Interaction of Aromatic Amino Acids in D and L Forms with 3,4-Dihydroxyphenylalanine Decarboxylase from Pig Kidney†

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ABSTRACT: The actions exerted by aromatic D-amino acids on 3,4-dihydroxyphenylalanine (Dopa) decarboxylase have been examined by measuring their effects on the enzymic activity and on the spectral properties of the pyridoxal 5′-phosphate bound enzyme. It has been shown that Dopa decarboxylase does not decarboxylate aromatic D-amino acids, thus indicating its stereospecificity. However, the enzyme does appear to bind these amino acids at the active site. Dopa, *m*-tyrosine, and *o*-tyrosine in D forms appear to inhibit the enzymic activity and to form stable intermediate complexes absorbing at 420 nm with the enzyme. 5-Hydroxytryptophan and tryptophan in D forms appear to exert a time-dependent inactivation and to form with the enzyme intermediate complexes absorbing at 420 nm undergoing time-dependent modification; the formation of pyridoxamine 5′-phosphate is also observed. The behavior of these latter two amino acids can be partially attributed to a transamination catalyzed by Dopa decarboxylase; a plausible mechanism of inactivation has not yet been elucidated. The interaction of the aromatic amino acids in D forms with the enzymes calls for critical revision of all previously obtained results by using the racemic forms as substrates. Therefore we here report on the kinetic parameters for Dopa, 5-hydroxytryptophan, *m*-tyrosine, and *o*-tyrosine in L forms, showing that unnatural substrates have a decarboxylation rate higher than that of the natural ones. We have also identified two enzyme–substrate intermediate complexes absorbing at 420 and 390 nm which are displayed by the low and high pH forms of the complexes, respectively. Considerations on the nature of this spectral change have been reported. The different p*K* value of the shift 420–390 nm, as determined for L-Dopa and L-5-hydroxytryptophan, appears to be related to the different chemical structure of the substrate. The authors also discuss the relevance of their findings toward a better elucidation of Dopa decarboxylase active site.

The issue of whether Dopa[1] decarboxylase and 5HTP decarboxylase are two distinct enzymes has been the subject of controversy for several years. However, more recent evidence (Christenson et al., 1970; Lancaster & Sourkes, 1972; Srinivasan & Awapara, 1978) favors the hypothesis that a single enzyme acts on both substrates, as well on tyrosine in para, meta, and ortho form, tryptophan, phenylalanine, and some of their methyl derivatives. For this reason, the enzyme is best described as aromatic L-amino acid decarboxylase (EC 4.1.1.28). Several authors, analyzing and comparing the kinetic parameters of the above-mentioned substrates (Christenson et al., 1970; Lancaster & Sourkes, 1972; Srinivasan & Awapara, 1978) and of their spectral enzyme–substrate intermediates (Fiori et al., 1975) involved in the catalytic process, made no distinction in using L and DL forms, the latter being, in some cases, the only ones commercially available. Since it is not yet clearly defined if D forms of these substrates are capable of binding to the active site of the enzyme, we decided to investigate their interaction with the enzyme.

We here report spectral and kinetic evidence for the binding of D forms of Dopa, *m*-tyrosine, and *o*-tyrosine to Dopa decarboxylase and for the time-dependent inactivation exerted by D-5HTP on the decarboxylase activity. We also report kinetic parameters for Dopa, 5HTP, *m*-tyrosine, and *o*-tyrosine in L forms showing that the rate of decarboxylation of *m*-tyrosine and *o*-tyrosine is higher than that of Dopa and 5HTP. Moreover, spectrophotometric studies relative to the effect of L-Dopa and L-5HTP on the enzyme-bound coenzyme have been carried out, suggesting that variations in substrate structure can result in variations in the properties of the intermediate complexes.

Materials and Methods

L-Dopa, D-Dopa, L-5HTP, D-5HTP, DL-*m*-tyrosine, DL-*o*-tyrosine, L-amino acid oxidase, D-amino acid oxidase, catalase, pyridoxal 5′-phosphate, and 2,4,6-trinitrobenzene-1-sulfonic acid were obtained from Sigma Chemical Co., and Dowex 50-X8 was purchased from Bio-Rad Laboratories. Aspartate aminotransferase was prepared by Prof. A. Giartosio (University of Rome, Italy). Spectral data were obtained with a Cary 219 spectrophotometer.

Enzyme Purification. Dopa decarboxylase from pig kidney has been highly purified according to Borri Voltattorni et al. (1979). Enzyme concentration is expressed as bound coenzyme determined by releasing the bound PLP into 0.1 M NaOH by using 6600 as the molar extinction coefficient for PLP concentration.

