Reaction of Dopa Decarboxylase with α-Methyldopa Leads to an Oxidative Deamination Producing 3,4-Dihydroxyphenylacetone, an Active Site Directed Affinity Label†

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ABSTRACT: Dopa decarboxylase (DDC) catalyzes the cleavage of α-methylDopa into 3,4-dihydroxyphenylacetone and ammonia, via the intermediate α-methyldopamine, which does not accumulate during catalysis. The ketone has been identified by high-performance liquid chromatography and mass spectroscopic analysis, and ammonia by means of glutamate dehydrogenase. Molecular oxygen is consumed during the reaction in a 1:2 molar ratio with respect to the products. The k_{cat} and K_m of this reaction were determined to be 5.68 min^{-1} and 45 μM, respectively. When the reaction is carried out under anaerobic conditions, α-methyldopamine is formed in a time-dependent manner and neither ammonia nor ketone is produced to a significant extent. The reaction is accompanied by a time- and concentration-dependent inactivation of the enzyme with k_{inact} of 0.012 min^{-1} and K_i of 39.3 μM. Free 3,4-dihydroxyphenylacetone binds to the active site of DDC and inactivates the enzyme in a time- and concentration-dependent manner with a k_{inact}/K_i value similar to that of α-methylDopa. D-Dopa, a competitive inhibitor of DDC, protects the enzyme against inactivation. Taken together, these findings indicate the active site directed nature of the interaction of DDC with 3,4-dihydroxyphenylacetone and provide evidence that the ketone generated by the reaction of DDC with α-methylDopa dissociates from the active site before it inactivates the enzyme. Inactivation of the enzyme by ketone followed by NaB^3H_4 reduction and chymotryptic digestion revealed that the lysine residue which binds pyridoxal 5′-phosphate (PLP) in the native enzyme is the site of covalent modification. Together with the characterization of the adduct released from the inactivated DDC, these data suggest that the enzyme is inactivated by trapping the coenzyme in a ternary adduct with ketone and the active site lysine. As recently reported for serotonin (5-HT) [Bertoldi, M., Moore, P. S., Maras, B., Dominici, P., and Borri Voltattorni, C. (1996) *J. Biol. Chem. 271*, 23954−23959], the conversion of dopamine (DA) into 3,4-dihydroxyphenylacetaldehyde and ammonia catalyzed by DDC is accompanied by irreversible loss of decarboxylase activity. However, the comparison between the absorbance, fluorescence, and CD features of DDC after 5-HT- or 3,4-dihydroxyphenylacetone-induced inactivation shows that a different covalent adduct is formed between either of these two molecules and DDC-bound PLP.

α-MethylDopa[1] [(2-methyl-3-(3,4-dihydroxyphenyl)-L-alanine] is considered a centrally acting antihypertensive agent, and it is currently believed that its pharmacological effects are due to the formation of α-methylnorepinephrine (derived from the product of decarboxylation, α-methyldopamine) which acts as an agonist for midbrain α_2-adrenergic receptors (*1*). However, despite this demonstrated antihypertensive action, the chemistry of its reaction with Dopa decarboxylase (DDC; EC 4.1.1.28) is not yet clearly defined.

The interaction of α-methylDopa with the pyridoxal 5′-phosphate (PLP) dependent enzyme DDC has been the subject of study for several decades. This α-methyl amino acid was shown to be slowly decarboxylated by DDC (*2*) and to inhibit the decarboxylation of L-Dopa (*3*). Characteristics of the inhibition of DDC by α-methylDopa are complex and depend on the experimental conditions (*4*, *5*). That a decarboxylation-dependent transamination may be responsible for the inactivation of DDC by α-methylDopa was first suggested by Borri Voltattorni et al. (*6*) and later studied in more depth by O'Leary and Baughn (*7*). However, the stoichiometry of this reaction appears to be abnormal and is not consistent with the proposed mechanism (*8*).

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; DDC, dopa decarboxylase; PMP, pyridoxamine 5′-phosphate; Dopa, 3,4-dihydroxyphenylalanine; α-methylDopa, 2-methyl-3-(3,4-dihydroxyphenyl)-L-alanine; DA, dopamine; 5-HT, 5-hydroxytryptamine; 5-HIA, 5-hydroxyindolacetaldehyde.

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In this study the reaction of DDC with α-methylDopa has been extensively characterized by kinetic and spectroscopic methods, and the reaction products have been definitively identified. It is demonstrated that α-methylDopa is cleaved to form 3,4-dihydroxyphenylacetone and ammonia, and evidence is provided that 3,4-dihydroxyphenylacetone generated at the active site is responsible for enzyme inactivation, behaving as an affinity labeling agent. The isolation and sequencing of a peptide containing the ketone-modified residue and the characterization of a PLP adduct released from the inactivated enzyme are herein reported. On the basis of these data, a mechanism of inactivation is presented.

The product amines serotonin (5-HT) and dopamine (DA) will also inactivate DDC; a mechanism-based inactivation has recently been proposed for the former (*9*). In light of the above-described data with α-methylDopa, the inactivation induced by 5-HT and DA has been further characterized. Evidence is provided that the kinetics of inactivation of DDC by these inhibitors is consistent with the conversion of 5-HT and DA to 5-hydroxyindolacetaldehyde (5-HIA) and 3,4-dihydroxyphenylacetaldehyde, respectively. However, the comparison between the spectral properties of DDC after 5-HT- or 3,4-dihydroxyphenylacetone-induced inactivation indicates that a different covalent adduct is formed between either of these two molecules and DDC-bound PLP.

EXPERIMENTAL PROCEDURES

Materials. L-Dopa, L-α-methylDopa, 5-HT, DA, PLP, bovine liver L-glutamic dehydrogenase (EC 1.4.1.3), horse liver alcohol dehydrogenase (EC 1.1.1.1), glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (EC 1.1.1.49), Hepes, α-ketoglutarate, and octanesulfonic acid were Sigma products. Sodium [^{3}H]borohydride (222.3 mCi/mmol) was purchased from New England Nuclear. All other chemicals were of the highest purity available. 3,4-Dihydroxyphenylacetone was synthesized according to Slates et al. (*10*) and further purified on HPLC. Centricon microconcentration filters (M_r 30 000) were from Amicon. Thin-layer chromatographies were performed on silica gel sheets and developed in 1-butanol/acetic acid/water (6/2/2, v/v). The liquid chromatography solvents (HPLC grade) were from Carlo Erba (Italy); the sequence-grade chemicals were from Perkin-Elmer (U.S.A.). Data for the determination of K_m, k_{cat}, k_{inact}, and K_i were fit to lines with the program Enzfitter (Elsevier).

Purification of DDC. Recombinant DDC was purified to homogeneity as previously described (*11*) with slight modification (*9*). The enzyme concentration was determined by using an E_M of 1.3×10^5 M^{-1} cm^{-1} (*12*).

Enzyme Assays. DDC activity was measured as described by Sherald et al. (*13*), as modified by Charteris and John (*14*). Production of ammonia by the reaction of DDC with α-methylDopa was determined by a spectroscopic assay using glutamate dehydrogenase, which forms glutamate from α-ketoglutarate and ammonia with the concomitant conversion of NADH to NAD^+. A 0.234-mL reaction mixture contained 10 μM DDC, 1400 μg of glutamate dehydrogenase, 1 mM α-ketoglutarate, and 300 μM NADH in 50 mM Hepes, pH 7.5 at 25 °C. The reaction was initiated with 26 μL of 1 mM α-methylDopa and monitored at 340 nm. The α-methylDopa concentrations were varied from 10 μM to 2 mM when kinetic parameters were determined. Production of ammonia by the reaction of DDC with DA was determined by the same assay under the same experimental conditions, at DA concentrations varying from 0.5 to 4 mM. Production of ammonia during the reaction of DDC with α-methylDopa, 5-HT, or DA was also measured under anaerobic conditions using 1-mL Reacti-Vials (Aldrich). Two vials, one containing the substrate and the other containing 50 mM Hepes, pH 7.5, were incubated at 25 °C and flushed with nitrogen for about 1 h. DDC was then added to the buffer solution, and the vials were flushed for another 15 min. Substrate was then withdrawn from the other vial and added to the enzyme solution in a final volume of 250 μL. A positive pressure of nitrogen was maintained over the solution throughout the reaction. At various times each reaction mixture was immediately boiled and centrifuged, and the supernatant was analyzed for ammonia content using the assay system described above. Each withdrawal was performed by using a microliter gastight syringe (Hamilton) inserted through the Teflon silicone septum.

