Insights into the Mechanism of Oxidative Deamination Catalyzed by DOPA Decarboxylase[†]

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ABSTRACT: The unusual oxygen-consuming oxidative deamination reaction catalyzed by the pyridoxal 5′-phosphate (PLP) enzyme DOPA decarboxylase (DDC) was here investigated. Either wild-type or Y332F DDC variant is able to perform such oxidation toward aromatic amines or aromatic L-amino acids, respectively, without the aid of any cofactor related to oxygen chemistry. Oxidative deamination produces, in equivalent amounts, a carbonyl compound and ammonia, accompanied by dioxygen consumption in a 1:2 molar ratio with respect to the products. Kinetic studies either in the pre-steady or in the steady state, together with HPLC analyses of reaction mixtures under varying experimental conditions, revealed that a ketimine accumulates during the linear phase of product formation. This species is reactive since it is converted back to PLP when the substrate is consumed. Rapid-mixing chemical quench studies provide evidence that the ketimine is indeed an intermediate formed during the first catalytic cycle. Moreover, superoxide anion and hydrogen peroxide are both generated during the catalytic cycles. On this basis, a mechanism of oxidative deamination consistent with the present data is proposed. Furthermore, the catalytic properties of the T246A DDC mutant together with those previously obtained with H192Q mutant allow us to propose that the Thr246-His192 dyad could act as a general base in promoting the first step of the oxidative deamination of aromatic amines.

An unusual oxidative deamination reaction catalyzed by the pyridoxal 5′-phosphate (PLP[1])-dependent enzyme DOPA decarboxylase (DDC, E.C. 4.1.1.28) toward aromatic amines (serotonin, dopamine, and α-methyldopamine) and D-tryptophan methyl ester has been identified and characterized in our laboratory during the past decade (*1–3*). The stoichiometry of this reaction indicates the production, in equivalent amounts, of aromatic aldehyde or ketoester, depending on the nature of the substrate, and ammonia with concomitant O_2 consumption in a 1:2 molar ratio with respect to the products (*2, 3*): aromatic amine (or D-tryptophan methyl ester) + $^1/_2O_2$ → aromatic aldehyde (or ketoester) + NH_3.

The intrinsic ability of DDC to catalyze an oxidative reaction is also validated by the occurrence of an analogous oxidative reaction, namely a decarboxylation-dependent oxidative deamination, in the Y332F variant of DDC in the presence of both L-DOPA and 5-hydroxy-L-tryptophan (*4*). In this case decarboxylation of the amino acid is stoichiometric with its oxidation, i.e. the release of CO_2 parallels the formation of aldehyde and ammonia. Concomitantly, O_2 is consumed in a 1:2 molar ratio with respect to products (*4*).

Although the capability of catalyzing, besides the main reaction, one or more side reactions is well established for PLP-dependent enzymes (*5*), such side catalytic activities generally relapse among those typical of PLP-chemistry (*5*). The novelty in DDC is the possibility of catalyzing a reaction involving dioxygen although the enzyme lacks of any cofactor or metal related to O_2 chemistry. Among PLP-enzymes, this ability is not confined only to DDC, since this oxidase reaction is also operative in other PLP-α-decarboxylases such as *Escherichia coli* glutamate decarboxylase and *Lactobacillus 30a* (*6*) as well as *Hafnia alvei* (*7*) ornithine decarboxylase. In this scenario the PLP plant enzyme phenylacetaldehyde synthase, that shows strong sequence homology to plant aromatic amino acid decarboxylases (*8*), could be considered since it catalyzes the PLP-dependent oxidative decarboxylation of L-phenylalanine to phenylacetaldehyde, ammonia, CO_2 and H_2O_2 (*8*). Furthermore, side reactions with ambient O_2 are reported to take place in the presence of various enzymes that do not possess cofactors/metals linked to O_2-related reactions: *S. typhimurium* acetolactate synthase isozyme II, baker's yeast pyruvate decarboxylase, *Zymomonas mobiliz* pyruvate decarboxylase and mammalian α-ketoglutarate dehydrogenase complex (*9–11*). It should be noted that a considerable group of them uses thiamine diphosphate (*12*), leading to the consideration that both PLP and thiamine diphosphate could have been underappreciated as biological catalysts (*12*).

The common feature of all these enzymes is the ability to generate a carboanionic species which could be prone to react with dioxygen (*9*). A mechanism for the DDC oxidative

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; DDC, DOPA decarboxylase; NADH, nicotinamide adenine dinucleotide, reduced form.

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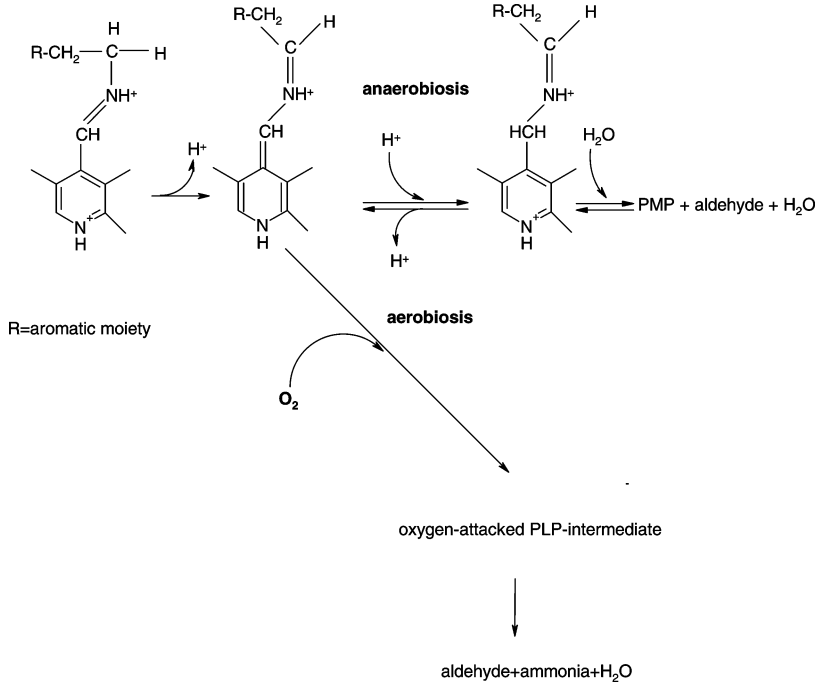
Scheme 1: Reaction Scheme of the Oxidative Deamination Mechanism of Wild-Type or Y332F Mutant DDC in the Presence of Aromatic Amines



