Chemical mechanism of D-amino acid oxidase from *Rhodotorula gracilis*: pH dependence of kinetic parameters

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The variation of kinetic parameters of D-amino acid oxidase from *Rhodotorula gracilis* with pH was used to gain information about the chemical mechanism of the oxidation of D-amino acids catalysed by this flavoenzyme. D-Alanine was the substrate used. The pH dependence of $V_{\max}$ and $V_{\max}/K_m$ for alanine as substrate showed that a group with a pK value of 6.26–7.95 (pK₁) must be unprotonated and a group with a pK of 10.8–9.90 (pK₂) must be protonated for activity. The lower pK value corresponded to a

group on the enzyme involved in catalysis and whose protonation state was not important for binding. The higher pK value was assumed to be the amino group of the substrate. Profiles of pK_i for D-aspartate as competitive inhibitor showed that binding is prevented when a group on the enzyme with a pK value of 8.4 becomes unprotonated; this basic group was not detected in $V_{\max}/K_m$ profiles suggesting its involvement in binding of the β -carboxylic group of the inhibitor.

INTRODUCTION

D-Amino acid oxidase (DAAO, EC 1.4.3.3) from the yeast *Rhodotorula gracilis* catalyses the oxidative deamination of D-amino acids to give the corresponding α -keto acids, hydrogen peroxide and ammonia. The enzyme from *Rhodotorula gracilis* is a dimer of identical subunits and contains one molecule of tightly, non-covalently bound FAD per monomer [1].

Recently, the amino acid sequence of the enzyme has been determined [2]. The enzyme has 368 amino acids and some of them have been suggested to play a role in binding and/or catalysis. To date, Arg²⁸⁵, a well conserved residue in all DAAO has been proposed to be involved in binding either the carboxyl group of the substrate or the negative charge close to the N(1)–C=O(2) locus of the flavin [3]. His³²⁹ has been suggested to play a role in the α -proton abstraction from the substrate as in pig kidney enzyme [2,4,5]. Among the other residues proposed to be in the active site of pig kidney DAAO by chemical modification, only Tyr²²⁸ and His³⁰⁷ (Tyr²²³ and His³²⁹ in *R. gracilis*) are conserved in *Rhodotorula gracilis* DAAO [2]. Moreover, the enzyme contains two aspartic residues conserved in all DAAO, namely Asp⁴⁰ and Asp¹⁹¹. In glycolate oxidase, another flavo-protein oxidase, an aspartic residue was proposed to play a role in the catalytic mechanism of the enzyme as a part of a charge relay involving the histidine responsible for proton abstraction of the substrate [6].

Kinetically, DAAO from *Rhodotorula gracilis* has been extensively characterized [7]. The rate determining step in the kinetic mechanism corresponds to the reductive half-reaction in contrast to the enzyme from pig kidney, where the slow step is product release from the oxidized enzyme [8]. Whereas DAAO from *Rhodotorula gracilis* is extensively characterized, little use has been made of the pH dependence of steady-state kinetic parameters, which provides information about the ionizable groups involved either in substrate binding or catalysis [9]. The present paper reports evidence about the chemical mechanism of DAAO from *Rhodotorula gracilis* by examining the pH dependence of kinetic constants of the enzyme and the variation with pH of K_i for D-aspartic acid as a competitive inhibitor.

MATERIALS AND METHODS

Materials

D-Alanine, D-aspartic acid, and 2,4-dinitrophenylhydrazine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents and solvents were of analytical grade and purchased from Merck (Darmstadt, Germany).

Enzyme purification

D-Amino acid oxidase was isolated and purified from *Rhodotorula gracilis* (American Type Culture Collection, strain number 26217) cultures as previously described [4].

Kinetic assays

The activity of D-amino acid oxidase was determined at 35 °C in 100 μ l air saturated incubation mixtures containing 87.5 ng of enzyme and 10 mM D-alanine in 50 mM potassium phosphate buffer at pH 8.5. The released pyruvic acid was determined after 10 min by reacting with 2,4-dinitrophenylhydrazine and the corresponding hydrazone was monitored at 450 nm. The product formation proceeded linearly with time for this period under all the conditions used in kinetic experiments.

The pH dependence of the kinetic parameters was determined in phosphate/pyrophosphate (neutral acid) or Mes/bis-Tris-propane (cationic) 50 mM in the temperature range 20–45 °C. The enzyme was stable in the temperature and pH range assayed. The ionic strength at each pH was adjusted to 250 mM by addition of NaCl in amounts calculated using a Visual Basic program developed in our laboratory, which allows analysis of buffer systems with up to four tetraprotic species. Solvent perturbation studies with 20 % DMSO were performed in both neutral acid and cationic buffer systems. The organic solvent was added after determination of the pH in the assay mixture. DMSO (20 %) caused no loss of enzyme activity. The pH dependence of K_i was analysed using D-aspartic acid as a competitive inhibitor of DAAO. Assays were run at variable

Abbreviation used: DAAO, D-amino acid oxidase.