3,4-Dihydroxyphenylacetaldehyde formation during the reaction of DDC with DA was assayed by coupling the aldehyde produced to the NADH-dependent alcohol dehydrogenase-catalyzed reaction. Routinely, a 0.27-mL reaction mixture contained 10 μM DDC, 96 μg of alcohol dehydrogenase, and 300 μM NADH in 50 mM Hepes, pH 7.5. The reaction was initiated by the addition of 30 μL of 40 mM DA, and the decrease in absorbance at 340 nm due to the conversion of NADH to NAD^+ was monitored.

O_2 consumption during the reaction of DDC with α-methylDopa, 5-HT, or DA was recorded using a Yellow Springs electrode (Yellow Spring Instruments). The reaction was carried out at 25 °C in air-saturated Hepes, pH 7.5.

H_2O_2 content was measured as previously described (*9*).

Inactivation Assays. The inactivation incubation mixtures contained enzyme (10 μM) and freshly diluted inactivator (α-methylDopa, 3,4-dihydroxyphenylacetone, 5-HT, or DA) at varying concentrations at 25 °C in 50 mM Hepes, pH 7.5. At various time intervals aliquots were taken and tested for residual decarboxylase activity as described. In protection experiments, the indicated substrate analogue was included prior to the addition of inactivator. Where indicated, the inactivation was performed in the presence of alcohol dehydrogenase and the incubation mixture contained, in addition to 10 μM DDC and 1 mM 5-HT, 96 μg of alcohol dehydrogenase, 300 μM NADH, 5 units of glucose-6-phosphate dehydrogenase, and 3.3 mM glucose 6-phosphate in 50 mM Hepes, pH 7.5, in a total volume of 100 μL. Under these experimental conditions, 1 unit of glucose-6-phosphate dehydrogenase oxidizes 0.6 μmol of glucose 6-phosphate to 6-phospho-D-gluconate per minute.

Identification and Quantification of the Products of the Reaction of DDC with α-MethylDopa by HPLC. Separation of the products of the reaction of DDC with α-methylDopa was done isocratically on a 5-μm LiChrospher 100 RP-18 column (4.6 × 100 mm, Merck) using an LKB instrument coupled with an SP44 00 Chromoject integrator. The solvent was H_2O/methanol/acetic acid (75/24/1, v/v/v) containing 6 mM octanesulfonic acid at a flow rate of 1 mL min^{-1}, while detection was at 280 nm. DDC (10 μM) was incubated with 100 μM α-methylDopa in 50 mM Hepes, pH 7.5, at 25 °C. Aliquots were removed at time intervals, and trichloroacetic

acid was added to a final concentration of 5%. Samples were then centrifuged and loaded directly along with appropriate blanks. For quantification, the areas were measured and converted to absolute amounts by using standard reference curves.

Detection of Pyridoxamine 5′-Phosphate (PMP) with Apoaspartate Aminotransferase. Determination of PMP after reaction of DDC with α-methylDopa was performed according to the method of Bossa and Barra (*15*). Samples were prepared by reduction with $NaBH_4$ followed by heat denaturation.

NaB^3H_4 Reduction of DDC. To a solution containing 1.5 mg of native or ketone-inactivated DDC was added approximately 0.17 mg of sodium [^{3}H]borohydride (223 mCi/mmol), and the mixture was incubated at 25 °C for 30 min. This was followed by the addition of cold sodium borohydride to a final concentration of 1.4 mM, and incubation was continued for an additional half-hour. The solution was extensively dialyzed against 5 mM Hepes, pH 7.5, and lyophilized.

Reductive Alkylation, Chymotryptic Digestion, HPLC Peptide Purification, and Sequencing. The lyophilized material was dissolved in 6 M guanidine hydrochloride solution containing 0.1 M Tris-HCl, 1 mM Na EDTA, and 2 mM DTT at pH 8 and incubated under nitrogen for 3 h at 25 °C. A fresh solution of iodoacetic acid, neutralized with NaOH, was added to give a final concentration of 10 mM, and carboxymethylation was allowed to proceed in the dark for 30 min at room temperature. After extensive dialysis against water, the solution was lyophilized. The carboxymethylated preparation (1 mg) was finally suspended in 0.3 mL of 0.1 M NH_4HCO_3 solution, 0.03 mg of chymotrypsin was added, and the solution was incubated at 37 °C. After 3 h the peptides were purified using a Beckman System Gold chromatograph on a macroporous reverse-phase column (Supelcosil LC18.DB, 4.6 × 150 mm, 3 mm, Supelco) and eluted with a 20-min gradient from 1 to 17.5% acetonitrile in 0.2% (by volume) trifluoroacetic acid, followed by a 70-min gradient from 17.5% to 49% of the same solvent at a flow rate of 0.6 mL/min. Absorbance at 214 nm was monitored to detect peptides. The fractions were lyophilized and resuspended, and 1/8 aliquot was counted in a liquid scintillator (model LS6800, Beckman.). The radioactive fraction was further purified using the same column but a different gradient. The amino acid sequence was determined by automated Edman degradation using a Perkin-Elmer ABI 476 sequencer.

Mass Spectrometry. Mass spectrometric analysis of 3,4-dihydroxyphenylacetone obtained by synthesis or by reaction of DDC with α-methylDopa was carried out using an HP 59980A instrument (Hewlett-Packard Co., Palo Alto, CA). Samples were introduced into the ion source as solutions in methanol through the particle beam interface (HP) using an HP 1050 HPLC pump. The mass spectrum of 3,4-dihydroxyphenylacetone obtained by chemical synthesis, recorded under scan conditions in the 50−500 mass range, showed prominent ions at *m*/*z* 123 and 166. The ratio of the two ions (*m*/*z* 166/123) determined by integration of the peak area in the ion chromatogram was 0.16. Both synthetic and presumed ketones were analyzed by the selected ion monitoring technique (SIM) focusing ions at *m*/*z* 123 and 166.

MALDI mass spectra of the adduct released by 3,4-dihydroxyphenylacetone-inactivated DDC were recorded on a Perseptive Biosystem VOYAGER DE MALDI-TOF mass spectrometer using sinapinic acid (10 mg/mL) in a solution of 50% acetonitrile in 0.05% trifluoroacetic acid as matrix. The sample was dissolved in water (20 μL), and 1 μL was loaded onto the sample plate, allowed to air-dry, and treated with 1 μL of matrix followed by air drying.

Spectrophotometric Measurements. Absorption spectra were carried out using a Jasco V-550 spectrophotometer at 25 °C. CD spectra were obtained with a Jasco 710 spectropolarimeter with a thermostatically controlled cell at 25 °C at a protein concentration of 10 μM. Spectra were recorded at a scan speed of 50 nm/min with a bandwidth of 2 nm and averaged automatically, except where indicated. Fluorescence spectra were taken with a Kontron SFM 25 spectrofluorometer at a protein concentration of 0.6 μM.

RESULTS

Identification of the Products of the Reaction of DDC with α-MethylDopa. When assayed for decarboxylase activity, the reaction of 10 μM DDC with 100 μM α-methylDopa at 25 °C in 50 mM Hepes, pH 7.5, does not produce detectable amounts of α-methyldopamine. Aliquots of an identical reaction mixture were withdrawn at time intervals and deproteinized, and their supernatants were subjected to fractionation on a reversed-phase column in HPLC. Only two peaks absorbing at 280 nm were found, which have been identified as 3,4-dihydroxyphenylacetone and α-methylDopa in that they co-elute with the corresponding standards (data not shown). Moreover, mass spectroscopic analysis by SIM of the former peak and of synthetic 3,4-dihydroxyphenylacetone showed ion ratio intensities (*m*/*z* 166/123) of 0.15 and 0.17, respectively.