Preparation of D and L Isomers of m-Tyrosine and o-Tyrosine. D-*m*-Tyrosine and D-*o*-tyrosine were prepared from the corresponding racemic forms by the action of L-amino acid oxidase and catalase in 0.1 M potassium phosphate buffer, pH 6.6, under O_2 stream. The enzymatic degradation of the D-amino acid form was followed by measuring at time intervals the formation of the keto acid form with 2,4-dinitrophenylhydrazine. The end point of the reaction was achieved when, even after further addition of enzymes, no more keto acid was detected. Then the reaction mixture, brought to pH 3, was poured on a Dowex 50-X8 column. After elution of the keto acid with 0.2 M pyridine buffer, pH 3.1, the D-amino acid was eluted with 2 M pyridine buffer, pH 5; this solution was then lyophilized. L-*m*-Tyrosine and L-*o*-tyrosine were prepared from the corresponding racemic forms by the action of D-amino acid oxidase and catalase in a 0.05 M pyrophosphate buffer, pH

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[1] Abbreviations: Dopa, 3,4-dihydroxyphenylalanine; 5HTP, 5-hydroxytryptophan; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane.

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8.3, under O_2 stream. The procedure described above has been used to follow the reaction and to obtain the separation of L forms from the keto acid. The purity of these preparations was revealed by (1) the absence of reaction of D and L forms with L-amino acid oxidase and D-amino acid oxidase, respectively, (2) total conversion of D and L forms into the keto acid form by D-amino acid oxidase and L-amino acid oxidase, respectively, and (3) the absence of keto acid on addition of 2,4-dinitrophenylhydrazine.

Assay of Enzymic Activity. Enzymic activity was measured by the assay method of Sherald et al. (1973), according to the modification introduced by Charteris & John (1975). The standard reaction contained, in a final volume of 250 μL, 100 μL of 0.1 M potassium phosphate buffer (pH 6.8) and various concentrations of the substrate and enzyme. Care has been taken to use adequate experimental conditions to keep the enzymic reaction linear during the first 5 min of reaction in order to minimize the inactivation of Dopa decarboxylase.

After 5 min of incubation at 25 °C, the reaction was stopped by heating at 100 °C for 1 min. Benzene (1.5 mL) and 2,4,6-trinitrobenzene-1-sulfonic acid (1 mL of a 4.3 mM solution in 0.1 M potassium phosphate buffer, pH 7.5) were added, and the reaction forming trinitrophenylamine derivative was carried out at 42 °C for 1 h with continuous shaking. The concentration of trinitrophenylamine derivative in the benzene layer was measured with a Beckman DB-GT spectrophotometer by using 12400 and 12800 as the molar extinction coefficients for trinitrophenyldopamine and (trinitophenyl)-5-hydroxytryptamine, respectively (Streffer, 1967).

Inhibition Experiments. Two types of inhibition experiments were performed. The first was carried out by running regular enzyme assays in the presence of inhibitor. The second was designed to determine by "preincubation" whether a compound would produce time-dependent inhibition, i.e., inactivation, and was therefore performed by treating enzyme with inhibitor at 25 °C in the absence of substrate. At time intervals, aliquots of the preincubation solution were removed and diluted (usually 50-fold) into a normal assay mixture containing substrate (L-Dopa), and enzymic activity was determined in the usual fashion.

Detection of PLP and PMP with Apoaspartate Aminotransferase. Determination of PLP and PMP in the enzyme after reaction with D-5HTP was performed according to the method of Bossa & Barra (1968); detection of PMP was carried out with the same method, after treatment with KBH_4 (Holzer & Schreiber, 1962) to reduce PLP.

Results

Behavior of Dopa Decarboxylase with Dopa, m-Tyrosine, and o-Tyrosine in D *Forms.* The addition of D-Dopa changes the spectral properties of the enzyme-bound coenzyme by increasing the intensity of the 420-nm peak and by decreasing the intensity at 335 nm. In Figure 1 the spectra of Dopa decarboxylase in the presence of increasing amounts of D-Dopa at pH 6.8 and 8.4 are reported. The differences in absorbance at 420 nm between the enzyme alone and enzyme plus increasing amounts of D-Dopa, used as a criterion for D-Dopa–enzyme complex formation, have been plotted as a function of D-Dopa concentration in a double-reciprocal plot as shown in the insets of Figure 1. The dissociation constants, K_D, measured in this way are reported in Table I.

