Aromatic amino acid methyl ester analogs form quinonoidal species with Dopa decarboxylase

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Abstract This study reports for the first time that binding of aromatic methyl ester analogs to Dopa decarboxylase in the native and inactive nicked forms causes the appearance of a dead-end quinonoidal species absorbing at 500 nm, in addition to an external aldimine absorbing at 398 nm. The equilibrium mixture of these species varies depending on both the analog structure and the enzyme form. The above mentioned intermediates are also characterized with respect to their CD properties and the equilibria for their formation are determined as a function of pH. The results have provided evidence that the establishment of proper contacts between the active site and hydroxyl groups of the ligand are indispensable in order to limit unwanted side reactions.

Key words: Dopa decarboxylase; Pyridoxal 5′-phosphate; Quinonoid; Methyl ester

1. Introduction

Amino acid substrates and analogs are frequently used to study the structural basis for their binding and for the dissection of mechanistic pathways along with the identification of the intermediates involved. Due to the distinct spectroscopic properties of enzyme bound pyridoxal 5′-phosphate (PLP), the interaction of ligands with the enzyme can be conveniently followed by UV-visible spectroscopy. The PLP-dependent enzyme Dopa decarboxylase (DDC; E.C. 4.1.1.28) catalyzes the conversion of L-aromatic amino acids into their corresponding amines and possesses coenzyme absorbances at 335 and 425 nm due to the internal aldimine formed between PLP and the ε-amino group of Lys^{303} [1,2]. The spectral changes occurring upon the addition of a number of ligands to DDC have been generally described [3]. When appropriate aromatic amino acid acids or analogs are complexed with the enzyme an external aldimine is formed, presumably via *geminal*-diamine intermediates, and show absorbances at around either 430 or 390 nm, depending on the nature of the ligand and the experimental conditions [4,5]. L-Dopa methyl ester (DME) is a substrate analog possessing an extremely high affinity for DDC [3]. In this study, the interaction of the enzyme with DME and other aromatic amino acid methyl esters has been extensively characterized. The use of these substrate analogs has permitted the fortuitous visualization of a quinonoid species and allowed for a critical analysis of the contribution of ligand side-chain interactions with the active site relative to the orientation and maintenance of the enzyme-ligand complex.

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2. Materials and methods

2.1. Materials

L-Dopa, DME, L-tyrosine methyl ester (free base) (TME), L-phenylalanine methyl ester (PME), and trypsin (type XIII from bovine pancreas; TPCK treated) were from Sigma. TME (hydrochloride) was also purchased from Fluka. All other chemicals were of the highest purity available. Recombinant Dopa decarboxylase was purified to homogeneity from *E. coli* expressing pKKDDCΔ4 as described [6] and was used throughout. When indicated, the enzyme used was purified from pig kidney [7]. Enzyme concentration was determined using a molar extinction coefficient of 1.30×10^5 M^{-1} cm^{-1} [8].

2.2. General

Enzyme activity was determined as described [9]. For all experiments potassium phosphate buffer was used at I_c=0.1, containing 10^{-4} M DTT. Absorbance spectra were taken with a Jasco V-550 spectrophotometer. CD spectra were obtained with a Jasco J-710 spectropolarimeter at a protein concentration of about 1 mg/ml using a 1 cm path length. Routinely, five spectra were recorded at a scan speed of 50 nm/min and averaged automatically. Limited proteolysis was performed as described [10]. All experiments were carried out at 25°C.

2.3. Data processing

Data were fit using the non-linear regression program MicroCal Origin (MicroCal Software, Inc.). For DME, the dissociation constant, K_d, was determined using Eq. 1 [11]. ΔA_{398} is the fractional absorbance change at 398 nm, ΔA_f, the saturation value of A, and n, the number of binding sites. Three parameters were fit: n, ΔA_f, and K_d. In all cases values of n approached unity, as expected for one binding site per monomer.

$$\Delta A_{398} = \Delta A_f/2(b-\sqrt{b\times b-4\times c}) \quad (1)$$

where $b = 1+([DME]+K_d)/n[DDC]$ and $c = [DME]/n[DDC]$

Typically, the enzyme concentration was about 10 μM, expressed as the monomer. For the other ligands, the K_d was determined using Eq. 2. The pK values obtained from the pH dependence of the K_d values were fit either to Eq. 3 or Eq. 4, describing one or two pK values respectively. $K_{d,lim}$ is the pH-independent value of K_d.

