Ornithine and glutamate decarboxylases catalyse an oxidative deamination of their α -methyl substrates

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Ornithine decarboxylase (ODC) from *Lactobacillus* 30a catalyses the cleavage of α -methylornithine into ammonia and 2-methyl-1-pyrroline; glutamate decarboxylase (GAD) from *Escherichia coli* catalyses the cleavage of α -methylglutamate into ammonia and laevulinic acid. In our analyses, 2-methyl-1-pyrroline and laevulinic acid were identified by HPLC and mass spectroscopic analysis, and ammonia was identified by means of glutamate dehydrogenase. Molecular oxygen was consumed during these reactions in a 1:2 molar ratio with respect to the products. The catalytic efficiencies (k_{cat}/K_m) of the reactions catalysed by ODC and GAD were determined as 12500 and 9163 M⁻¹·min⁻¹ respectively. When the reactions were performed under anaerobic conditions, no ammonia, 2-methyl-1-pyrroline or laevulinic acid was produced to a significant extent. The formation of ammonia and O₂ consumption (in a 1:2 molar ratio with respect to

ammonia) were also detected during the reaction of ODC and GAD with putrescine and γ -aminobutyrate respectively. Taken together, these findings clearly indicate that ODC and GAD catalyse an oxidative deamination of their decarboxylation products, a reaction similar to that catalysed by dopa decarboxylase (DDC) with α -methyl-dopa [Bertoldi, Dominici, Moore, Maras and Borri Voltattorni (1998) *Biochemistry* 37, 6552–6561]. Furthermore, this reaction was accompanied by a decarboxylation-dependent transamination occurring for GAD, DDC and ODC with a frequency of approx. 0.24%, 1% and 9% respectively compared with that of oxidative deamination.

Key words: α -decarboxylases, pyridoxal 5'-phosphate, decarboxylation-dependent transamination.

INTRODUCTION

Decarboxylation-dependent transamination is considered to be an inactivating side reaction catalysed by many amino acid α -decarboxylases. The occurrence of this abortive reaction has indeed been described for glutamate decarboxylase (GAD; EC 4.1.1.15) [1–4], dopa decarboxylase (DDC; EC 4.1.1.28) [5–7], ornithine decarboxylase (ODC; EC 4.1.1.17) [8] and methionine decarboxylase [9,10]; it has been observed that, whereas the rate of decarboxylation of α -methyl substrates is much lower than that of natural substrates, the proportion of decarboxylations leading to transamination is much higher for the α -methyl substrates. It has been proposed that the retention of this minor reaction in pyridoxal 5'-phosphate (PLP)-dependent decarboxylases could be of some evolutionary advantage because it could provide the basis of a control mechanism. The mechanism by which this reaction is presumed to occur entails an incorrect protonation of the quinonoid intermediate (at C-4') that is formed by decarboxylation of the bound PLP–substrate Schiff base [6]. This protonation leads to an inactive form of the enzyme. Thus this side reaction seems to be an inevitable consequence of the decarboxylation mechanism, resulting from the inability of the enzyme to control with absolute specificity the site of protonation of the quinonoid intermediate.

However, quantitative determinations of pyridoxamine 5'-phosphate (PMP) and 3,4-dihydroxyphenylacetone or 5-hydroxyindolaldehyde, the putative transamination products of the reaction of DDC with α -methyl-dopa or serotonin re-

spectively, were clearly not compatible with the mechanism previously proposed [11]. In fact, we have recently demonstrated that DDC catalyses under aerobic conditions an oxidative deamination of aromatic amines with the production of ketone or aldehyde and ammonia in equivalent amounts, and the consumption of molecular oxygen in a 1:2 molar ratio with respect to the products [12,13].

To learn whether the oxidative deamination reaction is operative for other α -decarboxylases, we performed qualitative and quantitative analyses of the products of the reactions of ODC from *Lactobacillus* 30a with α -methylornithine and of GAD from *Escherichia coli* with α -methylglutamate. The products and the stoichiometry of these reactions have been extensively characterized. The results obtained show that ODC and GAD catalyse an oxidative deamination identical with that already described for DDC with α -methyl-dopa [13]. Moreover, our results support the occurrence of a concomitant half-transamination reaction catalysed by DDC, ODC and GAD. The partition ratio between oxidative deamination and transamination for these α -decarboxylases is reported.

