5). This is similar to the situation with 5-hydroxytryptophan (5-HTP) and α-methyldopa (6) for which a decarboxylation-dependent transamination had been proposed (7, 8). However, it has been demonstrated recently that in the case of serotonin (5-HT), dopamine, and α-methyldopa this minor reaction catalyzed by DDC is actually an oxidative deamination and not a decarboxylation-dependent transamination. Indeed, ammonia and the corresponding aldehyde or ketone are produced in equivalent amounts, while O2 is consumed in a 1:2 molar ratio with respect to these products (9, 10). In the light of these results, a reinvestigation of the interaction of DDC with D-aromatic amino acids appears to be necessary.

Previous studies have provided evidence that partial trypsinolysis of DDC leads to the exclusive cleavage of the Lys334-His335 peptide bond (2). The nicked and native proteins have identical spectroscopic features and coenzyme content. The partially proteolyzed protein will bind aromatic amino acids in L- and D forms but lacks decarboxylase activity (11). Loss of decarboxylase activity would appear to be incompatible with the apparent preservation of the three-dimensional structure of the active site of the proteolyzed enzyme. However, it has been observed recently that the binding of aromatic amino acid methyl ester analogs to DDC in the native and nicked forms varies depending on both the analog structure and the enzyme form. This would suggest that the binding of these analogs to the nicked enzyme does not occur as to the native enzyme (12). To gain some insight into the nature of the interaction of substrates and substrate analogs with the nicked enzyme, spectral and kinetic studies of the interaction of this enzymatic species with various ligands have been performed and compared with those of native enzyme.

Here, we present spectral and kinetic evidence for the occurrence of concomitant half-transamination and Pictet-Spengler reactions during the interaction of native or nicked DDC with D-aromatic amino acids. We also report data showing that half-transamination and Pictet-Spengler reactions take place when the native enzyme reacts with aromatic amines under

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Recombinant pig kidney Dopa1 decarboxylase (DDC; EC 4.1.1.28) is a homodimer containing two molecules of pyridoxal 5'-phosphate (PLP) per protein dimer (1). Its primary structure has been determined (2), and according to the classification of Sandmeier et al. (3), DDC is a group II decarboxylase. This allows the identification of some functionally important residues of the enzyme by multiple sequence alignment with other group II decarboxylases combined with site-directed mutagenesis (4). The enzyme is a stereospecific α-decarboxylase that catalyzes the conversion of L-aromatic amino acids into their corresponding amines (5). D-Aromatic amino acids have also been shown to bind to the active site of the enzyme. Whereas aromatic amino acids with a catechol-related structure in the D form have been shown to inhibit enzymatic activity and to form stable intermediate complexes with the enzyme absorbing at 430 nm, the indole-related D forms exert a time-dependent inactivation and form intermediate complexes absorbing at 430 nm which undergo time-dependent modification (5). The behavior of the latter amino acids has been ascribed to a half-transamination reaction, although the stoichiometry of this reaction appeared to be abnormal (5). This is similar to the situation with 5-hydroxytryptophan (5-HTP) and α-methyldopa (6) for which a decarboxylation-dependent transamination had been proposed (7, 8). However, it has been demonstrated recently that in the case of serotonin (5-HT), dopamine, and α-methyldopa this minor reaction catalyzed by DDC is actually an oxidative deamination and not a decarboxylation-dependent transamination. Indeed, ammonia and the corresponding aldehyde or ketone are produced in equivalent amounts, while O2 is consumed in a 1:2 molar ratio with respect to these products (9, 10). In the light of these results, a reinvestigation of the interaction of DDC with D-aromatic amino acids appears to be necessary.