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concentrations of D-alanine (0.1–10 mM) with D-aspartic acid varied from 0 to 13 mM, at 35 °C for 10 min. Results in Figures and Tables are the mean value of triplicate experiments.

Statistical analysis of data

Values of kinetic constants were determined by fitting initial velocity data to the Woolf–Augustinsson–Hoffstee equation [10] by a weighted least-squares method developed in our laboratory. Substrate saturation kinetics were fitted to eqn. (1). Competitive inhibition data were fitted to eqn. (2).

$$v = \frac{V_{\max} S}{K_m + S} \quad (1)$$

$$v = \frac{V_{\max} S}{K_m(1 + I/K_i) + S} \quad (2)$$

Data for pH profiles showing a decrease in $\log V$ or $\log V/K$ with a slope of 1 as the pH is lowered and a slope of -1 as it is raised were fitted to eqn. (3).

$$Y = \frac{C}{(1 + [H^+]/K_1) + (K_2/[H^+])} \quad (3)$$

The enthalpies of ionization of enzyme residues were determined by fitting the pK values obtained from pH profiles at different temperatures to eqn. (4).

$$pK = \frac{\Delta H_{\text{ion}}}{2.3 \cdot R \cdot T} \quad (4)$$

pK_i for D-aspartic acid as competitive inhibitor was calculated by fitting the data from the corresponding pH profiles to eqn. (5).

$$Y = \frac{C}{1 + K_2/[H^+]} \quad (5)$$

In eqns. (1) and (2), v and $V_{\max}$ are initial and maximum velocity respectively, K_m is the Michaelis constant, S is the substrate concentration, I is the inhibitor concentration and K_i is the inhibition constant. In eqns. (3) and (5), K_1 and K_2 are dissociation constants of groups that must be deprotonated and protonated respectively for activity, Y is $V_{\max}$ or $V_{\max}/K_m$ and C is the value of Y attained at the optimum state of protonation. In eqn. (4), ΔH_{ion} is the enthalpy change of dissociation, R the gas constant and T the absolute temperature.

D-Alanine gave parallel line patterns in double reciprocal plots indicating that the steady-state rate equation is eqn. (6).

$$V = \frac{V[AB]}{K_a[B] + K_b[A] + [AB]} \quad (6)$$

RESULTS

Previous kinetic studies on DAAO from *Rhodotorula gracilis* were consistent with the kinetic mechanism shown in Scheme 1 [7]. The sequential mechanism represented by loop B best described the experimental results for neutral amino acids [7].

According to previous results [7,11] with D-alanine as substrate, initial rate data were best fitted to eqn. (6) which describes a parallel line pattern consistent with the reductive half-reaction being irreversible ($K_2 = 0$ in scheme 1) (results not shown). The finding of a parallel line pattern in double reciprocal plots is a diagnostic of a binary complex mechanism (Ping-Pong) or a limiting case of a ternary complex mechanism where some specific rate constants are sufficiently small.

Scheme 1

Figure 1 $V_{\max}$ (a) and $V_{\max}/K_m$ (b) profiles of the reaction of DAAO with D-alanine

Individual data points represent fits of initial velocities at saturating O_2 and varying D-alanine concentrations to eqn. (1). The curve through the data is an iterated fit to eqn. (3).

The $V_{\max}$ and $V_{\max}/K_m$ values for alanine were determined in the pH range 5.5–10.5. Figure 1 shows the pH variation of $V_{\max}$ and $V_{\max}/K_m$. The maximum velocity decreased both at low and

Figure 2 Temperature dependence of pK values from $V_{\max}$ (a) and $V_{\max}/K_m$ (b) profiles

Experiments similar to those in Figure 1 were carried out at four additional temperatures and the calculated values of pK_1 (●) and pK_2 (○) were plotted.

high pH with limiting slopes of 1 and -1 respectively and the data were fitted to eqn. (3) (Figure 1a). Data indicated that a group with an apparent pK value of 6.26 ± 0.04 (pK_1) must be unprotonated and a group with an apparent pK value of 10.80 ± 0.15 (pK_2) must be protonated for activity. The $V_{\max}/K_m$ profile (Figure 1b) showed a similar shape; the data indicated an apparent pK of 7.95 ± 0.05 (pK_1) on the acidic side and an apparent pK of 9.90 ± 0.15 (pK_2) on the basic side. When the observed pK s from the $V_{\max}/K_m$ profile were corrected by assuming that only the zwitterionic form of the substrate was active, the pK value on the acidic side was unaltered whereas no pK could be observed on the basic side, indicating that the amino group of the substrate was responsible for the observed pK_2 .

To gain information about the groups whose protonation state affects activity, the temperature dependence of $V_{\max}$ and $V_{\max}/K_m$ profiles was examined (Figure 2). Experiments were carried out at 20, 32, 35, 40 and 45 °C. No irreversible thermal inactivation was observed at these temperatures in the pH range used. Data were fitted to eqn. (4) in order to obtain the corresponding ionization enthalpies (ΔH_{ion}):

$$\Delta H_{\text{ion}} = 2.77 \pm 0.37 \text{ kcal/mol } (pK_1) \text{ and}$$

$$\Delta H_{\text{ion}} = 6.08 \pm 0.25 \text{ kcal/mol } (pK_2) \text{ from } V_{\max} \text{ profiles}$$

Table 1 Effect of 20% (v/v) DMSO on pK values in either neutral or cationic acid buffers

pK values and their standard deviations were obtained by statistical analysis of the parameter data in accordance with eqn. (3).