The same spectral behavior has been observed by adding D-*m*-tyrosine and D-*o*-tyrosine to the enzyme; on the basis of the spectra obtained at pH 6.8 at different concentrations of these compounds (data not shown), their respective affinity constants have been measured, and the values obtained are

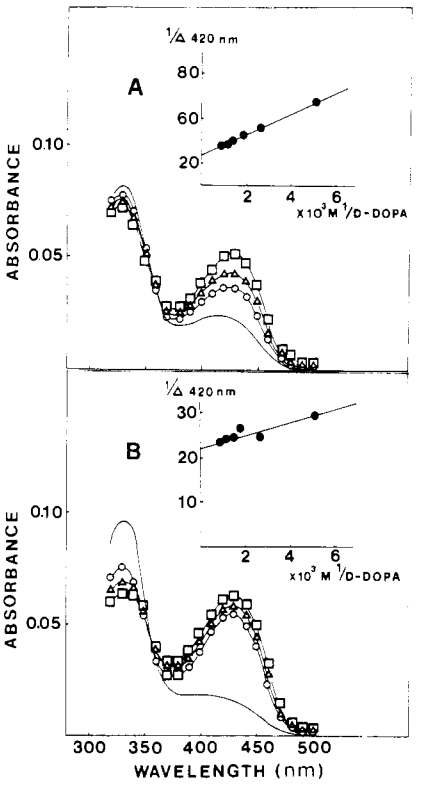


FIGURE 1: Spectra of (—) the 10 μM enzyme, of (○) the enzyme plus 196 μM D-Dopa, of (△) the enzyme plus 384 μM D-Dopa, and of (□) the enzyme plus 1075 μM D-Dopa in (A) 0.1 M potassium phosphate buffer, pH 6.8, and in (B) 0.1 M triethanolamine–HCl buffer, pH 8.4. (Inset) Absorbance at 420 nm of the enzyme plus D-Dopa as a function of its varying concentrations presented as double-reciprocal plot.

Table I: Kinetic Parameters for L-Amino Acids and Kinetic Constants for D-Amino Acids of Dopa Decarboxylase from Pig Kidney

amino acid	K_m (M)	V_{max} (nmol min^{-1} mg^{-1})	K_I[a] (M)	K_D[a] (M)
L-Dopa	1.8×10^{-4}	2386		
L-5HTP	2.3×10^{-4}	422		
L-*m*-tyrosine	5.2×10^{-4}	4944		
L-*o*-tyrosine	1.2×10^{-4}	5647		
D-Dopa			4.6×10^{-4}	3.3×10^{-4} (0.6×10^{-4})[b]
D-*m*-tyrosine			1.0×10^{-3}	1.4×10^{-3}
D-*o*-tyrosine			2.2×10^{-3}	3.2×10^{-3}

[a] K_I and K_D represent the apparent dissociation constants measured as described in the text. [b] The value in parentheses is obtained at pH 8.4 from the reciprocal plot of Figure 1B.

listed in Table I. Dopa and *m*- and *o*-tyrosine in D form do not act as substrates for the enzyme. Since these compounds affect the absorption peaks of the PLP-bound enzyme, we have investigated their effects on the enzymic activity. Decarboxylation of L-Dopa was competitively inhibited by D-Dopa, D-*m*-tyrosine, and D-*o*-tyrosine in concentrations less than 2 mM. The inhibitor constants, K_I, calculated at pH 6.8 are shown in Table I. At higher concentrations of the D-amino acid, a noncompetitive type of inhibition was observed.

Inactivation of Dopa Decarboxylase by D-*5HTP.* D-5HTP does not behave as a substrate of Dopa decarboxylase. The addition of D-5HTP to an enzymic solution at pH 8.4 causes the immediate appearance of the absorption at 420 nm and a slight decrease of the peak at 335 nm, indicating the active

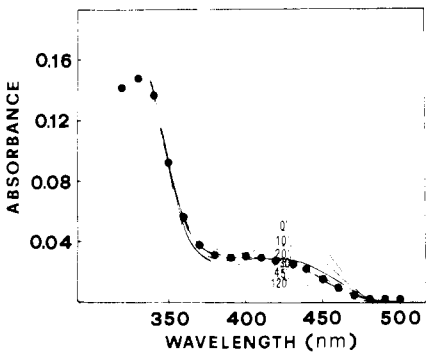


FIGURE 2: Spectra in 0.1 M triethanolamine–HCl buffer, pH 8.4, of (●) the 16 μM enzyme and of (—) the enzyme plus 5 mM D-5HTP at various times.

site directed nature of the interaction. Then the PLP absorption at 420 nm is bleached concomitantly with the increase of absorption at 325 nm (Figure 2). The observed time-dependent modification of the holoenzyme spectrum after addition of D-5HTP suggests the occurrence of a reaction between the enzyme and the D-amino acid. The active site implication has been further investigated by following the decarboxylase activity of the enzyme toward L-Dopa in the presence of D-5HTP. Incubation of Dopa decarboxylase with various concentrations of D-5HTP results in a time-dependent loss of enzymic activity (Figure 3).