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Here, we present spectral and kinetic evidence for the occurrence of concomitant half-transamination and Pictet-Spengler reactions during the interaction of native or nicked DDC with D-aromatic amino acids. We also report data showing that half-transamination and Pictet-Spengler reactions take place when the native enzyme reacts with aromatic amines under
an aerobic conditions and when the nicked enzyme reacts with these ligands under aerobic conditions. In Scheme 1 the Pictet-Spengler reaction of PLP with 5-HTP or Dopa is reported. Moreover, protection against limited tryptic proteolysis of native DDC by aromatic amino acids and amines has been examined as well as the effect of these ligands on the spectral properties of bound PLP. Together, these data suggest that DDC forms different intermediate complexes, possibly resulting in the stabilization of different conformational states. Herein, the possible relationship of these conformations with the reaction specificity of DDC is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—5-HTP, D-tryptophan, D-Dopa, 5-HT, dopamine, PLP, pyridoxamine 5′-phosphate (PMP), indole-3-pyruvic acid, bovine liver L-glutamic dehydrogenase, and horse liver alcohol dehydrogenase were purchased from Sigma. All other chemicals were of the highest purity available.

**Enzyme Purification**—Recombinant DDC was purified to homogeneity from *Escherichia coli* expressing pKKDDC445 as described (1, 9) and was used throughout. The enzyme concentration was determined using a molar extinction coefficient of 1.30 × 10^3 M^−1 cm^−1 (13). Limited tryptic digestion of DDC was performed as described (11).

**Enzyme Assays and Inactivation Assays**—DDC activity was measured as described by Sherald et al. (14), as modified by Charteris and John (15). The inactivation incubation mixtures contained native DDC (10 μM) and freshly diluted inhibitor (D-5-HTP or D-Dopa) at varying concentrations (0.05–5 mM) at 25 °C in 100 mM potassium phosphate, pH 7.5. At various time intervals, aliquots were removed and assayed for residual decarboxylase activity. Production of ammonia or 5-hydroxyindoleacetaldehyde by the reaction of DDC with 5-HT was determined by spectroscopic assays using the coupled system with glutamate dehydrogenase or alcohol dehydrogenase, respectively, as already described (9). Reaction of DDC with L-5-HTP, L-Dopa, or 5-HT under anaerobic conditions was performed using 1-ml Reacti-Vials (Aldrich) and was used throughout. The enzyme concentration was determined using a molar extinction coefficient of 1.30 × 10^3 M^−1 cm^−1 (13). Limited tryptic digestion of DDC was performed as described (11).

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**Kinetic Measurements**—The rates of the reaction of DDC with D-Dopa or D-5-HTP in 100 mM potassium phosphate buffer, pH 7.5, at 25 °C were obtained by observing the decrease of the 430 nm absorbance band. The kinetic measurements were performed at several aromatic amino acid concentrations using an excess of D-aromatic amino acid over enzyme concentration (2 μM) and were shifted slightly with respect to those of the reagents. All of these signals are consistent with the rapid and nearly complete formation of a Schiff base between PLP and the α-NH2 group of the amino acid. Subsequently these resonances decreased, and new ones arose, indicating that a nucleophile attack of the C-2 or C-6 of D-5-HTP or D-Dopa, respectively, to the imine carbon atom of PLP had occurred (data not shown). Comparison of these spectral features and of the chemical shift with those for the reaction of PLP with Dopa and tryptophan in the L form (17, 18) indicates that the compound formed are cyclized Schiff bases, the result of Pictet-Spengler condensation (Scheme 1).

These cyclic adducts were prepared by mixing 1 mM PLP with 10 mM D-5-HTP or D-Dopa in 100 mM potassium phosphate buffer, pH 7.5. After a 3-h incubation at 25 °C, the mixtures were processed by HPLC (see below). With 5-HT or dopamine, PLP forms cyclized Schiff bases that had been isolated using the HPLC methods described below and characterized by means of absorbance spectroscopy.