Reaction of the same mixture of DDC with α-methylDopa causes the production of ammonia, as revealed by a coupled assay system utilizing glutamate dehydrogenase. In Figure 1 the production of ammonia and 3,4-dihydroxyphenylacetone along with the consumption of α-methylDopa is shown. It can be observed that there is a concurrent production of equivalent amounts of 3,4-dihydroxyphenylacetone and ammonia which parallels the consumption of α-methylDopa. Furthermore, as shown in Figure 1, molecular oxygen is consumed during the reaction in a 1:2 molar ratio with respect to the produced ammonia or 3,4-dihydroxyphenylacetone. The steady-state kinetics of the formation of ammonia (using the auxiliary enzyme glutamate dehydrogenase) has been measured, with resulting values for k_{cat} of 5.68 ± 0.5 min^{-1} and for K_m of 45 ± 5.6 μM (Table 1). This latter value is higher than the K_i value of to 1.2 μM previously determined for α-methylDopa (*5*).

Under anaerobic conditions, the reaction of 10 μM DDC with 100 μM α-methylDopa produces ammonia (and 3,4-dihydroxyphenylacetone) in amounts less than 5% with respect to those found under aerobic conditions and, when assayed for decarboxylase activity, shows a time-dependent production of α-methyldopamine (data not shown).

During the course of the reaction of DDC with α-methylDopa, no detectable PMP or H_2O_2 was found.

Inactivation of DDC by α-MethylDopa and 3,4-Dihydroxyphenylacetone. Incubation of DDC with α-methylDopa at

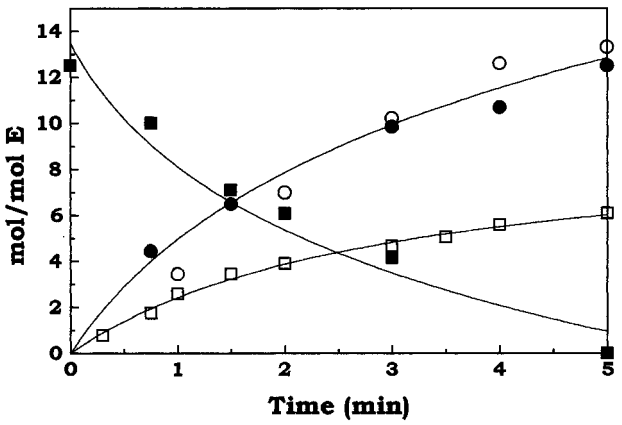


FIGURE 1: Consumption of α-methylDopa and O_2 and formation of ammonia and 3,4-dihydroxyphenylacetone during the reaction of DDC with α-methylDopa. DDC (10 μM) was incubated in 50 mM Hepes, pH 7.5, with 100 μM α-methylDopa. For α-methylDopa and 3,4-dihydroxyphenylacetone determination, samples of 20 μL were withdrawn at the indicated times and denatured with trichloroacetic acid. After removal of the precipitated protein by centrifugation, the supernatants were subjected to HPLC and the areas of the peaks were converted to absolute amounts (as described in the text). (●) 3,4-Dihydroxyphenylacetone; (■) α-methylDopa; (○) ammonia, measured by a continuous assay (Experimental Procedures); and (□) O_2 consumed. Data shown are means of three independent experiments; SE mean (not shown) in each case was less than 10% of the mean value.

25 °C in 50 mM Hepes, pH 7.5, resulted in both time- and concentration-dependent inactivation, as shown in Figure 2. The rate of inactivation saturates at high concentrations of α-methylDopa, and the apparent K_i of 39.3 ± 0.4 μM is consistent with the formation of a Michaelis complex between the enzyme and α-methylDopa. At saturating levels of α-methylDopa, k_{inact} was found to be 0.012 ± 0.001 min^{-1} (inset of Figure 2). However, it should be noted that the inactivation reaction is very slow relative to the rate of catalysis producing ammonia and 3,4-dihydroxyphenylacetone; while complete conversion of 100 μM α-methylDopa by DDC (10 μM) occurs in 6 min, complete inactivation requires more than 3 h. Therefore, 3,4-dihydroxyphenylacetone was tested as an inhibitor of DDC. When DDC was incubated with 3,4-dihydroxyphenylacetone, decarboxylase activity decreased as a function of time and ketone concentration (Figure 3). Inactivation followed pseudo-first-order kinetic behavior at each fixed concentration of ketone. A reciprocal plot of the pseudo-first-order constants at each inhibitor concentration, taken from the slopes of the lines in Figure 3, gave a straight line (inset of Figure 3) which demonstrates saturation kinetics with a K_i of 30.2 ± 3.8 μM and indicates the presence of a binding step prior to inactivation with a k_{inact} of 0.011 ± 0.001 min^{-1}. The k_{inact}/K_i value, which represents the efficiency of inactivation of DDC by 3,4-dihydroxyphenylacetone, is very similar to that calculated for α-methylDopa (Table 1). D-Dopa protects against inactivation by 3,4-dihydroxyphenylacetone. When the enzyme is incubated with 2 mM D-Dopa and 100 μM 3,4-dihydroxyphenylacetone, the rate of inactivation is 13% of that seen for incubation with ketone alone (Figure 3). This result is consistent with 3,4-dihydroxyphenylacetone competing with D-Dopa for binding to the active site.

After a 3-h incubation of DDC (10 μM) with 100 μM α-methylDopa or 100 μM 3,4-dihydroxyphenylacetone, the enzyme has lost about 90% activity. The enzyme is apparently irreversibly inactivated by ketone. There is no observable return of decarboxylase activity when 3,4-dihydroxyphenylacetone is removed by either gel filtration or prolonged dialysis. Incubation of almost completely inactivated enzyme with PLP after removal of 3,4-dihydroxyphenylacetone does not result in a return of enzyme activity. There was no adventitious cleavage, cross-linking, or aggregation of the ketone-inactivated DDC, since both the native and inactive enzymes behaved identically by gel electrophoresis either in the native or in the denatured state (data not shown).

Changes in Absorption and CD Spectra. Previous studies indicate that two spectral changes accompany the reaction of DDC with α-methylDopa: a rapid decrease of the absorption band at 420 nm and a corresponding increase at 320 nm (*6*). However, the spectral changes accompanying this reaction are more complex. As shown in Figure 4A, the addition of 100 μM α-methylDopa to DDC in 50 mM Hepes, pH 7.5, causes an immediate decrease of the absorption band at 420 nm and the appearance of a species absorbing maximally at 350 nm. This peak rapidly disappears, and a new absorbance at 335 nm can be observed. This absorption then slowly shifts with time to 340 nm. When spectra are recorded in the presence of 2 mM α-methylDopa, the 350-nm band disappears with a $t_{1/2}$ of 4.2 min (data not shown). The results are consistent with those previously obtained by stopped-flow spectrophotometry studying early events in the interaction of α-methylDopa with DDC purified from pig kidney in 100 mM Hepes at pH 7, in which three processes are observed (P. Dominici, A. Peracchi, P. S. Moore, and C. Borri Voltattorni, unpublished results). The first phase of the reaction is characterized by an increase at 420 nm followed by a decrease in the 420-nm band concomitant with an increase at 350 nm. The third and final phase consists of a decrease of the 350-nm species.

As shown in Figure 4B, the addition of 100 μM 3,4-dihydroxyphenylacetone to the enzyme (10 μM) resulted in a immediate decrease of the 420- and 335-nm absorbance bands. The peak at 335 nm slowly increases and shifts to 340 nm (1−10 min). Thereafter, there is a slow decrease in the absorbance at 340 nm that is complete within 1 h.