The inactivation can be largely prevented by adding PLP to the preincubation mixture and partially reversed by the presence of exogenous coenzyme in the reaction mixture as shown in Figure 3. The inactivated enzyme (90%) does not recover activity after extended dialysis or gel filtration; the addition of PLP restores the enzymic activity up to 55%. The spectral and kinetic behavior of the enzyme with D-5HTP suggests that the action of D-5HTP is due to a transamination. So that this hypothesis could be tested, an assay for PMP and PLP has been performed on D-5HTP-treated enzyme: after a 2-h reaction with 5×10^{-3} M D-5HTP at pH 8.4, only 27–30% of the original content of PLP is recovered as PMP, and an appreciable fraction of the coenzyme (60–70%) remains in the aldehydic form. These data, similar to those obtained in the presence of other substrates which are transaminated by Dopa decarboxylase (Barboni et al., 1981), indicate that the inactivation by D-5HTP could be explained, at least in part, on the basis of transamination.

A behavior corresponding to that described for the enzyme treated with D-5HTP at pH 8.4 can be observed at pH 6.8 where slower inactivation rate and spectral modification are obtained. Similar results are obtained with D-tryptophan.

Kinetic Data of L *Isomers.* Considering the inhibitory action of D isomers of Dopa, 5HTP, *m*-tyrosine, and *o*-tyrosine, the initial velocities of decarboxylation of the corresponding L isomers have been determined at pH 6.8.

It is well-known that Dopa, *m*-tyrosine (O'Leary & Baughn, 1977), and *o*-tyrosine (Rudd & Thanassi, 1981) undergo a decarboxylation-dependent transamination in the presence of Dopa decarboxylase which results in its inactivation. 5HTP appears not to follow the decarboxylation-dependent transamination at any significative extent (Rudd & Thanassi, 1981). Therefore, as described under Materials and Methods, we have determined the initial velocities for L-Dopa, L-*m*-tyrosine, and L-*o*-tyrosine in experimental conditions where enzyme-bound PLP is minimally converted to nonfunctional PMP by the decarboxylation-dependent transamination. The V_{max} values thus obtained are as close as possible to the correct values.

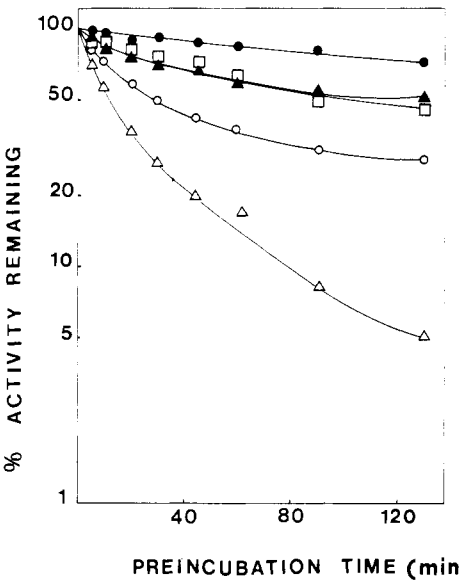


FIGURE 3: Time-dependent inhibition of Dopa decarboxylase caused by preincubating it with D-5HTP. Controls, which lack inhibitor, do not lose activity during the course of the experiments. Preincubation mixture contains 16 μM enzyme and the indicated concentrations of D-5HTP: (▲) 0.1 mM; (○) 0.5 mM; (△) 5 mM; (●) 5 mM D-5HTP and 0.2 mM PLP; (□) 5 mM D-5HTP in the presence of 1 μM PLP in the reaction mixture.

The K_m and V_{max} values derived from Lineweaver–Burk plots of the data are reported in Table I. It is apparent that L-Dopa undergoes decarboxylation better than L-5HTP; furthermore, it is noteworthy that L-*o*-tyrosine and L-*m*-tyrosine behave as better substrates than L-Dopa.