**Spectral Measurements**—All of the spectral measurements were carried out using 100 mM potassium phosphate, pH 7.5, at 25 °C. Absorption spectra were recorded in a Jasco V-550 spectrophotometer. Circular dichroism (CD) measurements were carried out in a Jasco J-710 spectropolarimeter at a protein concentration of 10 μM. Spectra were recorded at a scan speed of 50 nm/min with a band width of 2 nm and averaged automatically except where indicated. NMR spectra were obtained on a Bruker AM-400 machine operating at 400.131 MHz. A spectral width of about 20 ppm was used, and a repetition rate of 4.5 s was applied. After a 90° pulse (6.2 μs) free induction decays were stored on 16,000 bytes of memory and then, after multiplication for a sine bell shifted of ±3, were Fourier-transformed. For every experiment a typical average of 32 scans was obtained. The ppm scale was referred on tetramethylsilane on the basis of the resonance frequency of water at 300 K. Resonance caused by the residual water protons present in deuterated water was suppressed by presaturation irradiating for 1.66 s before pulsing. In the time-dependent experiments time was taken as the mean between the start and the end of averaging using the same number of scans.

**Characterization of the Reaction of PLP with D-5-HTP or D-Dopa and Chemical Synthesis of Pictet-Spengler Adducts**—The addition of D-5-HTP or D-Dopa at varying concentrations (1–10 μM) to 20 μM PLP in 100 mM potassium phosphate buffer, pH 7.5, results in marked absorption spectral changes that consist of an immediate shift of the maximum at 388 nm of free PLP to an absorption maximum at about 410 nm followed by a time-dependent decrease in the 388 nm and 410 nm absorbance bands. The reactions, monitored at 388 nm, follow pseudo first-order kinetics with second-order rate constants of 47.5 ± 0.003 mM^−1 min^−1 and of 28.98 ± 0.02 mM^−1 min^−1 for the reaction of PLP with D-5-HTP and D-Dopa, respectively (data not shown). The reaction was also studied with NMR spectroscopy. The assignment of resonances was straightforward on the basis of the NMR spectra of isolated compounds. Upon addition of a substoichiometric amount (1:10) of PLP to D-5-HTP or D-Dopa the NMR spectrum changes markedly. A very fast decrease up to the complete disappearance of the resonance because of the formyl proton of PLP was observed in both reactions. Moreover, in the same time course the resonances caused by the protons of D-5-HTP or D-Dopa decreased, and new resonances became clearly visible. These resonances display a spectral shape similar to those of D-5-HTP or D-Dopa, a low intensity similar to that of PLP, and are shifted slightly with respect to those of the reagents. All of these signals are consistent with the rapid and nearly complete formation of a Schiff base between PLP and the α-NH2 group of the amino acid. The reaction of PLP with D-5-HTP and D-Dopa, respectively (data not shown).

**Chemical Synthesis of Pictet-Spengler Adducts**—Chemical synthesis of the Pictet-Spengler adducts was carried out using procedures described (17, 18) indicates that the compounds formed are cyclized Schiff bases, the result of Pictet-Spengler condensation (Scheme 1).

These cyclic adducts were prepared by mixing 1 mM PLP with 10 mM D-5-HTP or D-Dopa in 100 mM potassium phosphate buffer, pH 7.5. After a 3-h incubation at 25 °C, the mixture was processed by HPLC (see below). With 5-HT or dopamine, PLP forms cyclized Schiff bases that had been isolated using the HPLC methods described below and characterized by means of absorbance spectroscopy.
HPLC Detection of PLP, PMP, Pictet-Spengler Adducts, and Indole-3-pyruvic Acid—The Pictet-Spengler adducts, obtained by synthesis as described above, were isolated from unreacted reagents using a 5-μm Pinnacle ODS (250 x 4.6 mm) (Restek) column connected to a Waters 625 LC HPLC control system. The eluant was 50 mM potassium phosphate buffer, pH 2.2, at a flow rate of 0.6 ml/min. A Waters UV detector set at 295 nm was employed. In addition to peaks corresponding to the retention time of PLP and D-5-HTP, this peak has a retention time of 44 min and an absorption spectrum with maxima at 275, 295, and 325 nm. For the reaction of PLP with D-5-HTP, this peak has a retention time of 44 min and an absorption spectrum with maxima at 275, 295, and 325 nm (data not shown).