The CD spectrum of holoDDC displays positive dichroic signals at 420 and 335 nm, thus providing a convenient method to examine the possibility of the intermediate being bound to the active site of the enzyme. Upon the addition of α-methylDopa (2 mM) to the enzyme (10 μM), the CD spectrum was altered, forming a new spectrum with a positive dichroic maximum at 350 nm (Figure 5A) corresponding to the absorption peak seen in Figure 4A. The CD spectrum recorded after 15 min exhibits a positive dichroic band at 340 nm (Figure 5A). Upon addition of 3,4-dihydroxyphenylacetone (100 μM) to DDC (10 μM), the 420-nm CD band decreased and concomitantly a positive CD band at 340 nm was observed which remains unaltered with time (Figure 5B). 3,4-Dihydroxyphenylacetone does not modify the visible and the near-UV CD spectra of the apoenzyme, and addition of PLP to this protein resulted in the appearance of the 420- and 335-nm signals, typical of the holoenzyme (data not shown).

Reaction of DDC with DA and 5-HT. As recently reported for 5-HT (*9*), DDC catalyzes the conversion of DA into 3,4-

Table 1: Kinetic Parameters of Reaction and Inactivation of DDC in the Presence of α-methylDopa, 3,4-Dihydroxyphenylacetone, DA, and 5-HT[a]

	k_{cat} (min^{-1})	K_m ($\times 10^{-6}$ M)	k_{cat}/K_m (M^{-1} min^{-1})	k_{inact} (min^{-1})	K_i ($\times 10^{-6}$ M)	k_{inact}/K_i (M^{-1} min^{-1})
α-methylDopa	5.68	45	126200	0.0120	39.3	305.0
3,4 dihydroxyphenylacetone				0.0110	30.2	364.0
DA	3.03	2480	1221	0.0059	2080.0	2.8
5-HT[b]	0.48	470	1021	0.0230	400.0	57.5

[a] All experiments were carried out in 50 mM Hepes, pH 7.5, at 25 °C. [b] Bertoldi et al. (1996).

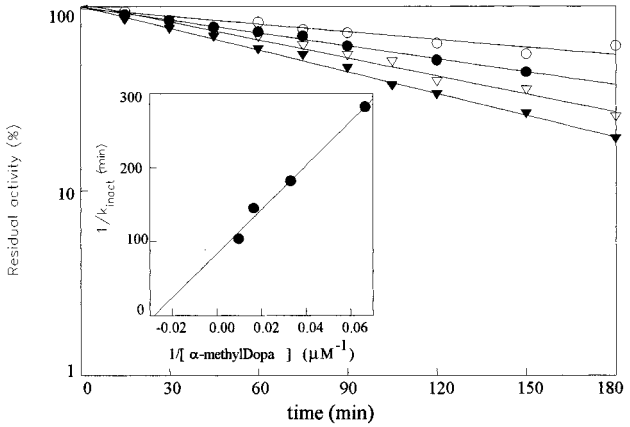


FIGURE 2: Concentration-dependent inactivation of DDC by α-methylDopa. At the indicated times, aliquots of reaction mixtures containing various amounts of α-methylDopa were assayed for residual decarboxylase activity as described under Experimental Procedures: (○) 15 μM α-methylDopa; (●) 30 μM α-methylDopa; (▽) 60 μM α-methylDopa; (▼) 100 μM α-methylDopa. Inset: Double-reciprocal plot of the apparent rate of inactivation as a function of α-methylDopa concentration.

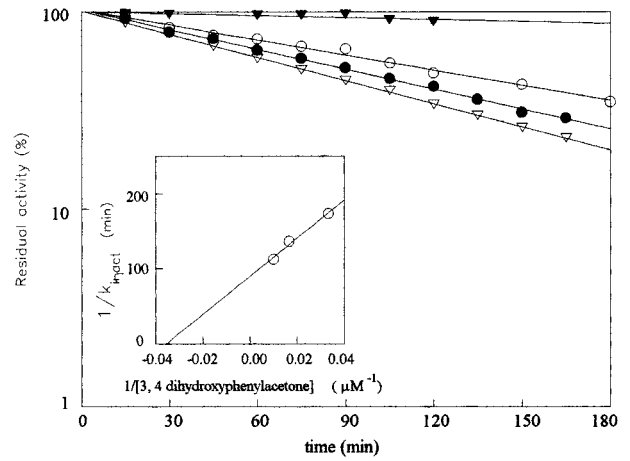


FIGURE 3: Concentration-dependent inactivation of DDC by 3,4-dihydroxyphenylacetone. At the indicated times, aliquots of reaction mixtures containing various amounts of 3,4-dihydroxyphenylacetone were assayed for residual decarboxylase activity as described under Experimental Procedures: (○) 30 μM 3,4-dihydroxyphenylacetone; (●) 60 μM 3,4-dihydroxyohenylacetone; (▽) 100 μM 3,4-dihydroxyphenylacetone; (▼) reactions to which 2 mM D-Dopa was added prior to the addition of 100 μM 3,4-dihydroxyphenylacetone. Inset: Double-reciprocal plot of the apparent rate of inactivation as a function of 3,4-dihydroxyphenylacetone concentration.

dihydroxyphenylacetaldehyde and ammonia, which have been identified and quantified by means of the auxiliary enzymes alcohol dehydrogenase and glutamate dehydrogenase, respectively. The k_{cat} and K_m of this reaction were determined to be 3.03 ± 0.08 min^{-1} and 2.48 ± 0.14 mM, respectively (Table 1). Concurrent with this reaction, DA

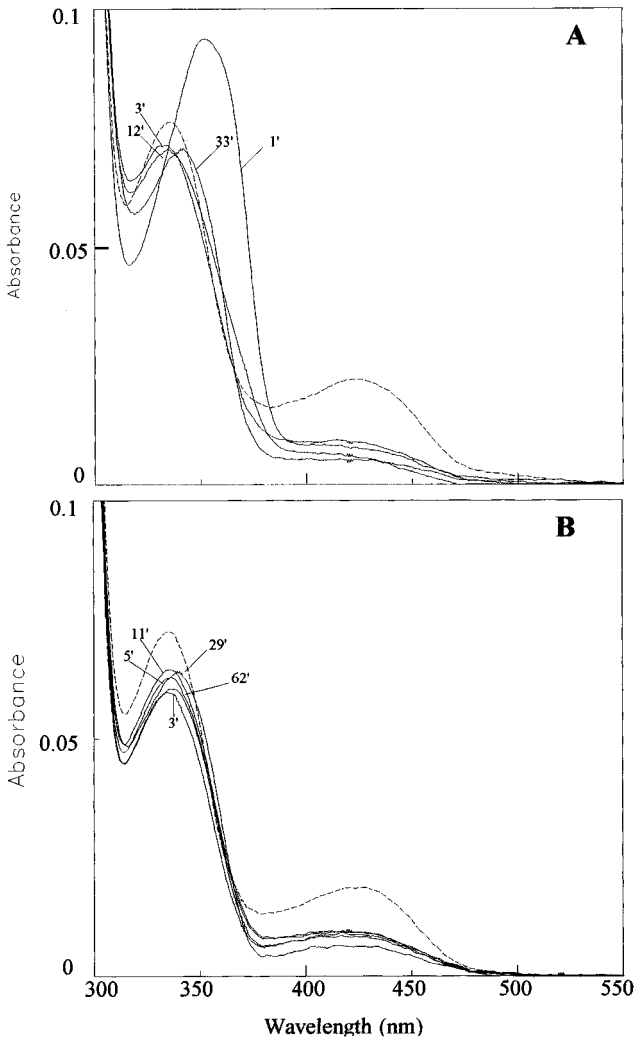


FIGURE 4: Time-dependent spectral changes occurring on addition of α-methylDopa or 3,4-dihydroxyphenylacetone to DDC. (A) The enzyme (10 μM) (---) in 50 mM Hepes, pH 7.5, was treated with α-methylDopa (100 μM), and spectra were recorded at 1, 3, 12, and 33 min (as indicated). (B) The enzyme (10 μM) (---) in 50 mM Hepes, pH 7.5, was treated with 3,4-dihydroxyphenylacetone (100 μM), and spectra were recorded at 3, 5, 11, 29, and 62 min (as indicated).

inactivates DDC in both a time- and a concentration-dependent fashion and exhibits saturation of the inactivation at high concentration. The K_i and k_{inact} were determined to be 2.08 ± 0.03 mM and 0.0059 ± 0.0003 min^{-1}, respectively. The k_{inact}/K_i value of 2.8 M^{-1} min^{-1} of DA is lower than that calculated for 5-HT (*9*) (Table 1).