deamination has been already proposed (*13*) (Scheme 1). According to this mechanism, the quinonoid intermediate formed after the abstraction of the α-proton from the PLP-substrate external aldimine intermediate could be susceptible to be attacked by either O_2 or H^+ at its C4′ depending on the aerobic or anaerobic conditions, respectively. As a consequence of the O_2 attack, an imine complex is formed and undergoes a spontaneous decomposition yielding aromatic aldehyde and ammonia. We have also reported that oxidative deamination does not occur in the absence of O_2 where it is replaced by a half-transamination (*13*). Under anaerobic conditions, the H^+ attack to C4′ of the pyridoxyl moiety would thus give rise to a ketimine intermediate that hydrolyzes to give the pyridoxamine 5′-phosphate (PMP)-enzyme and the related carbonyl compound.

Although our results are consistent with this mechanistic hypothesis, no evidence has been provided for the formation of any of the intermediates proposed except for the external aldimine (*13*) and the quinonoid (*3*). Moreover, the identity of either the species reactive toward oxygen or the chemical form in which dioxygen is reduced remains elusive. In this regard, it should be remembered that, according to the various reports concerning oxidative side reactions so far published, O_2 has been proposed to be reduced to either H_2O_2 (*7, 8*) or H_2O (*2, 4, 6*).

In this paper we have reinvestigated the oxidative deamination reaction in order to address the above-mentioned open questions. Being endowed with a consistent and therefore easily detectable decarboxylation-dependent oxidative deaminase activity (i.e., an amino acid oxidase-type reaction with the exception that CO_2 is released), the reaction of Y332F DDC mutant with L-DOPA ($k_{cat} \sim 4.5\ s^{-1}$ (*4*)) represents a good model system for these investigations.

Here, we report data suggesting that (1) a PMP-ketimine intermediate forms during the oxidative deamination, (2) both hydrogen peroxide and superoxide anion are formed during the reaction course and (3) O_2 controls the shift of the equilibrium between the quinonoid and the ketimine intermediates. From the above information, a detailed mechanism of oxidative deamination catalyzed by DDC is proposed. Moreover, site-directed mutagenesis studies lead to the proposal of a catalytic role for threonine 246 to act as a general base for the oxidative deamination reaction.

EXPERIMENTAL PROCEDURES

Materials. L-DOPA, 5-hydroxy-L-tryptophan, PLP, PMP, Hepes, nicotinamide adenine dinucleotide, reduced form (NADH), dopamine, serotonin, D-tryptophan methyl ester, bovine liver L-glutamate dehydrogenase, horse liver alcohol dehydrogenase, bovine liver catalase, superoxide dismutase from bovine erythrocytes, and trichloroacetic acid were purchased from Sigma. The liquid chromatography solvents were from Labscan. Ingredients for bacterial growth were from Difco. All other chemicals were of the highest purity available. 3,4-Dihydroxyphenylacetaldehyde was synthesized according to the method described (*14*), and its purity was checked by HPLC analyses.

Cloning, Expression and Purification of Wild-Type DDC and of Y332F and T246A Mutants. The DDC cDNA, either in its wild-type or mutated forms, was cloned into the pKKΔ3Δ4 vector, which was then used to transform SVS370 *E. coli* cells. The procedures used for wild-type and Y332F variant were already published (*4, 15*). T246A DDC was constructed using the QuickChange mutagenesis kit (Stratagene). The presumptive mutant was sequenced to verify that no other mutations were introduced during the procedure. The conditions used for expression and purification of wild-type and mutant variants of DDC are the same as described (*1, 4, 15, 16*). The purified enzymes were homogeneous as indicated by a single band on SDS−PAGE. The enzyme concentration was determined by using an ϵ_M of 1.3

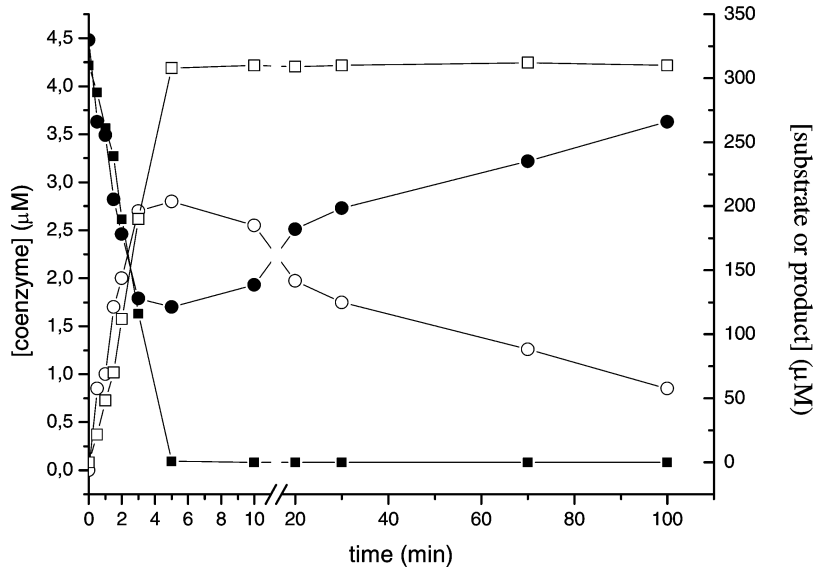


FIGURE 1: Consumption of L-DOPA, formation of 3,4-dihydroxyphenylacetaldehyde and PLP-PMP content during the reaction of Y332F DDC with L-DOPA. Y332F DDC (2.4 μM) was incubated in 50 mM Hepes, pH 7.5, with 320 μM L-DOPA. At the indicated times aliquots (150 μL) were withdrawn and denatured with 10% final trichloroacetic acid. After removal of the precipitated protein by centrifugation, the supernatants were subjected to HPLC analysis and the area of the peaks were converted to absolute amounts as described in Experimental Procedures. The symbols used are (■) L-DOPA, (□) 3,4-dihydroxyphenylacetaldehyde, (●) PLP and (○) PMP. Data shown are means of three independent experiments; the SE is less than 5%.