Spectral Changes of Dopa Decarboxylase with L-*5HTP and* L-*Dopa.* Fiori et al. (1975), working at a different pH, have identified two intermediate substrate–enzyme complexes absorbing at 420 and 390 nm for DL-*m*-tyrosine with a spectral transition pK of 7.6. Considering that D forms modify the spectral features of the enzyme, the results obtained with DL forms do not seem to represent the real intermediate substrate–enzyme complexes.

Due to the high rate of decarboxylation of L-*m*-tyrosine and L-*o*-tyrosine, the reaction of the enzyme with these substrates cannot be studied by conventional spectrophotometry. However, it has been possible to obtain spectral data of substrate–enzyme complexes for L-Dopa and L-5HTP, using a high concentration of both substrates and a low temperature (5 °C for L-Dopa, 10 °C for L-5HTP); thus the enzyme was maintained close to saturation for many minutes. As shown in Figures 4 and 5, an increased pH causes the decrease of the 420-nm form and the increase of the 390-nm band. The absorbance at 420 nm and at 390 nm has been measured for L-Dopa and L-5HTP as a function of pH. A transition (420 nm → 390 nm) was observed with a midpoint at 7.3 for L-Dopa (inset of Figure 4) and at pH 8.8 for L-5HTP (inset of Figure 5).

Discussion

There are conflicting reports concerning the interaction of aromatic amino acids in D forms with the enzyme. Inagaki & Tanaka (1978), using a partially purified L-aromatic amino acid decarboxylase of hog kidney, found that the decarboxylation of L-*threo*-(3,4-dihydroxyphenyl)serine specifically occurs with the L isomer and not with the D isomer. They also observed that the decarboxylation of L-*threo*-(3,4-dihydroxyphenyl)serine was inhibited to different extents by D-Dopa, D-*threo*-(3,4-dihydroxyphenyl)serine, and D-*erythro*-(3,4-di-

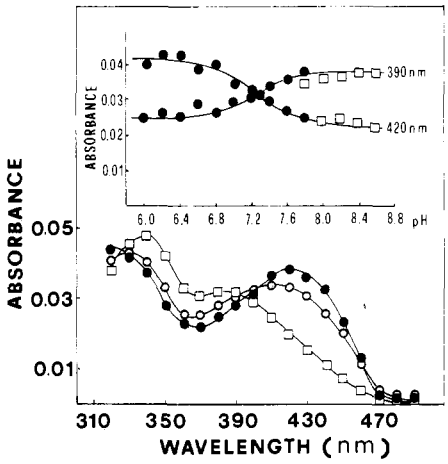


FIGURE 4: Spectra of 10 μM enzyme plus 2 mM L-Dopa run in 0.1 M potassium phosphate, pH 6.6 (●), 0.1 potassium phosphate, pH 7.2 (○), and 0.1 M Tris-HCl, pH 8.0 (□). (Inset) Absorbance at 420 nm and at 390 nm of the enzyme plus L-Dopa as a function of pH. (●) Potassium phosphate buffer; (□) Tris-HCl buffer.

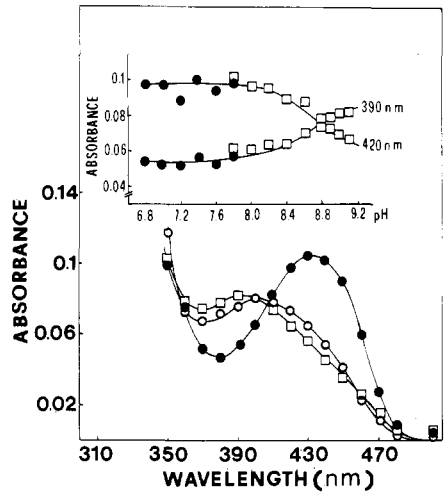


FIGURE 5: Spectra of 16 μM enzyme plus 5 mM L-5HTP run in 0.1 M potassium phosphate, pH 6.8 (●), 0.1 M Tris-HCl, pH 8.8 (○), and 0.1 M Tris-HCl, pH 9.1 (□). (Inset) Absorbance at 420 nm and at 390 nm of enzyme plus L-5HTP as a function of pH. (●) Potassium phosphate buffer; (□) Tris-HCl buffer.