MATERIALS AND METHODS

Materials

L-Dopa, L- α -methyl-dopa, L-ornithine, α -methylornithine, putrescine, L-glutamate, α -methyl-D,L-glutamate, laevulinic acid,

Abbreviations used: GAD, glutamate decarboxylase; DDC, dopa decarboxylase; ODC, ornithine decarboxylase; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate.

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PLP, bovine liver L-glutamic dehydrogenase (EC 1.4.1.3), gabase (the enzyme system 4-aminobutyrate:2-oxoglutarate aminotransferase + succinate semialdehyde NAD(P) oxidoreductase) from *Pseudomonas fluorescens* (EC 2.6.1.19; EC 1.2.1.16), Hepes, Mes and α -ketoglutarate were Sigma products. 2-Methyl-1-pyrroline was purchased from Aldrich. Dichloromethane and chloroform were obtained from J. T. Baker. Diethyl ether and trifluoroacetic acid were purchased from Carlo Erba. Diazomethane was generated in our laboratory from *N*-methyl-*N*-nitroso-*p*-toluenesulphonamide with a Diazald kit (Sigma-Aldrich). All other chemicals were of the highest purity available.

Purification of enzymes

DDC [12,14] ODC [15] and GAD [16] were purified as described previously. Protein concentrations (expressed as subunit concentration) were determined by absorbance, taking ϵ_{280} as $1.3 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for DDC dimer, ϵ_{412} as $5.4 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the ODC monomer and ϵ_{420} as $10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the GAD monomer.

Enzyme and inactivation assays

The reaction mixtures were prepared as follows: (1) 8 μM ODC was incubated with 0.2 or 10 mM α -methylornithine in 50 mM Hepes at 25 °C at pH 5.8; (2) 6.3 μM GAD was incubated with 0.5 or 10 mM α -methylglutamate in 50 mM Mes at 25 °C at pH 4.6. The production of ammonia by these reactions was determined with glutamate dehydrogenase, which forms glutamate from α -ketoglutarate and ammonia with the concomitant conversion of NADH to NAD⁺. Aliquots were removed from reaction mixtures at intervals. The reaction catalysed by ODC was stopped by heating the mixture at 100 °C for 2 min followed by centrifugation and adjustment of the supernatant to pH 7.5 with NaOH; the reaction catalysed by GAD was stopped by adjusting the pH of the enzymic solution to 8.6. Then aliquots of the reaction mixtures were added to a solution containing 1400 μg of glutamate dehydrogenase and 300 μM NADH in 50 mM Hepes, pH 7.5, and the decrease in $A_{340} \text{ nm}$ was measured. The α -methylornithine and α -methylglutamate concentrations were varied from 0.05 to 10 mM and from 1 to 10 mM respectively when kinetic parameters were determined.

The production of ammonia was also measured under anaerobic conditions with 1 ml Reacti-vials (Aldrich) as described previously [13].

O₂ consumption during the reaction of ODC with α -methylornithine or of GAD with α -methylglutamate under the experimental conditions described above was recorded with a Yellow Springs electrode (Yellow Springs Instruments, RDP Corporation, Dayton, OH, U.S.A.). The reaction was performed at 25 °C in air-saturated 50 mM Hepes, pH 5.8, for ODC, and in air-saturated 50 mM Mes, pH 4.6, for GAD.

H₂O₂ was measured as described previously [12].

Detection of 2-methyl-1-pyrroline, laevulinic acid and PMP by HPLC

The separations of 2-methyl-1-pyrroline and laevulinic acid produced during the reaction of ODC with α -methylornithine and of GAD with α -methylglutamate respectively were done isocratically on a 5 μm LiChrospher column 100 RP-18 (4.6 mm $\times$ 100 mm; Merck) for ODC and on a 7 μm Aquapore RP-300 column (4.6 mm $\times$ 250 mm, Brownlee[®] columns; Applied Biosystems) for GAD, with a Waters 625 LC system coupled with a Waters 745 B Data module (Millipore). For 2-methyl-1-pyrroline the solvent was water containing 0.05 %