$\times$ 10^5 M^{-1} cm^{-1} (*15*). PLP content of holoenzyme was determined by releasing the coenzyme in 0.1 M NaOH and by using an ϵ_M = 6600 M^{-1} cm^{-1} at 388 nm (*17*). The apparent equilibrium dissociation constant for PLP of mutant T246A was determined by measuring the initial velocity of L-DOPA decarboxylation (see below) obtained by mixing 6.5 μM T246A in the presence of varying PLP concentrations (from 0.02 to 10 μM). Data obtained were fitted to a quadratic equation.

Assay for Decarboxylase and Oxidative Deaminase Activity. Decarboxylase activity for either wild-type or T246A mutant was measured as described by Sherald et al. (*18*) and as modified by Charteris and John (*19*). Evolution of CO_2 and formation of ammonia and aldehyde for the reaction of Y332F mutant with L-DOPA were determined as already described (*4*). Reaction mixtures of wild-type and enzymic variants with aromatic amines were assayed for ammonia or aldehyde formation with the coupled assay systems of glutamate or alcohol dehydrogenase, respectively, as already reported (*1, 2, 4*).

HPLC Analyses. PLP and PMP were detected using a Jasco chromatographic system following the procedure already reported (*20*). Diode-array HPLC measurements were performed using a Jasco MD-2010 Plus Multiwavelength Detector connected to a Jasco PU-1580 HPLC pump. The eluent was 50 mM potassium phosphate, pH 2.35 or pH 7, and the column was a reverse phase C18 Discovery (Supelco).

Rapid Chemical Quench Studies. The chemical quench kinetic measurements were performed at 25 °C by mixing 100 μL portions of 100 μM Y332F DDC and 100 μL of 200 μM L-DOPA in 50 mM Hepes, pH 7.5. The reactions were quenched by mixing the solutions with an equal volume of 60% trichloroacetic acid in the quench syringe. Reaction times ranged from 50 ms to 4 s. The amount of PMP formed was determined by HPLC as indicated above.

O_2 Consumption and Detection of H_2O_2 or Superoxide Anion. Oxygen consumption was measured amperometrically with a Clark-electrode in a 0.7 mL cell as already reported (*2*) using an Instech System and DUO18 (World Precision Instruments, Inc.) software. The mixture containing enzyme (either wild-type or Y332F) plus substrate was magnetically stirred and thermostatically maintained at 25 °C. The formation of H_2O_2 or superoxide anion was detected by adding to the reaction mixture containing DDC (either wild-type or mutant) and substrate either catalase or superoxide dismutase and comparing the changes in O_2 consumption traces with respect to control conditions with no added catalase or superoxide dismutase. For all the experiments the ambient O_2 concentration was taken to be 260 μM. Anaerobiosis was maintained by continuously flushing nitrogen into a capped enzymic solution as described previously (*13*). All data analyses were performed by nonlinear curve fitting using Origin 7 (Origin Laboratory Corporation, Northampton, MA).

RESULTS AND DISCUSSION

Reaction of Y332F DDC with L-DOPA under Aerobic and Anaerobic Conditions. As previously reported (*4*), reaction of Y332F DDC (2.4 μM) with L-DOPA (320 μM) in 50 mM Hepes, pH 7.5, at 25 °C causes the production of ammonia and 3,4-dihydroxyphenylacetaldehyde along with the consumption of molecular oxygen in a 1:2 molar ratio with respect to products (*4*). Unexpectedly, we now notice that, during the linear phase of formation of the products, a conversion of the PLP cofactor into PMP can be observed (Figure 1). After complete consumption of L-DOPA a slow but consistent reconversion of PMP to almost all (~80−85%) the original PLP content could be seen. To determine if the coenzyme interconversion PLP ↔ PMP is an intrinsic characteristic of the oxidative deamination reaction or is related to the behavior of Y332F mutant DDC, 9.3 μM wild-

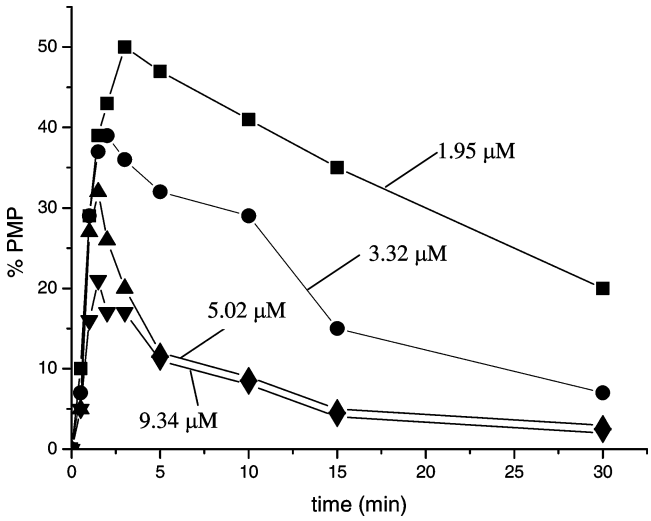


FIGURE 2: Time course of PMP formation during reaction of different concentrations of Y332F DDC with 300 μM L-DOPA. Y332F DDC at various concentrations was allowed to react with 300 μM L-DOPA. At definite times, aliquots were withdrawn and denatured with 10% final trichloroacetic acid. After removal of the precipitated protein by centrifugation, the supernatants were subjected to HPLC analysis, as described in Experimental Procedures, and the relative amount of PMP at the indicated enzyme concentrations (square for 1.95 μM enzyme concentration, circle for 3.32 μM, triangle up for 5.02 μM and triangle down for 9.34 μM) was determined.