Native or nicked DDC (8 μM) was incubated with 5 mM D-5-HTP in 100 mM potassium phosphate buffer, pH 7.5, at 25 °C. Aliquots were removed at time intervals, and trichloroacetic acid was added to a final concentration of 5% (v/v). The quenched solutions were centrifuged to remove protein, and the supernatants were analyzed using the above chromatographic system. Peaks, corresponding to PLP, PMP, and PLP-D-5-HTP adduct, were integrated with a Waters 745B data module. In the same manner, detection and quantification of these coenzymatic forms were carried out for reaction of native or nicked DDC with other ligands (D-Dopa, D-tryptophan, 1-5-HTP, or 5-HT). Standard curves of peak area as a function of concentration of coenzyme or coenzyme adducts were prepared using commercially available PLP and PMP or coenzyme adducts obtained by synthesis.

The separation of indole-3-pyruvic acid formed during the reaction of DDC with D-tryptophan was done isocratically on the 5-μm Pinnacle ODS column. The solvent was H2O/methanol/acetic acid (81.5/18/0.5, v/v/v) containing 1 mM octanesulfonic acid at a flow rate of 0.6 ml/min, and detection was at 295 nm. After incubation of 8 μM native DDC with 3 mM D-tryptophan for 15 h in 100 mM potassium phosphate, pH 7.5, at 25 °C, the reaction was stopped by adding trichloroacetic acid to a final concentration of 5% and then centrifuged to remove the precipitated protein. The supernatant and appropriate blanks were run. For quantification of indole-3-pyruvic acid, the area of the peak was measured and converted to absolute amounts by using a standard reference curve.

RESULTS

Reaction of Native DDC with D-Aromatic Amino Acids—As already reported for DDC purified from pig kidney (2), when recombinant DDC (10 μM) is preincubated with increasing concentrations of D-5-HTP (0.05–5 mM), the decarboxylase activity decreases as a function of time and D-5-HTP concentration following a pseudo first-order kinetics. Decarboxylase activity can be restored completely by the addition of exogenous coenzyme to the reaction mixture (data not shown).

Upon addition of D-5-HTP to DDC, an increased absorption centered at 430 nm appears immediately, and both the 420- and 335 nm dichroic bands decrease. These spectral changes can be attributed to the formation of the external aldimine. The 430 nm absorption and the 420 nm and 335 nm dichroic bands decrease with time, concomitant with the increase of the absorption in the 330 nm region (Fig. 1, A and B). During the reaction the absorption change at 430 nm follows a pseudo first-order behavior, and the apparent rate constant (kobs) shows dependence on the D-5-HTP concentration in a hyperbolic manner (Fig. 1A, inset). The value of the apparent dissociation constant and the maximum value of the rate constant are 1.07 ± 0.16 mM and 0.058 ± 0.003 min⁻¹, respectively. To determine if this reaction converted PLP into PMP or other coenzyme forms, at various times aliquots were withdrawn from a reaction mixture containing 8 μM DDC and 5 mM D-5-HTP in 100 mM potassium phosphate buffer, pH 7.5, and subjected to analysis by HPLC after total denaturation. These analyses confirmed that freshly purified native enzyme had all of its coenzyme in the PLP form. Moreover, time-dependent inactivation of DDC by D-5-HTP is accompanied by a decrease in PLP and a concomitant increase in PMP and PLP-D-5-HTP Pictet-Spengler adduct (Fig. 2A). This latter adduct has been identified as Pictet-Spengler type in that it has a spectrum (maxima at 325, 295, and 275 nm) and a retention time identical to those obtained with an authentic sample of PLP-D-5-HTP adduct formed by Pictet-Spengler reaction, as described above (data not shown).