As for α-methylDopa, the reaction of 5-HT and DA with DDC does not occur to a significant extent under anaerobic conditions and is accompanied by molecular oxygen consumption in a 1:2 molar ratio with respect to the products when carried out under aerobic conditions.

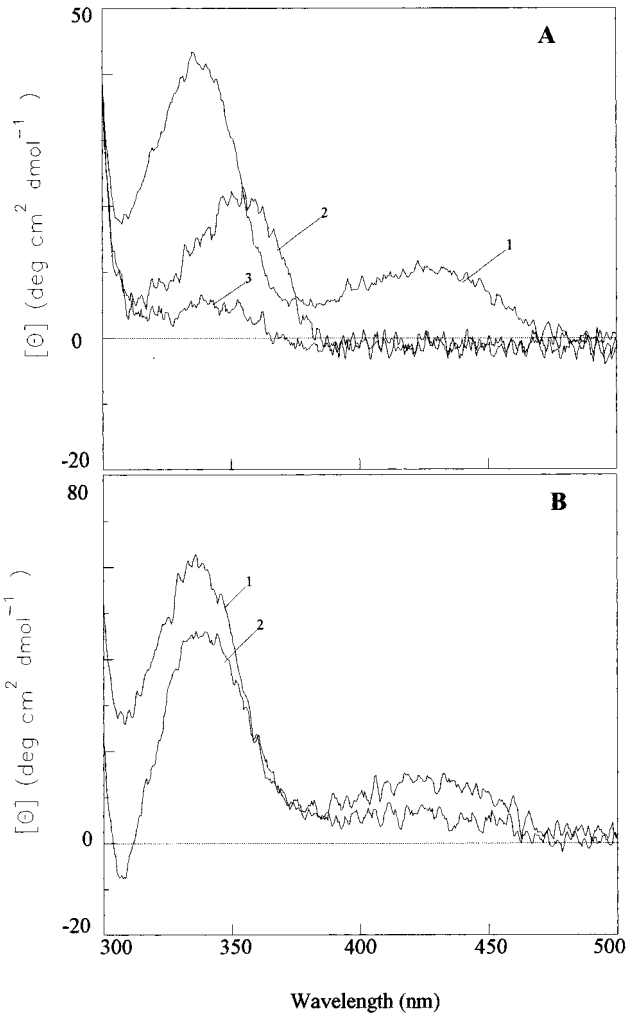


FIGURE 5: Changes in the visible circular dichroic spectrum of DDC upon its interaction with α-methylDopa or 3,4-dihydroxyphenylacetone. (A) The enzyme (10 μM) in 50 mM Hepes, pH 7.5 (1), and α-methylDopa (2 mM) were mixed; one spectrum was recorded immediately (2), and another was recorded 15 min after the addition of α-methylDopa to the enzyme. (B) The enzyme (10 μM) in 50 mM Hepes, pH 7.5 (1), and 3,4-dihydroxyphenylacetone (100 μM) were mixed, and one spectrum was recorded immediately (2).

In light of the data of the inactivation of DDC by α-methylDopa, we decided to examine the possibility that inactivation of DDC by 5-HT or DA might be attributed to the binding of the products of the reaction, 5-HIA and 3,4-dihydroxyphenylacetaldehyde, respectively, to the enzyme. Unfortunately, these compounds could not be tested as inhibitors of DDC due to the fact that they are commercially unavailable and are also extremely unstable. It was previously reported that inactivation of DDC by 5-HT is not prevented if 5-HIA is continuously removed from the reaction mixture by addition of 300 μM NADH and alcohol dehydrogenase (*9*). However, a different result is obtained when the same experiment is performed at higher concentration of NADH or by recycling the NADH during the inactivation period by adding the enzymatic system of glucose-6-phosphate dehydrogenase. In fact, under these experimental conditions, the rate constant for inactivation of DDC by 1 mM 5-HT is 14.4×10^{-3} min^{-1} when 5-HIA is permitted to remain and 6.6×10^{-3} min^{-1} when 5-HIA is removed by enzymic reduction. However, NADH or coupling enzymes at infinite concentration were unable to fully protect DDC from inactivation. In this regard, it must be noted that tryptophol does not bind or inhibit DDC. Similarly, under the above experimental conditions, inactivation of DDC by DA was partially prevented when 3,4-dihydroxyphenylacetaldehyde was continuously removed from the reaction mixture (data not shown).

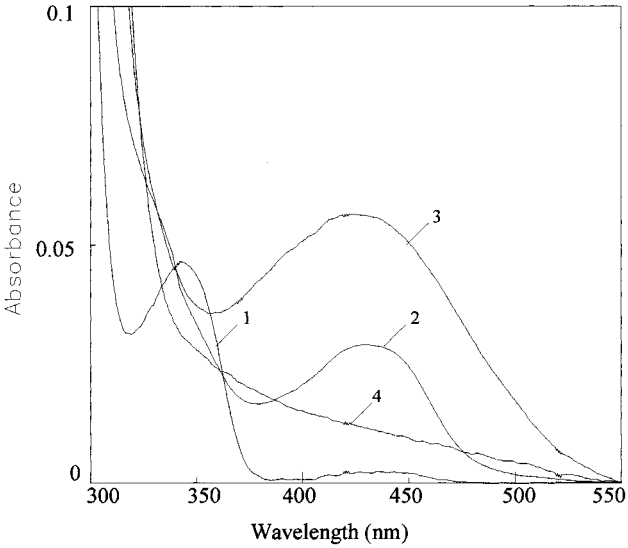


FIGURE 6: Absorption spectra of DDC after inactivation by 3,4-dihydroxyphenylacetone. Curve 1, enzyme inactivated for 3 h after removal of unreacted 3,4-dihydroxyphenylacetone by gel filtration. Curve 2, enzyme from curve 1 after storage at 37 °C in the dark for 10 h. Curve 3, enzyme from curve 1 after addition of sodium hydroxide to pH 13 and incubation for 30 min. Curve 4, enzyme from curve 1 reduced by $NaBH_4$ followed by addition of sodium hydroxide at pH 13 and incubation for 30 min.

Properties of the 3,4-Dihydroxyphenylacetone-Inactivated DDC and Comparison with Those of the 5-HT-Inactivated Enzyme. Following inactivation of DDC by 100 μM 3,4-dihydroxyphenylacetone as described in Figure 3 and removal of unreacted ketone by PD-10 column gel filtration, the enzyme exhibits an absorption band at 340 nm (Figure 6) associated with a positive dichroic signal centered at the same wavelength. When excited at 340 nm, the inactive enzyme has an emission fluorescence intensity identical with that of the native enzyme excited at the same wavelength. When excited at 278 nm, this inactivated enzyme displays an emission maximum at 326 nm, as for native DDC (data not shown). When the inactivated enzyme was adjusted to pH 13, an intense yellow color developed in 30 min with a maximum absorption at 424 nm, which is absent when the inactivated enzyme was first treated with $NaBH_4$ and then adjusted to alkaline pH (Figure 6). These spectroscopic features of the PLP adduct after alkaline treatment appear to be similar to those observed after alkaline treatment (pH 11−12) of aspartate aminotransferase or glutamate decarboxylase after inactivation by L-serine *O*-sulfate (*16*, *17*) and of some mutants of the $\alpha_2\beta_2$ complex of tryptophan synthase from *Salmonella typhimurium* after inactivation by β-chloro-L-alanine (*18*). Using an E_M of 8 mM^{-1} cm^{-1} reported for the 424-nm species (*16*, *17*), it was calculated that 80% inactivated DDC contains 1.4−1.5 mol of this adduct per mole of dimeric enzyme. Surprisingly, the 424-nm species was not separated from the proteic material by passage over a Centricon 30 tube in that the filtrate showed no consistent absorption. This behavior might be due to the dissociation

of the catecholic hydroxyl groups at high pH which could allow their interaction with proteic groups.