$$\Delta A_{500} = \Delta A_f[ligand]/(K_d + [ligand]) \quad (2)$$

$$K_d = K_{d,lim}(1 + 10^{pK-pH}) \quad (3)$$

$$K_d = K_{d,lim}[(1 + 10^{pK_1-pH})(1 + 10^{pK_2-pH})] \quad (4)$$

3. Results

3.1. Binding of DME to DDC

The addition of saturating concentrations of DME to the enzyme results in the immediate conversion of the coenzyme absorption bands at 335 and 425 nm to a stable external aldimine that absorbs at 398 nm ([3]; Fig. 1A). This is accompanied by the disappearance of the dichroic band at 425 nm and an extremely drastic reduction of the signal at 335 nm (Fig. 1B). Spectrophotometric titrations using the fractional

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absorbance changes at 398 nm were used to determine the dissociation constant for the DME-enzyme-PLP complex as a function of pH. As shown in Fig. 2, the resulting plot of K_d versus pH fit best to an equation describing two ionizations with an average of 7.3. (These pK values are too close together to permit an accurate estimation of their values). The pH-independent value of the DME dissociation constant was 50 nM.

3.2. *The TME-enzyme-PLP complex is an equilibrium between Schiff base and quinonoidal species*

The addition of TME to the enzyme leads to the immediate appearance of an external aldimine absorbing at 398 nm and reduction of the band at 335 nm. This was unexpectedly accompanied by a band with an absorbance maximum at 500 nm, indicative of a quinonoid species (Fig. 1). These absorbances remained unchanged for several hours. The 500 nm species was not dichroic and a slight negative Cotton effect was seen at around 400 nm in addition to the positive band at 335 nm (Fig. 1B). Several lines of evidence ruled out the possibility that the 500 nm species was an artifact. First, identical amounts of the species were observed among different enzymatic preparations, in different buffers, and with TME

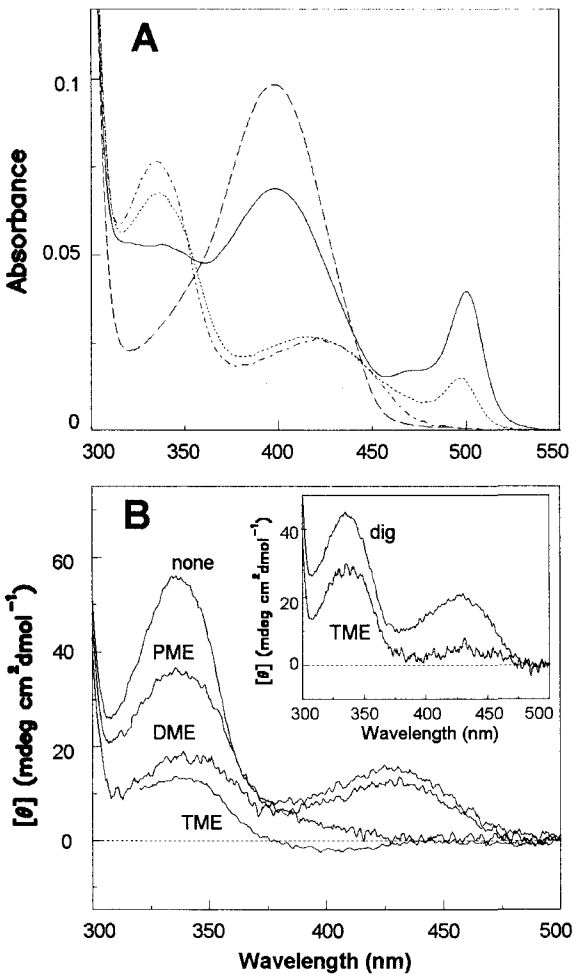

Fig. 1. (A) Absorbance spectra of 17 μM enzyme (– · –) and in the presence of 100 μM DME (– – –), 25 mM TME (——), or 25 mM PME (· · ·). (B) CD spectra were carried out as for the absorbance spectra and are as indicated. Inset: CD spectra of the nicked enzyme and in the presence of 25 mM TME as indicated.