The addition of D-Dopa to DDC causes the immediate increase of the absorbance at 430 nm with a concomitant decrease at 335 nm. However, it has been noted that in contrast to a previous report (5), these absorbing species are not stable intermediate complexes but undergo very slow time-dependent changes consisting of a decrease of the absorption at 430 nm and an increase of the 335 nm absorbance band. The apparent rate constant, measured following the 430 nm absorbance change at 5 mM D-Dopa, is 0.019 ± 0.001 min⁻¹. When DDC was preincubated with 5 mM D-Dopa in 100 mM potassium phosphate buffer, pH 7.5, at 25 °C, a slow loss of decarboxylase activity as a function of time was observed with a rate constant of 0.019 ± 0.013 min⁻¹. After a 15-h reaction of DDC with D-Dopa, the solution was denatured, and fractionation of the supernatant was then performed by HPLC under the same experimental conditions described above. In addition to D-Dopa, peaks were found corresponding to 32% PLP, 16% PMP, and 52% PLP-D-Dopa adduct, with respect to the original coenzyme content of the enzyme (Table I). The latter peak has been identified as a Pictet-Spengler adduct, PLP-D-Dopa, in that its retention time and its absorbance spectrum are identi-
Pictet-Spengler adduct is formed during the reaction of DDC transamination (5). The finding that in addition to PMP, a fraction could be explained, at least in part, on the basis of an appreciable fraction of the coenzyme remained in an acid produced during the reaction of DDC with D-5-HTP or under similar experimental conditions (data not shown).

0.5 nmol of ketoacid is produced, a quantity nearly equimolar with the PMP (0.4 nmol) formed under the same experimental conditions (data not shown). The absorption spectrum with those of standard sample and quantitatively determined. 0.5 nmol of ketoacid is produced, a quantity nearly equimolar with the PMP (0.4 nmol) formed under the same experimental conditions (data not shown).

Fractional coenzyme content and activity during the reaction of DDC with d-5-HP (Fig. 2). Native (panel A) or nicked (panel B) DDC (8 μM) was incubated with 5 mM d-5-HP in 100 mM potassium phosphate buffer, pH 7.5, at 25 °C. At the indicated times aliquots were removed and denatured with trichloroacetic acid. After removal of the precipitated protein by centrifugation, the supernatants were subjected to HPLC analysis as described under “Experimental Procedures.” □, PLP; ◇, PMP; ○, PLP-d-5-HP adduct.

In previous studies it was observed that inactivation of DDC by d-5-HTP does not parallel the conversion of PLP into PMP, and an appreciable fraction of the coenzyme remained in an unidentified form. This led to the suggestion that the inactivation could be explained, at least in part, on the basis of transamination (5). The finding that in addition to PMP, a Pictet-Spengler adduct is formed during the reaction of DDC with d-aromatic amino acids has allowed for a more complete understanding of this reaction.

5-Hydroxyindolepyruvic acid or 3,4-dihydroxyphenylpyruvic acid produced during the reaction of DDC with d-5-HP or d-Dopa, respectively, could not be identified because standard samples are not available. However, indole-3-pyruvic acid produced after 15 h of reaction of DDC with d-tryptophan has been identified by comparing its retention time on HPLC and its absorption spectrum with those of standard sample and quantitatively determined. 0.5 nmol of ketoacid is produced, a quantity nearly equimolar with the PMP (0.4 nmol) formed under the same experimental conditions (data not shown).

Protection against Limited Proteolysis of Native DDC by Aromatic Amino Acids and by Aromatic Amines—Limited trypsin digestion of DDC yields two fragments of 38 and 14 kDa produced by cleavage of the Lys^{334}-His^{335} peptide bond (11). When DDC was preincubated with d-5-HTP or d-Dopa, the tryptic digestion proceeded at a rate similar to that of the free enzyme. In fact, for unliganded DDC and the d-5-HP/DDC or D-Dopa/DDC complexes, the gradual disappearance of the 52-kDa band (corresponding to the undigested enzyme) and the concomitant appearance of the 38- and 14-kDa bands (corresponding to the proteolytic fragments) have similar time courses that lead to the near complete disappearance of the 52-kDa band within 10 min. On the other hand, the cleavage was markedly retarded by L-5-HP, dopamine, or 5-HT. In the presence of saturating concentrations of L-5-HP, dopamine, or 5-HT, the band corresponding to the intact enzyme persists up to 30 min (data not shown). These ligands were used at concentrations at least 10-fold higher than their K_D values. These results indicate a difference in accessibility of the enzyme and of the enzyme-ligand complexes to trypsin.