Various attempts were made to release the adduct from the protein. Treatment of the inactivated enzyme with 6 M guanidine hydrochloride at pH 7 or 2.5, with trichloroacetic acid, or by heating at 100 °C for 10 min does not release the adduct from the protein. It was subsequently found that if inactivated DDC is allowed to stand at 37 °C in the dark, the solution becomes yellow as a new absorbance peak at 424 nm is formed at the expense of the 340 nm band (Figure 6). These spectral changes are complete within about 30 h. The visible CD spectrum of this protein is characterized by positive dichroic bands centered at 425 and 315 nm with a shoulder at 335 nm and remains unaltered after addition of 50 μM PLP (data not shown). After treatment at 100 °C for 10 min and centrifugation of a ketone-inactivated proteic solution which was allowed to stand for 30 h at 37 °C, the resulting supernatant displays an absorption spectrum characterized by absorption bands centered at 405 and 280 nm (data not shown), and about 90% of the adduct appeared to be released from the protein. When ketone-inactivated DDC was reduced with $NaBH_4$ and then subjected to the same treatment, no changes in the absorbance spectrum were observed over 2 days and, after boiling and centrifugation, the supernatant does not show absorption bands in the 500−260-nm region (data not shown).

When the 5-HT-inactivated enzyme was subjected to alkaline treatment, the 340-nm band disappeared and only an absorbance below 300 nm was observed. Furthermore, when this inactivated enzyme was excited at 278 nm, this enzymatic species fluoresced not only at 326 nm but also at 390 nm (data not shown). This PLP emission is probably due to the occurrence of energy transfer from tryptophan residue(s) to the PLP adduct. It must be noted that the 5-HT-inactivated DDC has absorption and dichroic properties identical with those of the 3,4-dihydroxyphenylacetone-inactivated DDC, but upon excitation at 340 nm it exhibits an emission intensity at 385 nm, significantly higher than that of the native enzyme when excited at the same wavelength (*9*). Moreover, when 5-HT-inactivated DDC was allowed to stand at 37 °C for more than 30 h, no modification of the absorbance spectrum was observed; after heat treatment, the supernatant does not exhibit any absorption in the 260−500-nm region. Taken together, these data provide significant evidence that the covalent adducts formed after 5-HT or 3,4-dihydroxyphenylacetone-induced inactivation are different. Spectroscopic analysis of the enzyme completely inactivated by DA is hampered by the fact that the inactivation is very slow and occurs on a time-scale in which DA begins to undergo oxidation. The presence of unreacted PLP in the partially inactivated enzyme complicates further characterization.

Characterization of the Adduct Released by 3,4-Dihydroxyphenylacetone-Inactivated DDC. The heat-extracted supernatant from ketone-inactivated DDC (prepared as described above) migrates on TLC with a different R_f with respect to that of PLP or 3,4-dihydroxyphenylacetone, it appears as a single spot which gives positive Gibbs and 2,4-dinitrophenylhydrazine tests, and it displays fluorescence when illuminated by a long-wave UV lamp. Moreover, this released adduct was analyzed by MALDI-MS. The MALDI spectrum showed the occurrence of a mass signal at *m*/*z* 397 which was absent in the spectra of the matrix alone recorded for comparison. This mass value corresponds to the expected molecular mass of the 3,4-dihydroxyphenylacetone−PLP aldol condensation adduct.

Isolation and Sequencing of the Peptide Containing the Modified Residue. RP-HPLC purification of the peptide mixture obtained by chymotryptic digestion of labeled and reduced inactivated DDC gave a single radioactive peak. After a second chromatographic run, although a considerable amount of radioactivity was lost, a labeled purified peptide was obtained, whose sequence was determined to be NFN-PH(X)W (data not shown). This sequence corresponds to the region 298−304 in the primary structure of DDC (*19*) encompassing the active site lysine. In the sixth cycle of the Edman degradation no PTH derivative was detected, thus indicating that the Lys residue was modified. It must also be noted that the retention time of the radiolabeled peptide was different from that of the same peptide from native DDC in which the Schiff base had been reduced.

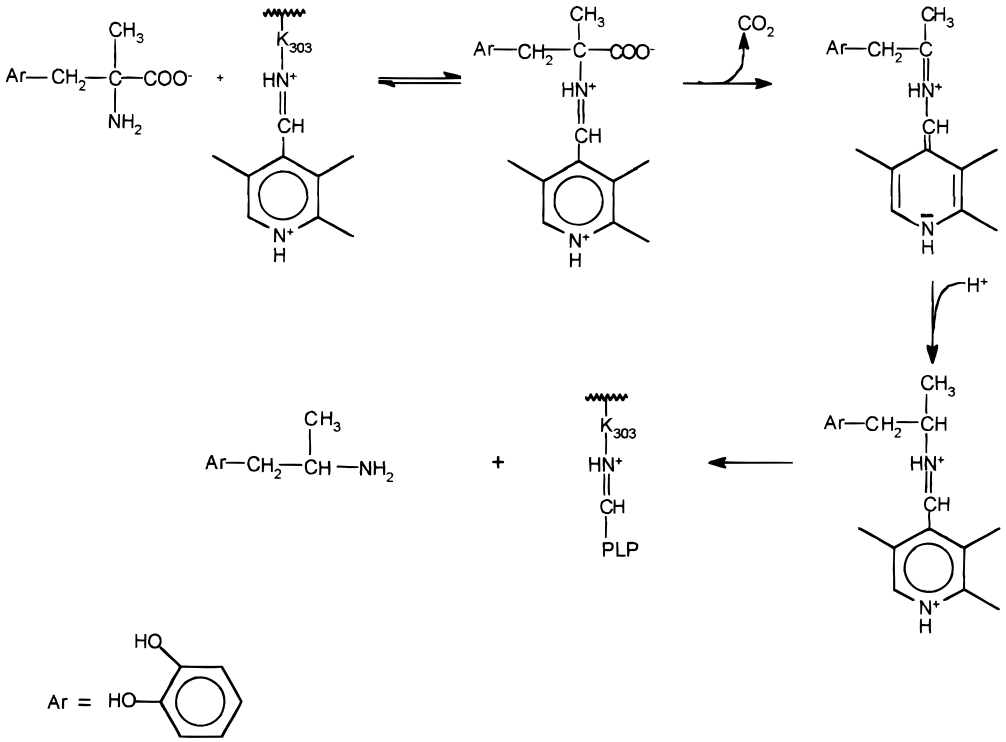
DISCUSSION

The present study seems to rule out that the reaction of DDC with α-methylDopa proceeds by decarboxylation-dependent transamination, as previously suggested (*6*, *7*). In fact, no production of PMP can be detected, and there is no significant reactivation of the α-methylDopa-inactivated enzyme upon the addition of PLP. Moreover, our data clearly indicate that the reaction of DDC with α-methylDopa produces equivalent amounts of 3,4-dihydroxyphenylacetone and ammonia, with no detectable formation of α-methyldopamine. These results strongly suggest that the reaction of α-methylDopa with DDC is a serial mechanism where α-methyldopamine, produced by decarboxylation (Scheme 1), is a transient intermediate which is subsequently converted to 3,4-dihydroxyphenylacetone and ammonia with a mechanism similar to that already described for the conversion of 5-HT to 5-HIA (*9*) and that for the conversion of DA to 3,4-dihydroxyphenylacetaldehyde. It should be noted that the catalytic efficiency of this reaction is much higher for α-methylDopa than that for 5-HT (Table 1). For each substrate, it has been demonstrated that (1) this reaction does not proceed to a significant extent under anaerobic conditions and (2) the electron acceptor is molecular oxygen, which is consumed in a 1:2 molar ratio with respect to the products. The accumulation of α-methyldopamine during the reaction of α-methylDopa with DDC under anaerobic conditions is in agreement with the former observation; indeed, these experimental conditions provide a convenient route for the synthesis of α-methyldopamine. Thus, aromatic amines act as substrates for this reaction, which is essentially an oxidative deamination reaction which, on the basis of its stoichiometry, can be written as follows:

$$\mathrm{Ar{-}CH_2CRHNH_2 + {}^1/_2O_2 \rightarrow Ar{-}CH_2CR{=}NH + H_2O}$$
$$\mathrm{Ar{-}CH_2CR{=}NH + H_2O \rightarrow Ar{-}CH_2CRO + NH_3}$$
$$\overline{\mathrm{Ar{-}CH_2CRHNH_2 + {}^1/_2O_2 \rightarrow Ar{-}CH_2CRO + NH_3}}$$

where Ar represents the aromatic moiety, and R = H or CH_3. Although the products of this reaction, aromatic aldehyde or ketone and ammonia, would also be produced by the action of monoamine oxidase, it must be pointed out that,