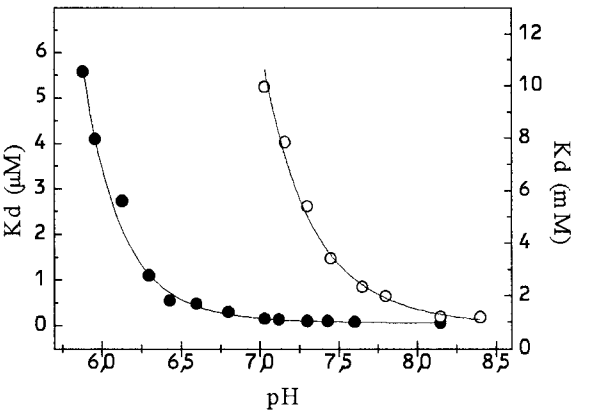

Fig. 2. Plots of K_d vs. pH for DME (●) and TME (○). Scale for the former is on the left-hand side and that for the latter on the right-hand side. The curves represent theoretical fits to the experimental points using Eq. 4. Fitting was also attempted using Eq. 3 but resulted in curves less compatible with the experimental data.

from different commercial sources. Second, the same species was observed in DDC prepared from different sources (i.e. recombinant vs. tissue-purified), and third, no 500 nm species was detected when TME was added to apoenzyme, to the $NaBH_4$ reduced enzyme, or when TME was mixed with PLP. Additionally, the 500 nm band has absorption characteristics typical of quinonoidal species, i.e. a shoulder at 27 nm on the low-wavelength side of the absorption maximum.

As for DME, similar spectrophotometric titrations following the 500 nm band were carried out as a function of pH. The resulting K_d values fit best to an equation describing two pK values with an average of 7.4 (Fig. 2) and a pH-independent value of 0.92 mM.

3.3. *L-Phenylalanine methyl ester also forms a quinonoidal species with DDC*

The addition of PME to DDC results in similar but much less-pronounced spectral changes as those seen with TME: the 325 nm band was slight reduced, the 425 nm absorbance was red-shifted, and a new absorbance at 498 nm appeared (Fig. 1A). The former two absorbance bands were associated with positive dichroic signals at the same wavelength (Fig. 1). PME binds in a less-dramatic pH-dependent manner than DME or TME (e.g. 1.0 mM at pH 8.15 and 2.7 mM at pH 7.08 for PME versus 1.2 mM at pH 8.15 and 10.4 mM at pH 7.03 for TME). The addition of 3,4-dihydroxyphenylacetic acid to the enzyme leads to the conversion of the internal aldimine to an absorption at 418 nm, attributed to a Michaelis complex [3]; its K_d was 35 μM and pH independent from pH 6–8.

3.4. *Binding of methyl ester analogs to nicked DDC*

Partial trypsinolysis of DDC leads to exclusive cleavage of the Lys^{334}–His^{335} peptide bond. This nicked protein possesses spectroscopic characteristics identical to the native enzyme, will bind aromatic amino acids, but is catalytically inactive [10]. When compared to the native enzyme, the addition of TME to the nicked protein results in a greater than 2-fold increase in the amount of the 500 nm species observed, accompanied by a decrease of the 398 nm species (Fig. 3) and a decrease of the positive dichroic signals (Fig. 1B, inset). At pH 7.6, its affinity for the nicked protein was roughly twice that for the native protein. When trypsin was added to the TME-

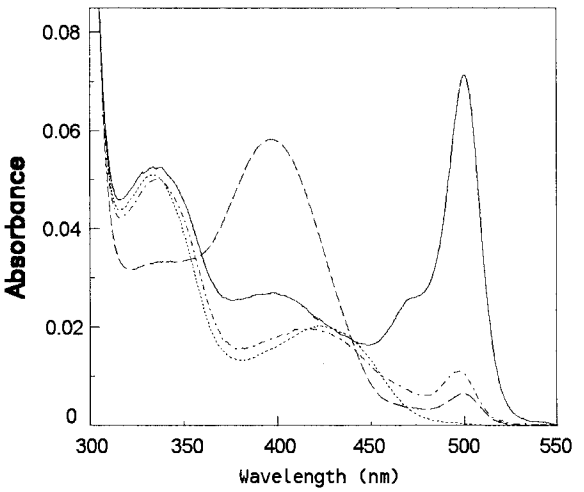

Fig. 3. Absorbance spectra of 12 μM enzyme treated with trypsin as described (· · ·) and in the presence of the same concentrations of ligands as indicated in Fig. 1. (– – –), DME; (———), TME; (– · –), PME. The spectrum for DME was taken 2.5 h after mixing.

enzyme complex, a time-dependent conversion of the 398 nm and the 500 nm bands was observed, leading to absorbance and CD spectra identical to those observed when TME is added to the proteolyzed enzyme (data not shown). Addition of PME to the nicked protein did not result in substantial spectroscopic differences when compared to the native protein and its affinity was unaltered. The DME-nicked enzyme complex shows a slow accumulation ($t_{1/2} = 66$ min) of a band absorbing at 500 nm (Fig. 3) and an extremely modest increase in the dichroic signal around 400 nm (data not shown). The affinity of this analog was increased about 10-fold with respect to the native enzyme.