Functional Properties of Trypsin-treated DDC and Comparison with Those of the Native Enzyme—Absorption and CD (Fig. 3) spectra of nicked inactivated DDC (<1% of residual decarboxylase activity) in the UV-vis region are essentially identical to those of the native enzyme. CD spectra taken in the far UV region were superimposable for the native and nicked DDC (data not shown). These data indicate that no alterations in the tertiary or secondary structures are produced by limited tryptic digestion. As for native DDC, the addition of L-5-HP to nicked enzyme causes an increase of the absorbance at 430 nm with a concomitant decrease at 335 nm. However, although binding of L-5-HP to native DDC results in the inversion of the 420 nm CD signal, i.e. disappearance of the original positive CD and its replacement by a negative CD shifted to 440 nm and in the increase of the 335 nm dichroic band, binding of L-5-HP to nicked DDC results in a marked decrease of both the positive 420- and 335 nm dichroic bands (Fig. 3). Moreover, after a 1-h reaction, 55% of the original PLP content of the nicked enzyme was converted into PLP-L-5-HTP Pictet-Spengler adduct. Upon addition of d-5-HTP to the nicked enzyme, absorption and CD spectral changes are nearly identical to those observed with the native enzyme described above. The change in the 430 nm absorbance with time follows a pseudo first-order behavior at 430 nm.

Reaction Specificity of Dopa Decarboxylase
droxyphenylacetaldehyde and ammonia in equimolar amounts, which consumes O$_2$ in a 1:2 molar ratio with respect to the products. Concurrent with this reaction, 5-HT or dopamine inactivates DDC with a $k_{\text{inact}}$ of 0.023 min$^{-1}$ and 0.0059 min$^{-1}$, respectively. Under anaerobic conditions, these products are formed in amounts less than 5% with respect to those found under aerobic conditions (9, 10). We have now obtained the following results. 1) The reaction of the native enzyme with 4 mM 5-HT under anaerobic conditions is characterized by a decrease of bound PLP and a concomitant increase of PMP and PLP-5-HT adduct with time (Table I). 2) The reaction of DDC with L-5-HTP under aerobic conditions gives a plot of 5-HT versus time nearly linear for at least 25 min. However, under anaerobic conditions the decarboxylation rate dropped to 40% of its initial value within 25 min. The addition of free PLP at that time restored the rate to its initial value. Likewise, under anaerobic conditions the decarboxylation rate dropped to 40% of its initial value within 25 min. The addition of free PLP at that time restored the rate to its initial value.

Taken together, these results indicate that under anaerobic conditions DDC undergoes half-transamination of aromatic amines accompanied by a Pictet-Spengler reaction and provide evidence that the observed loss of decarboxylase activity during the time course under anaerobic conditions is caused by the conversion of PLP into nonfunctional coenzyme forms (PMP and Pictet-Spengler adduct). The spectral changes observed upon addition of 5-HT or dopamine to nicked DDC consist of a modest absorption increase at 430 nm and no relevant change of the 430 nm dichroic peak. A very slow 420 nm absorbance decrease with time following a pseudo first-order behavior was observed for both of the aromatic amines (data not shown). At 2 mM 5-HT or dopamine the $k_{\text{obs}}$ was 0.009 ± 0.004 min$^{-1}$.

**TABLE I**

<table>
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<tr>
<th>Substrate$^a$</th>
<th>Reaction time</th>
<th>Native</th>
<th>PMP</th>
<th>Pictet-Spengler adduct</th>
<th>Nicked</th>
<th>PMP</th>
<th>Pictet-Spengler adduct</th>
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<td></td>
<td>h</td>
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<td>PMP</td>
<td>ND</td>
<td>PLP</td>
<td>PMP</td>
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<tr>
<td>D-Dopa (5 mM)</td>
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<td>52</td>
<td>47</td>
<td>10</td>
<td>43</td>
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$^a$ For D-5HTP see Fig. 2.

$^b$ PLP is converted into an inactive form (9, 10).