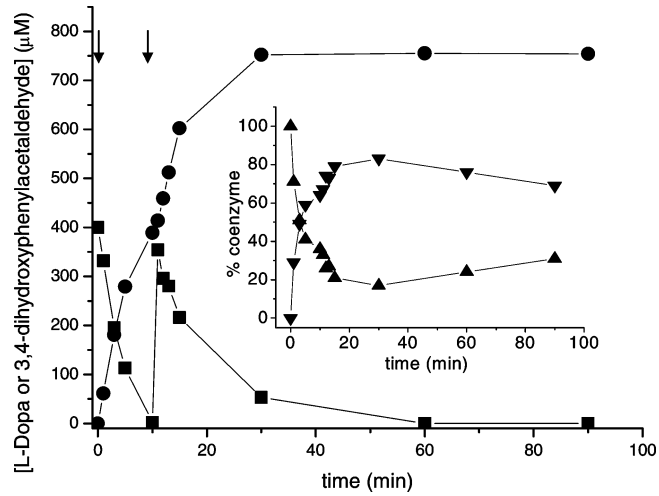


FIGURE 3: Time course of substrate depletion, product formation and coenzyme interconversion during the reaction of Y332F DDC in the presence of successive additions of L-DOPA in 50 mM Hepes pH 7.5. Y332F DDC (2.2 μM) was incubated with 400 μM L-DOPA for 10 min, a time sufficient to consume all substrate. Thereafter, an additional aliquot of 400 μM L-DOPA was added. Substrate addition is indicated by the arrows. At the indicated times, 150 μL of the reaction mixture was withdrawn and deproteinized with 10% trichloroacetic acid. After removal of the precipitated protein by centrifugation, the samples were analyzed by HPLC as described in Experimental Procedures and the relative amounts of L-DOPA (■), 3,4-dihydroxyphenylacetaldehyde (●), PLP (▲) and PMP (▼) were measured.

type DDC was allowed to react with 100 μM α-methyldopa, a reaction mixture known to give rise to ammonia and 3,4-dihydroxyphenylacetone, products of oxidative deamination, together with oxygen consumption (*2*). If this enzymatic solution was analyzed for cofactor content, a trend of interconversion between PLP and PMP similar to that displayed by Y332F with L-DOPA was observed (data not shown).

The course of the reaction of Y332F DDC mutant with L-DOPA (300 μM) has been followed at different enzyme concentrations. Although the shapes of the profiles for the interconversion of the two coenzymatic forms (PLP and PMP) are similar at all the enzyme concentrations tested (at each enzyme concentration there are two phases: at first, PMP increases at the expense of PLP, and then this tendency is reversed until approximately 85% of the PLP cofactor is regenerated), the amount of PMP formed relative to the initial PLP content of the enzyme, during consumption of L-DOPA, increases as the enzyme concentration decreases (Figure 2). At higher enzyme concentrations, where L-DOPA consumption is faster, the accumulation of the PMP-intermediate is less appreciable. It is noteworthy that in every case the PMP species is generated during the course of linear formation of products (data not shown).

The experiments performed above suggest the idea that the PMP species is a reaction intermediate. Thus, we decided to check whether the PMP species formed is reactive during the oxidative deamination reaction.

When 2.2 μM Y332F mutant was incubated with 400 μM L-DOPA, a decrease of L-DOPA level and a concomitant increase of 3,4-dihydroxyphenylacetaldehyde concentration occur with time. The initial velocities of these catalytic events were estimated to be 0.50 ± 0.01 and 0.46 ± 0.02 s^{-1}, respectively. At 10 min, when the substrate was almost consumed, and ~70% of the original coenzyme content was converted to a PMP form, an additional amount of L-DOPA was added to the reaction mixture to a final concentration of 400 μM. As shown in Figure 3, production of 3,4-dihydroxyphenylacetaldehyde and a concomitant consumption of L-DOPA takes place with initial velocities similar (0.50 ± 0.02 and 0.47 ± 0.01 s^{-1} for L-DOPA and aromatic aldehyde, respectively) to those measured in the presence of the first aliquot of L-DOPA. At times longer than 8 min after the addition of the second aliquot of the substrate, a decrease in the reaction rate (possibly due to an almost overall consumption of solution dioxygen whose initial concentration in aqueous solutions at 25 °C is ~260 μM) could be observed. If no additional L-DOPA is added, when the substrate is consumed, the PMP cofactor gradually reconverts into PLP (inset of Figure 3). On the contrary, if a third aliquot of 400 μM L-DOPA is added to the above mixture, whose O_2 content has been completely depleted, there is no formation of products and no consumption of this third aliquot, and almost all coenzyme shifts to the PMP form (data not shown). This behavior recalls what was already reported for wild-type DDC and other α-decarboxylases (*6*) in the presence of millimolar concentrations of their α-methyl substrates; in those experiments the PMP generation was attributed to a decarboxylation-dependent transamination (*6, 21*).

A further evidence of the correlation between PMP formation and dioxygen depletion comes from the analysis of the reaction mixture of 2.5 μM Y332F in the presence of 25 mM L-DOPA; the PLP-bound is rapidly converted to PMP (Figure 4), together with formation (data not shown) of about 500 μM aldehyde and ammonia (this result is expected since the stoichiometry of O_2 consumption is 1:2 with respect to products (*2*)). Thereafter the reaction stops and the enzyme remains in the PMP form. On the other hand, it should be

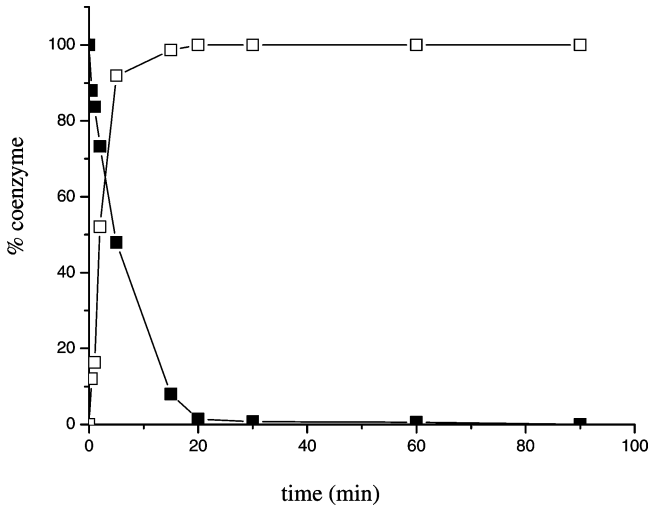


FIGURE 4: Time course of coenzymes content during the reaction of Y332F DDC in the presence of 25 mM L-DOPA. Y332F DDC (2.5 μM) was incubated with 25 mM L-DOPA in 50 mM Hepes, pH 7.5. At various times, aliquots were withdrawn and denatured with 10% final trichloroacetic acid. After removal of the precipitated protein by centrifugation, the supernatants were subjected to HPLC analysis as described in Experimental Procedures and the areas of the peaks were related to the percentile amount of the coenzymes. Symbols are (■) PLP and (□) PMP.