Scheme 1



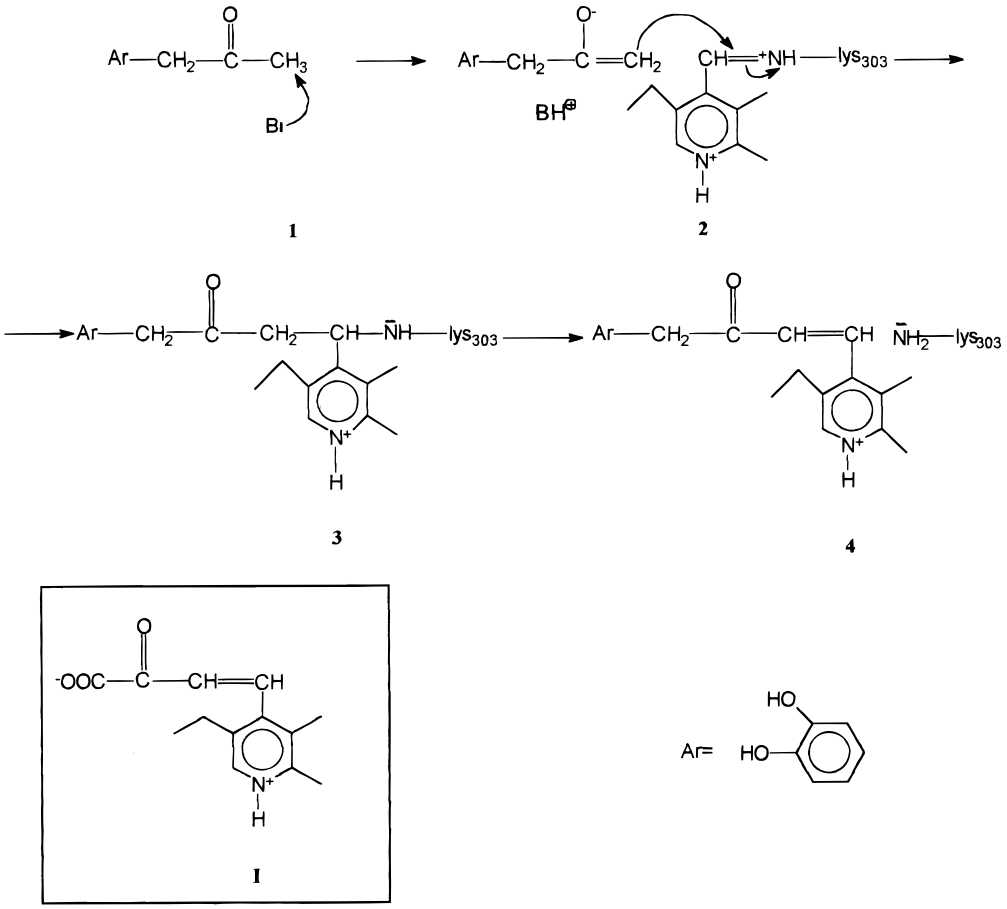
unlike monoamine oxidase, no production of H_2O_2 was observed during the reaction of DDC with 5-HT (*9*), DA, or α-methylDopa.

Since α-methyldopamine does not accumulate during the reaction under aerobic conditions, the rate constant for decarboxylation must be similar to or lower than that of the secondary reaction. The efficiency of this secondary reaction obviously depends on how long α-methyldopamine remains at the active site of DDC; the high rate of production of 3,4-dihydroxyphenylacetone and ammonia seems to exclude that α-methyldopamine formed as an intermediate immediately diffuses from the active site of the enzyme, at least under the in vitro experimental conditions tested. However, it has been reported that in human subjects the metabolites of orally administered ^{14}C-labeled α-methylDopa in urine are free and conjugated α-methylDopa; α-methyldopamine and 3-*O*-methyldopamine; and 3,4-dihydroxyphenylacetone in amounts equal to about 25%, 6%, and 3% of the dose, respectively (*20*). In vivo 3,4-dihydroxyphenylacetone formation from α-methylDopa administration has been described previously also in the rat (*21*). These findings might indicate that this secondary reaction catalyzed by DDC producing ketone and ammonia also occurs in vivo, although to a lesser extent than that in vitro. It is possible that the production of ketone and ammonia is relative to some side effects observed after administration of α-methylDopa.

In addition to determining the course of product formation, attempts were made to correlate this with the observed spectral changes taking place in the bound coenzyme. Several intermediates in the reaction of α-methylDopa with DDC can be detected by stopped-flow and conventional spectrophotometry, and structures might be assigned to these species on the basis of kinetic and spectral evidence. The most likely interpretation is that the initial rise at 420 nm represents the formation of the protonated external aldimine between PLP and α-methylDopa which is converted within manual mixing time to a 350-nm chromophore, which is probably the unprotonated Schiff base between PLP and α-methylDopa. An analogous 420 → 390 nm shift, attributed to the transition $ESH^+ \rightarrow ES$, has been proposed for the catalytic pathway of DDC with L-Dopa (*22*). The attribution of the 420-nm absorbance to an external aldimine between PLP and α-methylDopa is also supported by the spectral characterization of the interaction of α-methylDopa with DDC modified by limited proteolysis. Partial trypsinolysis of DDC leads to exclusive cleavage of Lys334−His335. The nicked protein possesses spectroscopic features identical with those of the native enzyme, is able to form external aldimine with substrates and substrate analogues, but is catalytically inactive (*23*). Upon addition of α-methylDopa to the nicked DDC, an increase at 420 nm is observed (data not shown).

Furthermore, our results demonstrate that DDC undergoes α-methylDopa-induced inactivation and that the mechanism of inactivation appears to involve generation of an active site directed affinity label, 3,4-dihydroxyphenylacetone. In fact, when 3,4-dihydroxyphenylacetone was tested as an inhibitor of DDC to determine if inactivation was occurring from an enzyme−product complex, we observed that ketone inactivates DDC in both a time- and a concentration-dependent manner which saturates at high concentrations of inhibitor and has a k_{inact}/K_i value similar to that of α-methylDopa (Table 1). This indicates that the same reaction of inactivation of DDC is being measured in the presence of α-methylDopa or 3,4-dihydroxyphenylacetone, the only differences being the source of the ketone inhibitor and that the efficiency of inactivation of α-methylDopa is determined solely by the reaction of the free ketone with the enzyme. This result strongly suggests that 3,4-dihydroxyphenylacetone generated during catalysis dissociates from the active site

Scheme 2



before inactivation occurs. That 3,4-dihydroxyphenylacetone binds to the active site is demonstrated by (a) the protection of inactivation of DDC by the ketone by an active site directed inhibitor of the enzyme, D-Dopa, and (b) the changes in the absorbance and CD bands of the visible spectrum of enzyme-bound PLP induced by the addition of 3,4-dihydroxyphenylacetone.