(v/v) trifluoroacetic acid at a flow rate of 0.6 ml/min; detection was at 220 nm. For laevulinic acid the solvent was 50 mM ammonium acetate, pH 6.3, at a flow rate of 0.8 ml/min; detection was at 270 nm. ODC (8 μM) was incubated with 0.2 or 10 mM α -methylornithine in 50 mM Hepes, pH 5.8, at 25 °C. Aliquots were removed at intervals. The reaction was stopped by heating for 2 min at 100 °C and then centrifuged to remove the precipitated protein. Supernatants and appropriate blanks were run. GAD (6 μM) was incubated with 0.5 or 10 mM α -methylglutamate in 50 mM Mes, pH 4.6, at 25 °C. Aliquots were removed at intervals and HClO₄ was added to a final concentration of 10 % (w/v). Samples were then centrifuged and loaded directly, together with the appropriate blanks. For quantification, the areas under the curves were measured and converted to absolute amounts by using standard reference curves.

The PLP and PMP contents during the reactions of DDC (50 mM Hepes, pH 7.5), ODC (50 mM Hepes, pH 5.8) and GAD (50 mM Mes, pH 4.6) with their α -methyl substrates were analysed with the HPLC method described previously [17]. The standard curve of peak area as a function of the concentration of coenzymes was prepared with the use of commercially available PLP and PMP.

MS

GLC-MS analyses of 2-methyl-1-pyrroline and laevulinic acid standards or obtained by reaction of ODC with α -methylornithine and of GAD with α -methylglutamate respectively were performed by using a TRIO 2000 GLC-electron ionization-MS apparatus equipped with a gas-liquid chromatograph GC 8000 series (Micromass, Manchester, U.K.) fitted with a capillary column (30 m $\times$ 0.25 mm internal diam., 25 μm film thickness) DB-5 (J&W Scientific, Folsom, CA, U.S.A.).

After 3 h of reaction of ODC with α -methylornithine, a few microlitres of 0.02 M NaOH was added to the enzymic mixture to increase the pH from 5.8 to 7.5. The mixture was extracted twice with 1 ml of chloroform. The sample was dried under a stream of nitrogen, then resuspended in 20 μl of chloroform; 1 μl of the suspension was injected into the gas-liquid chromatograph. A solution of authentic 2-methyl-1-pyrroline was subjected to the same treatment.

After 3 h of reaction of GAD with α -methylglutamate, the pH of the enzymic mixture was decreased to 3 by adding a few microlitres of 0.1 % (v/v) trifluoroacetic acid; the solution was extracted twice with 1 ml of diethyl ether. The organic phase was dried under a stream of nitrogen and treated at room temperature for 5 min with 500 μl of diazomethane. The solution was dried, then resuspended in 20 μl of dichloromethane; 1 μl of the suspension was injected into the gas-liquid chromatograph. A solution of authentic laevulinic acid was subjected to the same treatment.

The analysis of 2-methyl-1-pyrroline was performed with the following analytical conditions: injection was in a splitless mode with an injector temperature of 250 °C, an ion source temperature of 200 °C and a GLC-MS interface temperature of 250 °C; the temperature of the GC column was programmed from 40 to 150 °C at 10 °C/min after an initial period of 5 min. Mass spectrometric analysis was performed at 70 eV ionization energy; the mass range 40–250 mass units was analysed with a scan time of 0.6 s. Similar analytical conditions were used to analyse laevulinic acid; the temperature of the capillary column was raised from 50 to 280 °C at 12 °C/min after an initial period of 10 min.

RESULTS

As shown in Figures 1 and 2, equivalent amounts of ammonia and 2-methyl-1-pyrroline or laevulinic acid are produced during the reactions of 6 μ M ODC with 0.2 mM α -methylornithine or 6.3 μ M GAD with 0.5 mM α -methylglutamate respectively. Whereas ammonia production was detected by using a coupled assay with glutamate dehydrogenase, 2-methyl-1-pyrroline and laevulinic acid were detected on a reverse-phase column by

Figure 1 Formation of 2-methyl-1-pyrroline and ammonia and O₂ consumption during the reaction of ODC with α -methylornithine