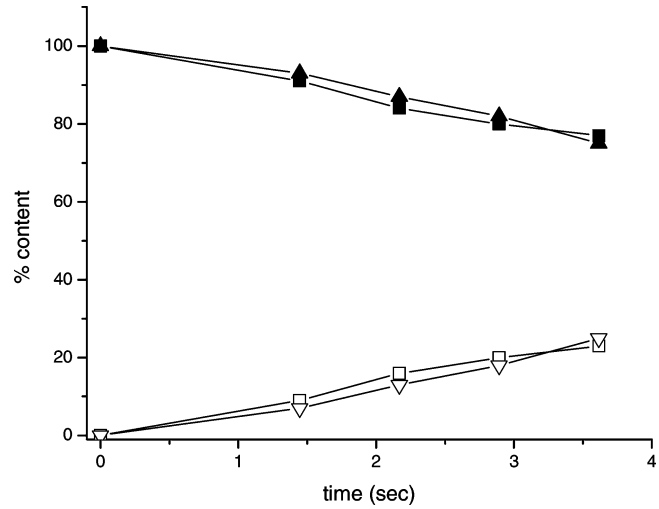


FIGURE 5: Single turnover analysis of the reaction of 50 μM Y332F DDC with 100 μM L-DOPA in 50 mM Hepes, pH 7.5. Y332F DDC (50 μM) was mixed with 100 μM L-DOPA, and at the indicated times the reaction was stopped by mixing with 20% final trichloroacetic acid. Thereafter, the samples were centrifuged to precipitate the denatured protein and the supernatants were subjected to HPLC analysis. Symbols are (■) PLP, (□) PMP, (▲) L-DOPA, and (▽) 3,4-dihydroxyphenylacetaldehyde.

recalled that if Y332F DDC (2.4 μM) is incubated with L-DOPA (0.35 mM) under anaerobic conditions, the PLP content of the mutant dropped to approximately 8% with respect to the original amount within 2 min, the remaining 92% was recovered as PMP, and no detectable consumption of substrate was observed (*4*). However, we have now observed that when O_2 is dissolved in such an anaerobic reaction mixture, L-DOPA undergoes a decarboxylation-dependent oxidative deamination and the enzyme, initially present predominantly in the PMP form, gradually converts into PLP. About 80−85% of the original content of the coenzyme is restored after one hour, a time longer than that occurring in an air-saturated buffer, with the reaction rate likely being limited by the time necessary for O_2 to be dissolved into the solution. It should be noted that neither PMP alone in aerobic conditions nor PMP in the presence of a high concentration of O_2 (directly bubbled into the solution from a bottle containing 100% O_2) gives rise to PLP formation.

The following experiments were carried out with the aim of identifying the intermediate(s) of the oxidative deamination reaction.

When 50 μM Y332F DDC is mixed with 100 μM L-DOPA under single turnover conditions in a quench flow apparatus up to 4 s (Figure 5) a gradual conversion of the PLP of the enzyme takes place concomitantly to PMP and aldehyde formation while L-DOPA is consumed. After 3.6 s, 23% of the original PLP has been converted into PMP and 25% of the original L-DOPA has been transformed into 3,4-dihydroxyphenylacetaldehyde. These results suggest that a PMP-intermediate forms during the first catalytic cycle. Again, 2.4 μM Y332F DDC was treated with 0.2 mM L-DOPA under anaerobic conditions (to allow the greatest amount of PMP intermediate to be accumulated, as observed above) for 5 min reaction time, and then the mixture was deproteinized by centrifugation for 5 min in a Centricon-30 device (Amicon) that was maintained capped and as long as possible anaerobic under a nitrogen atmosphere. During this time, O_2 does not efficiently dissolve into solution so that PMP is not converted into PLP. The filtrate was then recovered with a Gastight Hamilton syringe and loaded onto a HPLC column using a 50 mM potassium phosphate buffer pH 7.4 as eluent. Using a diode array device for quantitation, it was determined that the mixture contains 4.5 μM PMP, almost all initial L-DOPA and 5 μM 3,4-dihydroxyphenylacetaldehyde, thus strongly suggesting that the PMP-intermediate, that builds up during the first reaction cycle, is composed of a PMP moiety in equimolar amount with 3,4-dihydroxyphenylacetaldehyde. These results support the identification of the intermediate with a ketimine. All attempts to block the putative imine derivative using reducing agents such as $NaBH_4$ or $LiAlH_4$ were unfortunately unsuccessful. The finding that a ketimine intermediate accumulates and breaks down in a catalytically competent manner does not necessarily mean that it is an intermediate in the steady-state of the oxidative deamination reaction. It is likely that (1) the catalytic competence of the ketimine is a reflection of the inherent reactivity of the quinonoid species that converts into the ketimine by abstracting a proton from a neighboring acid residue (Scheme 1) and (2) the small amount of quinonoid that is present may then slowly react with O_2 to complete the reaction.

In order to unravel which species is responsible for the conversion of the ketimine back to the internal aldimine, we incubated apo-Y332F DDC or apo-wild-type DDC with PMP, or PMP + 3,4-dihydroxyphenylacetaldehyde or PMP + supernatant of oxidative deamination reaction mixture of Y332F with L-DOPA.