It has been noted that alkali treatment of the ketone-inactivated DDC gives rise to a spectrum absorbing maximally at 424 nm typical of a highly conjugated PLP derivative and that the same treatment, after $NaBH_4$ reduction of the inactivated enzyme, prevents the formation of this 424-nm species. A similar behavior was first observed after alkaline treatment of aspartate aminotransferase (*16*) or glutamate decarboxylase (*17*) inactivated by serine-*O*-sulfate and later for other PLP-dependent enzymes inactivated by substrate analogues which have a strong leaving group in the β position (*18*, *24*−*27*). All of these inactivation mechanisms involve the formation of an enamine intermediate generated by transimination of the elimination product by a lysine residue. Although not conclusively identified, a hypothetical structure has been assigned to the yellow compound released from the inactivated enzymes by hydroxide treatment, corresponding to the pyruvate-PLP adduct first synthesized by Schnackerz (*28*) (compound **I** in Scheme 2).

Sequence analysis of the isolated labeled peptide obtained by chymotrypsin digestion of NaB^3H_4-reduced native and ketone-inactivated enzymes proved unambiguously that in the fragment encompassing residues 298−304 the PLP-binding active lysine of the inactive enzyme is modified. Furthermore, conditions were found that allow for the release of the adduct from the inactivated protein and its subsequent characterization. From the available information, an inactivation mechanism of DDC by 3,4-dihydroxyphenylacetone can be suggested (Scheme 2). The methyl group of the ketone would lose a proton which can be accepted by an unidentified base (**1**), thus generating a reactive species similar to the enamine intermediate. The nucleophilic methylene group can attack the C-4′ of PLP (**2**), resulting in the formation of the covalent adduct **3**. The next step, probably due to a slow abstraction by a general base from the solvent or by an enzymic basic group of one of the protons on the C_3 of **3**, implicates the elimination of the ϵ-NH_2 of the enzyme's active site lysine, creating the highly conjugated derivative **4**. Release of this chromophore into solution occurs only when denaturation follows storage of **3** for many hours at 37 °C in the dark. Reduction of the C=O bond of **3** by borohydride would prevent the removal of the adjacent carbon-bound proton by attack of a base and thereby prevents the formation of **4**.

It has now been established that continuous removal of the product 5-HIA or 3,4-dihydroxyphenylacetaldehyde protects against inactivation of DDC by 5-HT and DA, respectively. However, the lack of full protection against 5-HT- or DA-induced inactivation leads to the consideration that, although the aldehyde can inactivate once desorbed from the enzyme, it may also do so prior to release. It would seem most probable that the mechanism of inactivation by 5-HT is more complex than previously believed (*9*) and that

the aldehydes behave as both mechanism-based inactivators and site-directed affinity labels. It is of interest that some of the spectroscopic properties of the covalent adduct formed between 5-HT and DDC are different from those formed between 3,4-dihydroxyphenylacetone and DDC. In fact, the adduct formed between 5-HT and DDC shows different fluorescent characteristics, and conditions have not yet been found for its release. Thus, a tentative working model for inactivation of DDC by 5-HT must await further investigation.

In conclusion, DDC is able to catalyze an oxidative deamination reaction which converts α-methylDopa, 5-HT, or DA into the corresponding aromatic ketone or aldehyde and ammonia. The comparison of their k_{cat} values suggests that the rate of their cleavage in ketone or aldehyde and ammonia is higher for aromatic compounds having a catechol as substituent on Cβ rather than an indolic ring. This is analogous to that already observed for the k_{cat} values of decarboxylation by DDC of catechol and indole substrates. The definition of this reaction enhances the already wide catalytic versatility of PLP. It will be interesting to establish the structural basis of this new catalytic property of the coenzyme. Studies are in progress to determine whether this type of reaction also takes place in other PLP-dependent α-decarboxylases. While the mechanism of inactivation accompanying the reaction producing aldehydes seems to be more complex than that previously suggested (*9*), the inactivation of DDC by α-methylDopa appears to result from the covalent linkage of 3,4-dihydroxyphenylacetone to PLP, and not to an enzymic residue, with C_3 of the ketone attached to the aldehydic carbon of the coenzyme in an aldol type adduct, thus trapping the PLP-lysyl aldimine of the active site. The observation that among all these aromatic compounds 3,4-dihydroxyphenylacetone is the most effective inhibitor of the enzyme may provide useful information for the design at active site-directed inhibitors of DDC.

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REFERENCES

1. Oates, J. A. (1966) in *The Pharmacological Basis of Therapeutics* (Hardman, J. G., Goodman Gilman, A., and Limbird, L. E., Eds.) 9th ed., pp 780−808, McGraw-Hill, London.
2. Weissbach, H., Lovenberg, W., and Udenfriend, S. (1966) *Biochem. Biophys. Res. Commun. 3*, 225−227.
3. Sourkes, T. L. (1971) *Nature 229*, 413−414.
4. Lovenberg, W., Barchas, J., Weissbach, H., and Udenfriend, S. (1963) *Arch. Biochem. Biophys. 103*, 9−14.
5. Smith, S. (1960) *Br. J. Pharmacol. 15*, 319−327.
6. Borri Voltattorni, C., Minelli, A., and Turano, C. (1971) *FEBS Lett. 17*, 231−235.
7. O'Leary, M., and Baughn, R. L. (1977) *J. Biol. Chem. 252*, 7168−7173.
8. Barboni, E., Borri Voltattorni, C., D'Erme, M., Fiori, A., Minelli, A., Rosei, M. A., and Turano, C. (1981) *Biochem. Biophys. Res. Commun. 99*, 576−583.
9. Bertoldi, M., Moore, P. S., Maras, B., Dominici, P., and Borri Voltattorni, C. (1996) *J. Biol. Chem. 271*, 23954−23959.
10. Slates, H. L., Taub, D., Kuo, C. H., and Wendler, N. L. (1964) *J. Org. Chem. 29*, 1424−1429.
11. Moore, P. S., Dominici, P., and Borri Voltattorni, C. (1996) *Biochem. J. 315*, 249−256.
12. Dominici, P., Moore, P. S., and Borri Voltattorni, C. (1993) *Biochem. J. 295*, 493−500.
13. Sherald, A. F., Sparrow, J. C., and Wright, T. R. F. (1973) *Anal. Biochem. 56*, 300−305.
14. Charteris, A., and John, R. A. (1975) *Anal. Biochem. 66*, 365−371.
15. Bossa, F., and Barra, D. (1968) *Ital. J. Biochem. 17*, 285−291.
16. Ueno, H., Likos, J. J., and Metzler, D. E. (1982) *Biochemistry 21*, 4387−4393.
17. Likos, J. J., Ueno, H., Feldhaus, R. W., and Metzler, D. E. (1982) *Biochemistry 21*, 4377−4386.
18. Ahmed, S. A., Ruvinov, S. B., Kayastha, A. M., and Miles, E. W. (1991) *J. Biol. Chem. 266*, 21548−21557.
19. Maras, B., Dominici, P., Barra, D., Bossa, F., and Borri Voltattorni, C. (1991) *Eur. J. Biochem. 201*, 385−391.
20. Au, W. Y. W., Dring, L. G., Grahame-Smith, D. G., Isaac, P., and Williams, R. T. (1972) *Biochem. J. 129*, 1−10.
21. Porter, C. C., and Titus, D. C. (1963) *J. Pharmacol. Exp. Ther. 139*, 77−87.
22. Minelli, A., Charteris, A. T., Borri Voltattorni, C., and John, R. A. (1979) *Biochem. J. 183*, 361−368.
23. Tancini, B., Dominici, P., Simmaco, M., Schininà, M. E., Barra, D., and Borri Voltattorni, C. (1988) *Arch. Biochem. Biophys. 260*, 569−576.
24. Badet, B., Roise, D., and Walsh, C. T. (1984) *Biochemistry 23*, 5188−5194.
25. Roise, D., Soda, K., Yagi, T., and Walsh, C. T. (1984) *Biochemistry 23*, 5195−5201.
26. Silverman, R. B., and George, C. (1988) *Biochem. Biophys. Res. Commun. 150*, 942−946.
27. Bhattacharjee, M. K., and Snell, E. E. (1990) *J. Biol. Chem. 265*, 6664−6668.
28. Schnackerz, K. D., Ehrlich, J. H., Giesemann, W., and Reed, T. A. (1979) *Biochemistry 18*, 3557−3563.

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