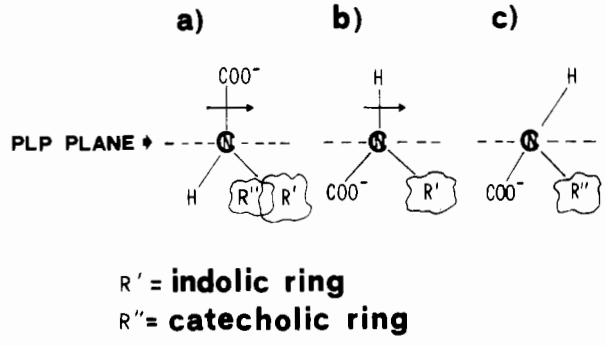
hydroxyphenyl)serine, suggesting that this inhibition could be the result of the interaction of D isomers with the enzyme. D-5HTP and D-tryptophan did not show inhibitory effects at least at the concentrations used (up to 1 mM). The same results have been obtained by authors who measured the decarboxylation of L-5HTP by rat brain, liver, and kidney homogenate in the presence of D-Dopa, D-*threo*-(3,4-dihydroxyphenyl)serine, D-5HTP, and D-tryptophan (Shikimi et al., 1978).

Moreover, in a recent report Maycock et al. (1980), analyzing the inactivation of Dopa decarboxylase by the L isomer of 2-(fluoromethyl)-3-(3,4-dihydroxyphenyl)alanine, suggest that the D isomer is likely to have some affinity for the enzyme. On the other hand, Rudd et al. (1979), studying the inhibitory action of *N*-(5′-phosphopyridoxyl) derivatives of several aromatic amico acids on a crude extract of Dopa decarboxylase from mouse liver, pointed out that the L-Dopa derivative shows approximately the same inhibition at half the concentration of the DL derivative, thus indicating that the enzyme has a stereochemical preference for the L isomer.

Our spectral and kinetic data on the interaction of D-amino acids with Dopa decarboxylase are indicative of their binding to the enzyme-bound coenzyme which results in intermediate complexes with different properties that seem to depend on the aromatic D-amino acid structure. The addition to the enzyme of aromatic amino acids with a catechol-related structure, in D form (i.e., Dopa, *m*-tyrosine, and *o*-tyrosine), induces a spectral modification of the enzyme-bound coenzyme, consisting in an increase of intensity of the 420-nm band and a decrease of the 330-nm band. These compounds give a spectrum similar to that of the enzyme–substrate complex, characterized by an increased absorption at 420 nm, already attributed to a 4′-N-protonated Schiff base structure (Fiori et al., 1975). While this absorption band disappears during the decarboxylation of the substrate (Fiori et al., 1975), the same peak formed with these D-amino acids does not undergo any further change in time. It can be inferred that these compounds are capable, as the substrate does, of forming a Schiff base with the aldehydic group of the coenzyme, but their positioning at the active site of the enzyme does not result in a right configuration for the catalysis. Actually, these compounds do not behave as substrates for the decarboxylase reaction. Their affinity constants, K_D, calculated from the spectra obtained at different D isomer concentrations, are quite consistent with the K_I obtained by competitive inhibition kinetics. The noncompetitive inhibition observed at a concentration higher than 2 mM can be regarded as further indication for the existence of a binding site(s) other than the active one, as already suggested by several authors (Bender & Coulson, 1977; Srinivasan & Awapara, 1978; Maycock et al., 1978; Borri Voltattorni et al., 1981; Rudd & Thanassi, 1981). As for the interaction of the enzyme with 5HTP and tryptophan in D forms, we observed a time-dependent modification of the holoenzyme spectrum, a time-dependent inactivation of the enzymic activity which could be reversed, although partially, by adding PLP. We also observed the formation of PMP in the enzyme treated with these amino acids. Taken together, these data can be indicative of the occurrence of a transamination between the enzyme and these D-amino acids. However, it should be noted that during the inactivation process other event(s), in addition to transamination, are possibly occurring since (1) 60–70% of the enzyme-bound PLP remains and 27–30% of the original content of PLP is recovered as PMP when more than 90% of the activity is lost, (2) the enzymic activity cannot be fully recovered by adding PLP to the inactivated enzyme, and (3) the time course of inactivation displays a biphasic nature. One could therefore envisage that, in addition to transamination, a suicide enzyme inactivation takes place. Further work will be needed to assess whether this type of inactivation meets the criteria for identifying a suicide enzyme inactivation. Moreover, it must be kept in mind that data obtained in the presence of other substrates which are transaminated by Dopa decarboxylase (Borri Voltattorni & Minelli, 1980; Barboni et al., 1981) are indicative of an abnormal stoichiometry of the reaction. Actually, the aldehyde or the ketone is produced in amounts far exceeding, on molar basis, that of the PLP, and only a small fraction (10–20%) of PLP is transformed into PMP. It has been proposed that PLP might be the catalyzer of the transamination reaction to some, not yet identified, amino group acceptors located on (or strongly bound to) the enzyme.