No detectable PLP formation could be observed when free PMP (5−50 μM) was added to a solution of 2.4 μM apo-DDC (either apo-Y332F or wild-type) in aerobiosis in the absence or presence of 0.2 mM 3,4-dihydroxyphenylacetaldehyde. On the contrary, the almost complete conversion of PMP to PLP could be achieved as follows: 2.5 μM apo-DDC (either wild-type or mutant) incubated with 5 μM PMP

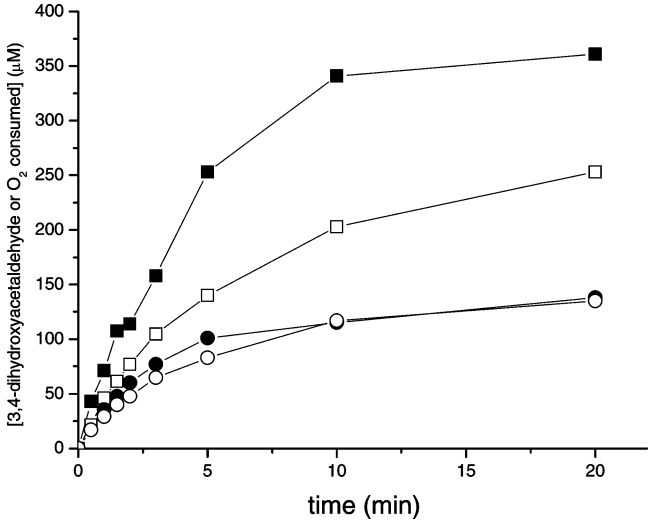


FIGURE 6: O_2 consumption during the reaction of Y332F DDC with L-DOPA. Y332F DDC (1.85 μM) was allowed to react with 0.35 mM L-DOPA in 50 mM Hepes, pH 7.5. The formation of 3,4-dihydroxyphenylacetaldehyde (■) and O_2 consumption (□) were monitored. O_2 consumption was also measured upon addition to a reaction mixture as described above of 500 units of catalase (●) or 50 units of superoxide dismutase (○).

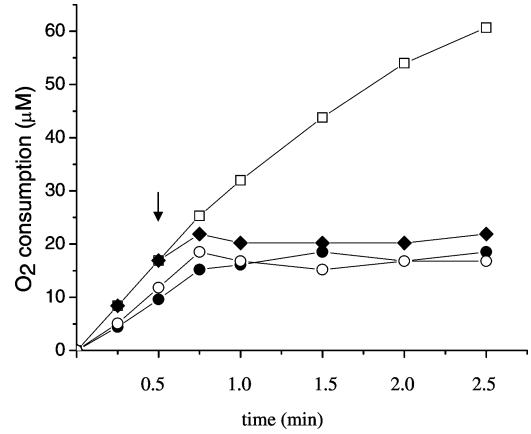


FIGURE 7: O_2 consumption during the reaction of Y332F DDC with L-DOPA in the absence or presence of carbiDOPA. Y332F DDC (1.85 μM) was allowed to react with 0.35 mM L-DOPA in 50 mM Hepes, pH 7.5. The O_2 consumption was monitored in the absence (□) or presence (◆) of 10 μM carbiDOPA added after 30 s of reaction time (arrow). O_2 consumption was also measured in a reaction mixture as described above containing either 500 units of catalase (●) or 50 units of superoxide dismutase (○) in the presence of 10 μM carbiDOPA added after 30 s of reaction time.

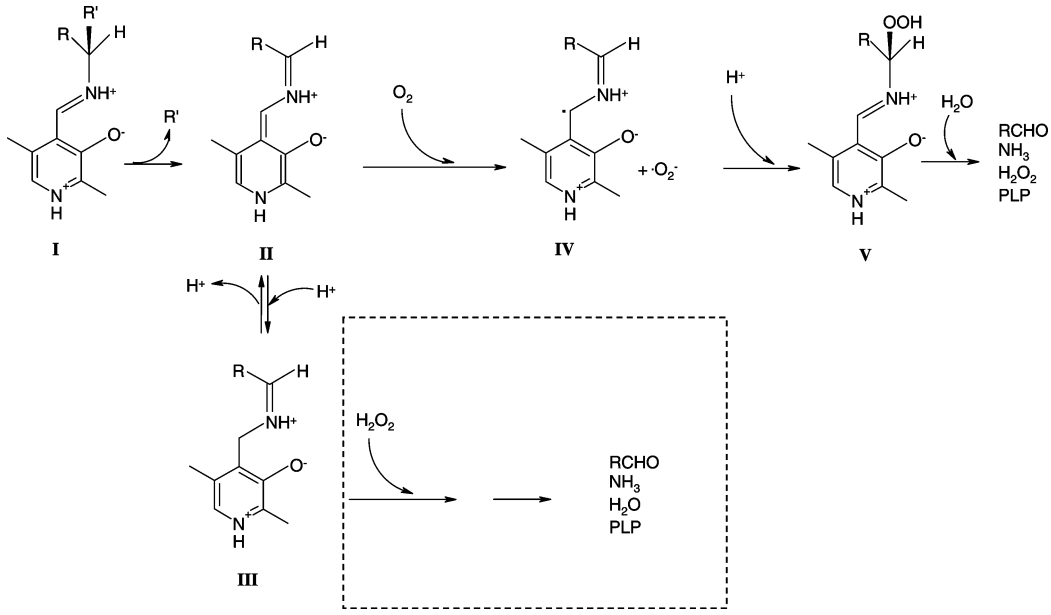
was treated for 10 min with the supernatant (obtained by deproteinization through a Centricon-30 (Amicon) device) of a reaction mixture of 2.4 μM Y332F DDC with 0.2 mM L-DOPA. Based on these results, it can be foreseen that O_2 converts to some reduced derivative that promotes the PMP to PLP conversion. Attempts to verify this hypothesis are currently underway.

Reduction of O_2 during the Reaction of Y332F DDC with L-DOPA. Reaction of 1.85 μM Y332F DDC with 0.35 mM L-DOPA was followed at the Clark electrode by measuring O_2 consumption. As already reported (*4*), the stoichiometry of O_2 consumption is peculiar being 1:2 with respect to product formation (Figure 6). However, if to the same reaction mixture 500 units of catalase (10 μL of a 25 mg/mL solution) or 50 units of superoxide dismutase (10 μL of a 3.1 mg/ml solution) were added and the O_2 consumption traces were monitored, a decrease in the rate of O_2 consumption was evident in both cases (Figure 6). The decrease in the rate of oxygen consumption was in both cases half of that measured in the absence of these enzymes. These data, together with the fact that neither L-DOPA alone nor the nonenzymatic complex PLP−L-DOPA is capable of generating either H_2O_2 or superoxide anion, strongly suggest that hydrogen peroxide and superoxide anion are generated during the oxidative deamination reaction. We realized that our previously reported attempts (*2, 4, 6*) to detect H_2O_2 failed because the peroxidase coupled system used (horseradish peroxidase in the presence of 2,2-azinobis-(3-ethylbenzthiazoline)sulfonate or diaminobenzidine) interfered with the aromatic chemical structure of the catechol or indole substrates of DDC, thus preventing any measurements from being reliable.