**FIG. 3.** CD spectral changes of native (solid line) and nicked (broken line) DDC upon reaction with L-5-HTP. Curve 1 represents native DDC (10 μM) in 100 mM potassium phosphate buffer, pH 7.5. To this mixture, 5 mM L-5-HTP was added and the CD spectrum immediately recorded (curve 2). Similarly, curve 1$'$ represents nicked DDC (10 μM) in the same buffer as above, and curve 2$'$ is the CD spectrum after the addition of 5 mM L-5-HTP.

**FIG. 4.** Spectral changes occurring upon addition of 5-HT to the native enzyme. The native enzyme (10 μM) in 100 mM potassium phosphate buffer, pH 7.5, was incubated with 2 mM 5-HT at 25°C. Absorbance (panel A) and CD (panel B) spectra were recorded at the indicate times. The broken line represents the absorbance (panel A) and CD (panel B) spectra of untreated 10 μM native DDC.
After a 15-h reaction of nicked DDC with 5-HT or dopamine, the original PLP was converted into PMP and PLP-5-HT or PLP-dopamine adducts, respectively (Table I). Neither ammonia nor aldehyde formation could be observed when the reaction of the nicked enzyme with 5-HT or dopamine was coupled with glutamate dehydrogenase or with alcohol dehydrogenase, respectively, in the presence of NADH. Moreover, no O₂ consumption could be detected. These data suggest that the truncated enzyme species is unable to catalyze oxidative deamination. These data indicate that, unlike the native enzyme, the nicked enzyme undergoes a slow transaminase reaction of aromatic amines accompanied by a Pictet-Spengler reaction occurring between the ligand and the PLP-bound enzyme.

**DISCUSSION**

Along with the main reaction, almost all PLP-dependent enzymes catalyze many side reactions. In this regard, DDC is not an exception in that in addition to catalyzing decarboxylation of L-aromatic amino acids, it has been demonstrated recently that the enzyme catalyzes oxidative deamination of aromatic amines, even at O₂ concentrations higher than atmospheric (data not shown). Taken together, these results indicate that, unlike the native enzyme, the nicked enzyme undergoes a subtle conformational change followed by a marked conformational change during the transaldimination process. The finding of lack of protection of DDC by D-aromatic amino acids against trypsin modification argues against this proposal. Our results suggest that a substantial conformational change of this region occurs only during a transaldimination that leads to an external aldimine competent for decarboxylation or for oxidative deamination, but not for transamination. This view is reinforced by the following points. 1) Although binding of aromatic amines to the active center of truncated DDC does occur, as shown by changes in the absorbance and CD bands at 430 nm, this enzymatic species fails to form an absorbance and a positive dichroic band around 400 nm. This observed red shift could indicate a change in the environment which does not necessarily imply a global change throughout the entire active site region. 2) Although the addition of saturating concentrations of L-5-HTP to the native or the nicked forms of the enzyme causes an identical increase of the absorbance at 430 nm for both enzymic species, it results in distinctly different changes of their coenzyme dichroic bands (Fig. 3). This suggests that binding of L-5-HTP to the native enzyme causes changes in the orientation of the coenzyme, with respect to the neighboring residues, different from those caused by the binding of the same ligand to the nicked enzyme. 3) Absorbance and CD spectral features of the intermediate D-5-HTP-enzyme complexes are identical for either native or nicked DDC, thus suggesting that binding of aromatic amino acids in the D form causes similar changes in the microenvironment of the coenzyme for both of the enzymatic species, different from that generated upon binding of the corresponding L-aromatic amino acids to the native enzyme.