Whatever the mechanism for the inactivation process exerted by D-5HTP and D-tryptophan could be, it is quite difficult to explain why some authors (Inagaki & Tanka, 1978; Shikimi et al., 1978) failed to detect any inhibitory effects on the enzymic activity by these D-amino acids; it is reasonable to hypothesize that the crude enzyme preparation they used could

Scheme I



underlie their results. The failure of Dopa decarboxylase to decarboxylate the tested D-amino acids indicates the enzyme reaction specificity for L-amino acids. Dunathan (1966) explained this PLP enzyme specificity in a model postulating that the conformation of the groups about the substrate α-carbon to nitrogen bond in the initial amino acid–PLP aldimine is such that the bond to be cleaved is oriented in an activated position in a plane perpendicular to the plane of the PLP ring. He also suggested that this orientation could be directed by site-specific binding of the substrate α-carboxylate in the active site. According to this view, the decarboxylation of aromatic L-amino acids can take place when the C_α-COOH is perpendicular to the plane of the cofactor system (Scheme Ia). It should be noted that Dopa decarboxylase does bind the aromatic amino acids in D forms. Among these amino acids, it has been observed that the indole-related D forms undergo a transamination. This observed reaction specificity is not predicted by the Dunathan model if α-carboxylate binding controls substrate geometry. This transamination, however, is consistent with the model if one assumes that site-specific binding of the amino acid side chain directs the conformation of the substrate bonds relative to the coenzyme. The proposal of the amino acid side chain as directing group to a single, activated position has been already invoked by Bailey et al. (1970) to explain the enzyme reaction specificity of α-dialkylamino acid transaminase. The substrates of Dopa decarboxylase have bulky groups as side chains as compared with the remaining groups attached to the α-carbon so that the requirement of the side chain for an active orientation seems to be an even more crucial factor. Under the assumption that the side chain of aromatic D-amino acids is oriented in the same relative position as the corresponding L forms, it can be argued that the α-carboxylate and α-hydrogen of the enantiomers occupy opposite sites when they are bound. As for the indole-related D forms, our results are compatible with a conformation most similar to that of the transition state in the proton elimination reaction.

The finding of lack of reactivity of catechol-related D forms does not agree with our view of substrate binding to Dopa decarboxylase. They do not undergo either decarboxylation or transamination, indicating that neither α-carboxylate nor α-hydrogen groups orient in active position. However, they still bind to the active site of the enzyme with dissociation constants of the same order of magnitude as the constants for the corresponding L-amino acids (Table I). It is not easy to envisage a different position of the side chain for the enantiomers of the catechol-related compounds, and at this stage only hypotheses can be advanced. We can suggest the existence of two subsites at the active site of the enzyme, with different groups involved in the binding of the side chains. A similar hypothesis has been already suggested for Dopa decarboxylase (Bender & Coulson, 1977; Srinivasan & Awapara, 1978). These two subsites could be partially overlapping.

When the D-indole compound binds to the enzyme, attachment of side chain at its own subsite would place the α-hydrogen in the activated position for cleavage (Scheme Ib); the side chain of the D-catechol compound binding at its own subsite should not result in an activated α-hydrogen position (Scheme Ic) probably due to steric hindrance between the catechol side chain and the α-carboxylate group. Such steric hindrance would not occur with L-catechol compounds whose α-carboxylate group is perpendicularly oriented to the plane of the cofactor system. This view is merely suggestive of the active site structural features of Dopa decarboxylase. The fact that indole compounds inactivate whereas catechol compounds do not is not sufficient evidence for the existence of different binding sites.

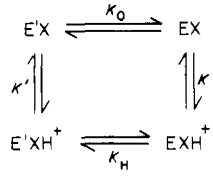
Conformational changes in the enzyme, induced by aromatic D-amino acids could also be taken into account, even if no evidence is, at the moment, available. The poorly understood specificity of Dopa decarboxylase active site makes any attempt to a correct interpretation difficult; further investigations on the functional groups involved in the binding with substrates and their analogues are needed.