The effect of the DDC-inhibitor carbiDOPA (3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methyl propionic acid, a known potent inhibitor of DOPA decarboxylase (*22, 23*) employed in Parkinson's disease therapy) on the formation of these reduced oxygen species has been analyzed. As shown in Figure 7, addition of 10 μM inhibitor to reaction mixtures, either in the presence or in the absence of catalase or superoxide dismutase, immediately stops the O_2 consumption. This implies that O_2 reduction to superoxide anion or hydrogen peroxide involves an active-site reaction intermediate that is catalytically competent.

Mechanism of Oxidative Deamination. On the basis of all above-reported results, a detailed mechanism of oxidative deamination could be proposed taking into account these new collected data (Scheme 2). The external aldimine intermediate, **I**, undergoes a decarboxylation or a deprotonation leading to a quinonoid species, **II**, that is protonated at C4′ producing the ketimine intermediate, **III**. Although it cannot be ruled out that this intermediate could be attacked by dioxygen, it is difficult to envisage how it could proceed. It seems much more likely, as postulated by Abell and Schloss regarding enzymes proceeding through a carbanion chemistry (*9*) and by us (*3, 13*) on DDC, that the more electron dense quinonoid intermediate, in equilibrium with the ketimine, is reactive toward O_2. We could assume that aerobiosis shifts the quinonoid−ketimine equilibrium toward quinonoid, while anaerobiosis shifts the equilibrium toward ketimine. The reaction between dioxygen and the quinonoid would give rise directly to a superoxide anion and semiquinone, **IV**. Superoxide is deprotonated since its pK_a value is 4.8 (*24, 25*) and in its anionic form is thus able to couple with the semiquinone giving rise to a peroxide species that is further protonated, as already suggested for DDC and other decarboxylases (*9, 12, 13*), and thus forming a hydroperoxy-PLP intermediate, **V**. This rearranges to produce aldehyde, ammonia and hydrogen peroxide. It should be taken into consideration that hydrogen peroxide could spontaneously disproportionate, generating oxygen and water. If one takes this into consideration, the 1:2 stoichiometry of oxygen consumption is the expected result, since hydrogen peroxide should form in equimolar amounts with respect to products. Altogether, we can suggest that there is a two-electron reduction in the first part of the reaction (when oxygen converts, through superoxide, to hydrogen peroxide) and a second two-electron reduction in the second part (when

Scheme 2: Oxidative Deamination Mechanism Catalyzed by Wild-Type or Y332F DDC in the Presence of Aromatic Amines or Aromatic L-Amino Acids, Respectively[a]



[a] R represents the CH_2-aromatic moiety, i.e. the catechol or the indole ring. R′ represents the carboxyl group or the hydrogen atom for the amino acidic or amine substrate, respectively. The dashed rectangle represents a hypothetical pathway of addition of hydrogen peroxide to the ketimine intermediate.

hydrogen peroxide converts into water). On this basis, one can possibly advance the hypothesis that hydrogen peroxide could be prone to attack as a nucleophile a ketimine intermediate generated in a second round of reaction. This proposal is merely suggestive and needs further experimental evidence (Scheme 2, dashed box).

This mechanism, consistent with our experimental data, unravels some important open issues regarding the oxidative deamination reaction catalyzed by DDC: (1) how 1 mol of O_2 could produce 2 mol of products; (2) the identification of a ketimine as a catalytic intermediate of the reaction; and (3) the shift of the equilibrium between the quinonoid and ketimine intermediates is possibly controlled by O_2.

Structural Basis for the Mechanism. The first step in this mechanism is a deprotonation or a decarboxylation depending on the chemical structure of the substrate. While CO_2 could be evolved without any acid−base catalyst, deprotonation of an aromatic amine requires an enzyme residue that should act as a base. An inspection of the active site of DDC (*23*) shows (Figure 8) that two residues are located in a proper position: the PLP-lysine 303 is placed at 2.96 Å from C4′ and 5.15 Å from Cα of the external aldimine intermediate (in this case it deals with external aldimine with carbiDOPA), while, on the other face of the cofactor, threonine 246 is located in good proximity (5.05 Å) to the Cα of the substrate moiety. PLP-Lys303 is a particularly good candidate to perform a deprotonation/protonation reaction, shifting the equilibrium from external aldimine toward ketimine. The role of this residue as acid−base catalyst (*26*) has been well identified in many aminotransferases and in some PLP-enzymes that catalyze transamination as side reaction. However, a catalytic role of Lys303 in the oxidative deamination reaction is excluded since the K303A variant is able to catalyze oxidative deamination of aromatic amines (M.B. et al., unpublished data). On the basis of this observation, the role of threonine 246 should be taken into consideration. Threonine is not expected to be ionized under most conditions, and neutral threonine could not act as general base. However, histidine 192 is in a good position (the iminic nitrogen of His192 is 4.58 Å from the OH moiety of Thr246) (Figure 8) to abstract a proton from threonine 246 which is then activated to abstract a proton from the Cα of the external aldimine. It should be pointed out that