4. Discussion

The visible absorbance and CD spectral changes that occur when these aromatic amino acid methyl ester analogs are added to native DDC indicate that they bind to the active site of the enzyme. Undoubtedly, their affinity for DDC is higher than that of the corresponding carboxyl derivatives; this is most evident when the affinity of DME is compared with that of L-Dopa [3]. It has been proposed that binding of the carboxyl group introduces a strain that is relevant for catalysis [3]. Similarly, Altson and Abeles [12] explained the tight binding of histidine methyl ester to *Lactobacillus* histidine decarboxylase by proposing that the carboxyl group is placed in a hydrophobic region and that, while this unfavorable interaction facilitates decarboxylation, it is no longer present with methyl ester derivatives. The comparison of the K_d for these analogs indicates that, while the 4′OH group has little if any effect on ligand affinity, the contribution of the 3′OH is impressive. In fact, the absence of 3′hydroxyl moiety results in a difference of 2×10^4 in the respective K_d for DME and TME and indicates that it is worth 5.8 kcal/mol.

Since none of the substrate analogs used in these studies has titratable moieties within the pH range examined, the observed pK values must represent the macroscopic ionization constants of enzyme side-chains which must be deprotonated for optimal Schiff base formation. The assignment of both of the ionizations involved in the binding of these ligands cannot be made with certainty. One of these ionizations can be most safely be attributed to an enzymic group involved in transimination. This unidentified residue with a pK value around 7.3 in the active site could accept a proton from the incoming α-amino group of the ligand which would then be competent to carry out nucleophilic attack of the C4′ internal Schiff base. In support of the above suggestion, the binding of 3,4-dihydroxyphenylacetic acid was observed to be pH independent.

It was surprising to find that the binding of these methyl ester analogs to DDC results in the appearance of an absorbance at 500 nm, in addition to an absorbance at 395 nm, attributed to quinonoidal and external aldimine species, respectively. To our knowledge, this is the first example of a quinonoidal species which has been observed in a PLP-dependent decarboxylase. From a chemical viewpoint, it would seem most likely that the proton on Cα is removed to give rise to the 500 nm band, as none of the other substituents on Cα would be good leaving groups. In this context the observed quinonoidal species would thus not be a catalytic intermediate on the mechanistic pathway for decarboxylation.

Despite the similarities in structure of these ligands, there are significant differences not only in their affinity, but also in the equilibrium mixtures of external aldimine and quinonoidal species and in their effect on the CD spectral properties of the bound PLP. As previously suggested [5], it is reasonable to believe that the amino acid side-chain of substrates is the directing group to an activated position. Under the plausible assumption that the side-chain of these ligands is oriented in the same relative position as the corresponding L-amino acids, these data suggest that this different behavior must necessarily reflect some differences in the interaction of the ligand side-chain with the enzyme and that the presence of both catecholic hydroxyl groups might be a driving force in establishing the proper alignment of catalytic groups with respect to the ligand-PLP complex. It follows then that specific interactions involving these hydroxyl groups affect covalent binding at the Schiff base linkage by imposing important constraints on the allowed orientation of the reacting ligand. It is proposed that this recognition triggers protein conformational transitions which would be obligatory during conversion of the internal aldimine to the external aldimine. This could then explain why binding of DME to native enzyme does not result in the appearance of the 500 nm band and why a quinonoid species appears upon binding of TME and PME. Thus, both the hydroxyl groups seem to be structural elements that are necessary in order to exclude or limit side reactions such as α-proton removal.

The different behavior of the nicked enzyme toward these methyl ester analogs can be explained by taking into account its inability to assume a catalytic conformation. In this case, DME induces a slow accumulation of the 500 nm band and TME favors the formation of this species at the expense of that absorbing at 398 nm. This indicates that even in the presence of both side-chain hydroxyl groups, binding does not occur as in the native enzyme. This is further demonstrated by the observations that (1) the nicked protein has a lower affinity for these ligands compared to the native enzyme and (2) upon formation of an external aldimine with TME, the coenzyme is in a different orientation in the native and nicked enzymes. The proposed enzymic transition accompanying the interconversion of the internal aldimine to the external aldimine would be impaired in the nicked enzyme, even in the

presence of both side-chain hydroxyl groups. The finding that the binding of PME to the nicked enzyme is unaltered with respect to the native enzyme is consistent with the above proposals.

In conclusion, these data confirm the importance of catecholic hydroxyl groups for optimal substrate binding and catalysis and suggest that the structure of the catecholic ring of aromatic amino acids may have significant effects on the preferred structures of intermediates in the reaction pathway catalyzed by DDC.

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