On the basis of these results, the occurrence of at least three and two different conformational states of the native and the nicked DDC enzymes, respectively, can be predicted (Scheme III). Both of the unliganded enzymatic species adopt the "open" conformation. Although binding of ligands leading to decarboxylation or oxidative deamination shifts the conformational equilibrium to the "closed" form, binding of ligands leading to half-transamination and Pictet-Spengler reactions shifts the equilibrium to the "half-open" form. We propose two models of transition between these conformational states (Scheme III). In...
the first model (A), the shift open → closed or open → half-open might take place in one step; in the second (B) two steps may be required for the transition open → closed. The closed form may be a compact structure that may be considered a critical one for catalysis (decarboxylation and oxidative deamination, but not transamination). In this conformational form, the Pictet-Spengler reaction does not occur. The closing of the active-site cleft would correspond to a ligand-induced conformational change that cannot occur in the nicked enzyme. Ligand-induced conformational changes closing the active-site pocket have been demonstrated (23–26) or proposed (27–30) for many PLP-dependent enzymes. Thus in the structural transition from the open to the closed form, a crucial role appears to be played not only by the region around the tryptic cleavage site, but also by the chemical nature of the ligand. Structural elements in the ligands that are required for this conformational shift can be suggested. The first may involve the catecholic or the 5-hydroxyindolic ring of L-aromatic amino acids or aromatic amines, whose specific interactions with the protein could impose important constraints in the alignment of catalytic groups at the active site. The fact that L-tyrosine, L-phenylalanine, and L-tryptophan are very poor substrates for DDC with respect to L-Dopa (31) and the recent finding that unlike that observed with L-Dopa and L-tyrosine methyl esters, the binding of L-phenylalanine methyl ester to the nicked enzyme is unaltered with respect to the native enzyme (12) and that this shift cannot be achieved without the integrity of the region around the specific tryptic cleavage of DDC. Therefore, according to this view, the active site of native DDC would be able to accommodate either CO₂ (conformation I, Scheme II) or dioxygen molecules at subsite A. A conformation (III) (Scheme II) orienting the Cα-H bond perpendicular to the plane of the cofactor system is productive for the transamination half-reaction of aromatic amines. Considering that the proposed mechanism for the re-action of DDC with 5-HT under aerobic conditions involves a conformation of the external aldimine undergoing deprotonation at Cα (9), it can be suggested that the conformation III (Scheme II) could also be productive for oxidative deamination, provided that in this conformation subsite A is capable of productively binding molecular oxygen. This hypothesis is reminiscent of the mutual competition between CO₂ and O₂ to the enediol(ate) intermediate formed from d-ribulose 1,5-bisphosphate at the active site of ribulose-1,5-bisphosphate-carboxylase/oxygenase (32). It is clear that this view is merely suggestive, and the poorly understood kinetic mechanism of this reaction makes any attempt to correct interpretation difficult. However, whatever the mechanism of oxidative deamination may be, our data indicate a different fate of an L-aromatic

(SCHEME III. Conformational states of the native and the nicked DDC enzymes.)
The half-open form of DDC has been attributed to a state of the enzyme in the native or nicked form, in which in addition to a half-transamination, a Pictet-Spengler reaction between the ligand and bound PLP takes place. In the absence of a crystal structure of DDC, the half-open form of this enzyme can only be presumed to be a looser structure with respect to the closed one. As for the occurrence of the Pictet-Spengler reaction, at present it is not possible to distinguish if the cyclized Schiff base is generated at the active site of the enzyme or if the external aldimine undergoes subsequent cyclization after release from the enzyme. Again, it is not easy to envisage why events leading to the Pictet-Spengler adduct are more frequent than transamination events with D-Dopa with respect to D-5-HTP and with dopamine with respect to 5-HT. We can speculate that different substrates stabilize slightly different conformers of the enzyme which differ in their preference for the pathway leading to transamination versus the pathway leading to cyclization. Nevertheless, the finding that D-5-HTP forms an external aldimine with either native or nicked enzyme in a conformation leading to transamination and Pictet-Spengler reactions indicates that a structural change involving the region around Lys304 is not required for transition between conformationally open and half-open states. The behavior resulting from the binding of aromatic amines to the nicked enzyme is in accord with this interpretation. Furthermore, the finding that the external aldimine of L-5-HTP with the nicked enzyme undergoes only a Pictet-Spengler reaction and not transamination indicates the nonproductive binding mode for L-5-HTP transamination, as suggested by the proposed active site model (Scheme II).

In conclusion, spectral and kinetic studies of the reactions of the native and nicked DDC enzymes with various ligands have allowed the elucidation of some structural elements of the protein and ligand which appear to be relevant to the reaction specificity of this enzyme.

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Reaction Specificity of Native and Nicked 3,4-Dihydroxyphenylalanine Decarboxylase
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