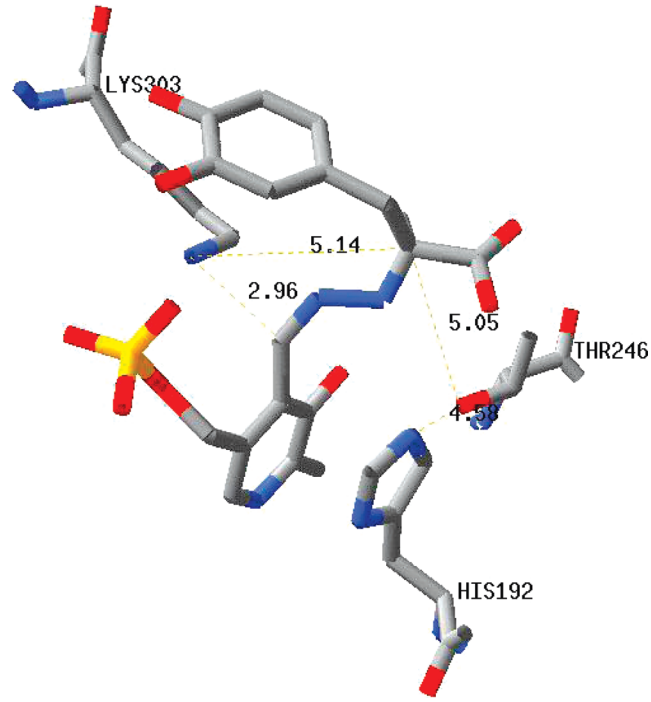


FIGURE 8: Active site of pig kidney DOPA decarboxylase in the carbiDOPA-PLP complex (PDB: 1JS3). The active site of the PLP−carbiDOPA complex of DDC shows residues involved in this investigation that are indicated by CPK colors. Figure was built using Swiss-Pdb-Viewer (*31*).

His192, a conserved residue of DDC, has been found to be essential for active site integrity (*27*). The spatial arrangement of these two residues is reminiscent of that displayed by urate oxidase, another enzyme catalyzing an O_2-dependent reaction without any cofactor participating in the catalytic reaction (*28*). In that case, the authors proposed the existence of a thr-lys catalytic dyad that could act as a general base in promoting the first step of the oxidase reaction (*28*).

Following the above hypothesis, we decided to characterize the functional properties of mutant T246A DDC and to reconsider the results already obtained with H192Q DDC concerning its role in the oxidative deamination reaction (*27*). A preliminary study on *E. coli* cell lysates of T246A DDC from rat liver (*29*) yielded a mutant enzyme that was 1% as active as the wild-type enzyme, thus suggesting that this residue is not essential for catalysis. However, the decrease in activity has been tentatively attributed to a conformational change induced by the mutation, although this effect was not investigated at all. Indeed, we now report that the absorbance features of purified T246A are essentially unchanged with respect to those of the wild-type protein. The CD far- and near-UV spectra are superimposable to those of wild-type DDC (data not shown), thus suggesting that T246A has maintained its structural integrity in secondary and tertiary structure as well as in active site topology. The only differences are related to a weaker PLP binding ability. In fact, the mutant binds 1.3 mol of PLP/mol of dimer, instead of 2 mol of PLP bound to dimeric wild-type DDC (data not shown). Furthermore, the apparent equilibrium dissociation constant for PLP, K_d, was found to be 0.09 ± 0.02 μM, a value approximately twice that measured for the wild-type protein (*30*).

The data of initial velocity for decarboxylation of both L-DOPA and 5-hydroxy-L-tryptophan versus substrate concentration curiously exhibit a substrate-inhibition pattern that requires a modified version of the Michaelis−Menten equation to be used ($v/[E] = k_{cat}/(1 + (K_m/[S]) + ([S]/K_I))$) leading to the following kinetic parameter values: $k_{cat} = 8.8 \pm 0.3$ min^{-1}, $K_m = 0.058 \pm 0.009$ mM and $K_I = 10 \pm 1$ mM for L-DOPA and $k_{cat} = 0.67 \pm 0.02$ min^{-1}, $K_m = 0.049 \pm 0.006$ mM and $K_I = 5.7 \pm 0.6$ mM for 5-hydroxy-L-tryptophan. The reason for the substrate inhibition is currently unknown. The values of the catalytic efficiency for decarboxylation for both substrates are 2−5% with respect to those of wild-type (*20*). When 20 μM T246A was incubated with 0.5 or 5 mM concentration of either dopamine or serotonin, no detectable consumption of aromatic amine was observed nor formation of ammonia or aldehyde, thus suggesting the inability of this mutant to catalyze the oxidative deamination of aromatic amines. Surprisingly, T246A DDC catalyzes the oxidative deamination of D-tryptophan methyl ester with a k_{cat} value approximately 5% with respect to the corresponding one measured for wild-type (*3*).

The comparison of the catalytic activities of T246A and of H192Q mutant (*27*) with respect to those of wild-type DDC, revealed that (a) both variants present a decarboxylase activity, even if the k_{cat} value (measured for mol of cofactor) is decreased by 11-fold for H192Q (*27*) and 29-fold for T246A with respect to wild-type (*20*); and (b) oxidative deamination reaction of aromatic amines occurs with a k_{cat} approximately 20-fold lower for H192Q with respect to wild-type, while it does not occur for T246A. The behavior of T246A in the presence of D-tryptophan methyl ester could be explained taking into account that the $C\alpha H^+$ of D-tryptophan methyl ester is rather acid and possibly does not require a base to be removed, being that it is in the α position with respect to a carbonyl moiety. This view is consistent with the appearance under steady-state conditions of the quinonoid, which thus accumulates during the reaction of wild-type DDC with D-tryptophan methyl ester (*3*).

These results suggest that Thr246 is an essential catalytic (acting as a general base) residue for oxidative deamination and His192 could act in concert with it by abstracting a proton from the hydroxyl moiety of Thr246. Given that the pK_a of free glutamine is much higher than that of His, in the H192Q mutant the H^+ abstraction from Thr should be impaired. However, we could speculate that the H^+ moiety of the OH-Thr could be displaced by a hydrogen bonding interaction with Gln, thus increasing the nucleophilic character of the oxygen atom and allowing it to behave, although not efficiently, as a general base. The possibility that H^+ abstraction could be accomplished by a water molecule located at the active site, or alternatively, by a small percentage of Thr246 in deprotonated form could also be considered.

The data presented here allow widening of the investigation to enzymes (PLP or not) capable of performing decarboxylation together with O_2-consuming reactions with the aim of finding a common structural basis. A recent report (*12*) suggests that several enzymes that possess PLP and catalyze oxygen consuming reactions producing reactive oxygen species may contribute to normal cellular signaling and also to cellular damage in neurodegenerative diseases. A deep knowledge of this new opening world of catalyzed PLP-catalyzed, O_2-dependent reactions is surely desirable.

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