ODC (8 μ M) was incubated with 0.2 mM α -methylornithine in 50 mM Hepes, pH 5.8, at 25 °C. For 2-methyl-1-pyrroline determination, samples of 220 μ l were withdrawn at the indicated times and heated for 2 min at 100 °C. After removal of the precipitated protein by centrifugation, the supernatants were subjected to HPLC and the areas under the peak were converted to absolute amounts, as described in the text. Symbols: ∇ , 2-methyl-1-pyrroline; $\blacktriangledown$, ammonia, measured by the glutamate dehydrogenase assay (as described in the Materials and methods section); $\bullet$, O₂ consumed. Abbreviation: E, enzyme.

Figure 2 Formation of laevulinic acid and ammonia and O₂ consumption during the reaction of GAD with α -methylglutamate

GAD (6.5 μ M) was incubated with 0.5 mM α -methylglutamate in 50 mM Mes, pH 4.6, at 25 °C. For laevulinic acid determination, samples of 220 μ l were withdrawn at the indicated times and HClO₄ was added. After removal of the precipitated protein by centrifugation, the supernatants were subjected to HPLC and the areas under the peak were converted to absolute amounts, as described in the text. Symbols: ∇ , laevulinic acid; $\blacktriangledown$, ammonia, measured by the glutamate dehydrogenase assay (as described in the Materials and methods section); $\bullet$, O₂ consumed. Abbreviation: E, enzyme.

HPLC; the latter compounds were identified by co-elution with the corresponding standards. In addition, the identification of 2-methyl-1-pyrroline and laevulinic acid was achieved by GC/MS analyses, which revealed the presence in these enzymic reaction mixtures of compounds whose retention times and mass spectra were identical with those of the corresponding authentic standards (results not shown). Furthermore, molecular oxygen was consumed during the reaction of ODC with α -methylornithine (Figure 1) and of GAD with α -methylglutamate (Figure 2) in a 1:2 molar ratio with respect to the products of the reactions. No H₂O₂ or PMP was detected during these reactions. Under anaerobic conditions, the reactions of ODC with α -methylornithine and of GAD with α -methylglutamate produced ammonia (and 2-methyl-1-pyrroline or laevulinic acid respectively) in amounts less than 5% with respect to those found under aerobic conditions.

The initial velocity of the reaction catalysed by ODC and GAD was dependent on their α -methyl substrate concentrations according to saturation kinetics (results not shown). The K_m values ($\pm$ S.D.) for α -methylornithine and for α -methylglutamate were determined as 0.092 ± 0.016 and 1.04 ± 0.27 mM respectively, with k_{cat} values of 1.15 ± 0.04 and 9.53 ± 0.63 min⁻¹ for monomeric ODC and GAD respectively. Thus the calculated k_{cat}/K_m was 12500 M⁻¹·min⁻¹ for ODC and 9163 M⁻¹·min⁻¹ for GAD.

The identities of the products and the stoichiometries of the reactions catalysed by ODC and GAD on their α -methyl substrates parallel those previously reported for the reaction of DDC with 100 μ M α -methyltyrosine [13] but seem to contradict many observations, from other laboratories, that are consistent with a mechanism of decarboxylation-dependent transamination [1–10]. Although the authors of most of these papers assume rather than demonstrate that PLP is converted into PMP, the direct demonstration of PMP formation and the determination of the stereochemistry of the proton transfer occurring during the reaction of GAD and methionine decarboxylase with their respective α -methyl substrates have been reported [4,9]. Because the concentration of α -methyl substrates used in the above cited papers was higher (10–25 mM) than that in our experiments, we decided to examine the products of the reactions catalysed by GAD, ODC and DDC by using their α -methyl substrates at a concentration of 10 mM. The reactions were characterized by a time-dependent production of ammonia and ketone, accompanied by a decrease in PLP and a concomitant increase in PMP concentration (results not shown). Thus, under the experimental conditions that we used, a half-transamination was detected in addition to the oxidative deamination. The rate constant of PMP formation for DDC, ODC and GAD has been determined (Table 1). An estimate of the ratio of oxidative deamination to decarboxylation-dependent transamination can be obtained by comparing the k_{cat} of oxidative deamination with the time course of PMP formation. The values obtained were 90:1, 412:1 and 12:1 for DDC, GAD and ODC respectively (Table 1).