Considering the binding of aromatic D-amino acids at the active site of Dopa decarboxylase, the following observations should be made: (1) Due to the inhibitory effect exerted by aromatic D-amino acids on the decarboxylase activity, real kinetic parameters can only be obtained in the presence of substrates in L forms; results of studies using some substrates in L forms and others in DL forms (Lancaster & Sourkes, 1972; Srinivasan & Awapara, 1978) are not to be compared. Therefore, this is the first report of the kinetic parameters of Dopa decarboxylase with its substrates in L forms, as listed in Table I; their values can be compared to one another as all are obtained with L forms of the substrate. It can be seen that unnatural substrates, i.e., *o*- and *m*-tyrosine, have a decarboxylation rate higher than that of the natural ones.

(2) Due to the ability of aromatic D-amino acids to modify the coenzyme absorption bands, the use of the substrate in L forms is needed to obtain the real spectral features of enzyme–substrate intermediate complexes. Fiori et al. (1975) have previously carried out a study, by conventional spectrophotometry, on the interaction of Dopa decarboxylase in the presence of 5HTP in L form and of *m*-tyrosine in racemic form. An enzyme–L-5HTP intermediate complex absorbing at 420 nm, essentially invariant in the pH range 6.8–8.4, has been identified. As for DL-*m*-tyrosine, in the above-mentioned pH range, two intermediate complexes absorbing at 420 nm and at 390 nm have been observed, which are displayed by the low and high pH forms of the DL-*m*-tyrosine–enzyme complex, respectively. It has been proposed that the 420- and the 390-nm absorption bands are due to 4′-N-protonated and 1-N-protonated–4′-N-unprotonated Schiff bases, respectively; therefore, the observed transition 420 nm → 390 nm should be caused by a deprotonation of the 4′-nitrogen of the substrate–enzyme complex.

A similar assignment has been attributed to this transition observed by stopped-flow spectrophotometry during the stages in the decarboxylation of L-Dopa (Minelli et al., 1979). In the present study, where substrates in L forms (Dopa and 5HTP) and a wider pH range have been used, two forms absorbing at 420 and 390 nm have been observed for both the substrates which are displayed by the low and high pH forms of the substrate–enzyme complex, respectively. It is possible that the structure which absorbs at 390 nm is the conjugate base arising from acidic dissociation of the structure which

Scheme II



absorbs at 420 nm, as already suggested (Fiori et al., 1975; Minelli et al., 1979).

However, examination of the spectral changes and titration curves shown in Figures 4 and 5 indicates that at pH values much lower than the apparent pK for the transition some 390-nm absorbing species is still present and that at pH values much higher than pK some 420 nm material remains. These observations suggest that the ionization is not directly responsible for the spectral transition but rather that it influences the equilibrium between two tautomers that absorb respectively at 420 nm and at 390 nm. In Scheme II, E'XH$^+$ and E'X represent protonated and unprotonated tautomers both absorbing maximally at 390 nm, and EXH$^+$ and EX represent the other tautomer (protonated and unprotonated) absorbing maximally at 420 nm. For such a scheme the sum of the 390-nm absorbing complexes is given by

$$[E'X] + [E'XH^+] = \frac{[EX_0]\left(1 + \frac{[H^+]}{K}\right)}{1 + \frac{[H^+]}{K} + K_0 + \frac{K_H[H^+]}{K}}$$

[EX_0) is the total complex concentration, K the dissociation constant for EXH$^+$, K_0 = [EX]/[E'X], and K_H = [EXH$^+$]/[E'XH$^+$]. Thus when [H$^+$] $\ll K$, the fraction present at 390-nm absorbing species is $1/(1 + K_0)$, and when [H$^+$] $\gg K$, this fraction becomes $1/(1 + K_H)$. We estimate very approximately that at acidic pH about a quarter is present as 390-nm absorbing species and that this becomes approximately two-thirds at high pH. Values suggested for K_0 and K_H are therefore 0.5 and 3, respectively. Yet, the values found for the apparent pK of the group responsible for the spectral change are different: 7.3 for L-Dopa and 8.8 for L-5HTP. These large variations on the properties of the intermediate substrate–enzyme complexes might be ascribed to the nature of the substrate. Different groups on the enzyme can be thought to be involved in the binding for each substrate: this view might strengthen, even if it does not prove, the suggested existence of two binding subsites for catechol- and indole-related structures.

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Registry No. Dopa decarboxylase, 9042-64-2; L-Dopa, 59-92-7; L-5HTP, 4350-09-8; L-*m*-tyrosine, 587-33-7; L-*o*-tyrosine, 7423-92-9; D-Dopa, 5796-17-8; D-*m*-tyrosine, 32140-49-1; D-*o*-tyrosine, 24008-77-3; D-5HTP, 4350-07-6; D-tryptophan, 153-94-6.

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