The formation of ammonia was also revealed during the reaction of ODC with 10 mM putrescine at pH 5.8 (0.25 nmol of ammonia/min per nmol of enzyme) as well as during the reaction of GAD with 100 mM γ -aminobutyrate at pH 4.6 (0.5 nmol of ammonia/min per nmol of enzyme), whereas O₂ was consumed in a 1:2 molar ratio with respect to ammonia (results not shown).

DISCUSSION

As claimed previously, DDC, ODC and GAD catalyse, in addition to decarboxylation, a decarboxylation-dependent transamination leading to reversible inactivation. However, this side

Table 1 Rate constants for the oxidative deamination and the half-transamination of DDC, ODC and GAD in the presence of their α -methyl substrates

k_{obs} is the rate constant for the conversion of PLP into PMP at saturating concentrations of α -methyl substrate.

Enzyme	Oxidative deamination, k_{cat} (min^{-1})	Half-transamination, k_{obs} (min^{-1})	Partition ratio (deamination/transamination), $k_{\text{cat}}/k_{\text{obs}}$
DDC	2.84*	0.0315	90
ODC	1.15	0.0924	12
GAD	9.53	0.0231	412

* As determined in [13].

reaction occurs less frequently (from 0.2% to 9%) than another side reaction catalysed by these α -decarboxylases. In fact, our results clearly indicate that ODC and GAD catalyse the decarboxylation of their α -methyl substrates and the following oxidative deamination of their decarboxylation products, similarly to that already observed for DDC [12,13]. The finding of 2-methyl-1-pyrroline, instead of 5-amino-2-pentanone, the product expected from the oxidative deamination of the decarboxylation product of α -methylornithine, can be explained by the fact that the ketone exists primarily as the cyclic imine [8]. For both these α -decarboxylases 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. This oxidation side reaction could have physiological relevance in that it might provide a key step in the control of amine levels in cells.

It should be noted that an ornithine oxidase activity catalysed by ODC from *Hafnia alvei* has been reported recently by Sakai et al. [18]. However, these authors found that, in addition to CO_2 , H_2O_2 was formed in equimolar amounts with ammonia and γ -aminobutyraldehyde among the products of the reaction of the enzyme with L-ornithine. Moreover, putrescine oxidase activity was undetectable.

It is noteworthy that DDC, ODC and GAD belong to the fold-type I superfamily [19,20]. This suggests that they share an as yet unidentified structural basic motif that is responsible for this new catalytic property of the coenzyme. In fact, among PLP-dependent reactions, the oxidative deamination catalysed by these enzymes seems unique: this is the first evidence that a PLP-dependent enzyme catalyses such an oxidation without the simultaneous reduction of the coenzyme to its amine form. With each transaminase half-reaction, PLP undergoes a net two-electron reduction or oxidation, depending on the direction. The reaction of α -decarboxylases with amines yields a product that has undergone a two-electron oxidation at $\text{C}\alpha$: amine or α -methylamine is thus oxidized to the corresponding aldehyde or ketone respectively, with the release of ammonium ions. Because the oxygen-dependent nature of the chemistry performed by these enzymes is evident, O_2 must have an essential role even if the real factor controlling the O_2 reactivity in these enzymes remains unknown. Although the crystal structure of only *Lactobacillus* 30a ODC has so far been determined [21], there is no evidence for the presence of redox-active metals or redox cofactors in these enzymes. This fact, together with the lack of H_2O_2 as a product of the reaction, makes it very difficult to establish how this reaction might proceed with molecular oxygen.

Among oxygen-utilizing enzymes, the chemical mechanism of the urate oxidase reaction has not been established; this is particularly interesting because the enzyme neither contains nor requires a cofactor, as confirmed by the recently determined crystal structure of *Aspergillus flavus* urate oxidase complexed with azaxanthine [22]. At present, the chemical mechanism suggested for the oxidation of serotonin by DDC [12], in which O_2 acts as an electron acceptor from an intermediate of the reaction, must be considered only an attractive possibility. For the future, experiments will be focused on an extensive kinetic analysis of the mechanism of the reaction described here.

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