	Neutral buffer		Cationic buffer	
	– DMSO	+ DMSO	– DMSO	+ DMSO
$\log V_{\max}$				
pK_1	6.59 ± 0.05	6.37 ± 0.02	6.78 ± 0.06	6.74
pK_2	10.40 ± 0.20	—	10.52 ± 0.28	—
$\log V_{\max}/K_m$				
pK_1	7.84 ± 0.06	7.09 ± 0.08	7.38 ± 0.08	7.39 ± 0.13
pK_2	9.98 ± 0.18	10.27 ± 0.32	9.93 ± 0.07	10.06 ± 0.18

Figure 3 Variation of pK_i with pH for D-aspartic acid

Experiments were carried out as in Figure 1 with D-aspartic acid varied from 0 to 13 mM. K_i values were calculated by fitting data to eqn. (2) and pK_i values were plotted versus pH and fitted to eqn. (5) with correction for the ionization of inhibitor ($pK_{\text{amino}} = 9.82$).

$$\Delta H_{\text{ion}} = -5.07 \pm 0.32 \text{ kcal/mol } (pK_1) \text{ and}$$

$$\Delta H_{\text{ion}} = 11.07 \pm 0.70 \text{ kcal/mol } (pK_2) \text{ from } V_{\max}/K_m \text{ profiles}$$

Further evidence on the nature of the catalytic groups was obtained by analysing the effect of organic solvents on the pK values in both neutral and cationic acid buffers (Table 1). Addition of 20% DMSO caused a decrease in the pK_1 value observed in neutral acid buffer, whereas this pK remained unchanged in cationic acid buffer, indicating that the group responsible for this pK is the cationic acid type. On the other hand, the addition of 20% DMSO removed the observed pK_2 in the $V_{\max}$ profile either in neutral and cationic acid buffers and did not change significantly in the $V_{\max}/K_m$ profile.

The pK_i profile for an inhibitor shows the effect of ionizations on binding only and has the virtue of giving correct pK values [12]. $V_{\max}$ and $V_{\max}/K_m$ profiles show the effect of ionizations on catalysis as well as on binding and thus a comparison of the two types of profiles is very useful to identify the groups responsible for catalysis as opposed to those only involved in binding. The pH dependence of K_i was studied with D-aspartate as a competitive inhibitor of the reaction with D-alanine (Figure 3). Data were fitted to eqn. (5); the pK_i value was 8.40 ± 0.17 indicating

that a group with a pK of 8.4 must be protonated for the inhibitor to bind. The observed pK has been corrected for the ionization of the inhibitor.

DISCUSSION

The plots of $\log V_{\max}/K_m$ and $\log V_{\max}$ showed changes in slope at both low and high pH. The one at low pH corresponds to a pK value of 6.26 in the $V_{\max}$ profile and 7.95 in the $V_{\max}/K_m$ profile. Therefore, a group on the enzyme or in the substrate must be unprotonated for activity. As the substrate has no ionizable group with this pK value, it follows that a group on the enzyme is essential for activity. The results of solvent perturbation studies (Table 1) indicated that this group is a cationic acid. However, the corresponding ΔH_{ion} points to a neutral acid. There are two aspartic residues conserved in DAAO isolated from all species thus far, namely Asp⁴⁰ and Asp¹⁹¹ [2]. In glycolate oxidase, another flavoprotein oxidase, an aspartic residue is proposed to play a role in the catalytic mechanism of the enzyme as a part of a charge relay involving the histidine responsible for proton abstraction from the substrate [6]. Recently [4], we have shown the existence of an essential histidine in the active site of DAAO from *Rhodotorula gracilis*; it would be possible to postulate one of two conserved aspartic residues as part of a charge relay similar to that proposed for glycolate oxidase. Results of solvent perturbation studies presented here indicated a cationic group as being responsible of pK_1 , although ΔH_{ion} of the group pointed to a carboxylic residue. It is possible that the observed pK may be the result of several contributions from groups of different types. In this sense, for the carboxylate-imidazole system of chymotrypsin, the proton is added to the side of the histidine that is accessible to water and the net charge of the complex is that of a neutral acid. Thus, the test with organic solvent points to a cationic acid, whereas the value of ΔH_{ion} corresponds to a carboxylic residue [13–15]. The difference in ΔH_{ion} values from $V_{\max}$ and $V_{\max}/K_m$ profiles can be ascribed to the solvation of the acidic group. When ES is formed, it decreases the solvation of the carboxylic residue, increasing ΔH_{ion} of the group with respect to the value seen in the $V_{\max}/K_m$ profile, which corresponds to that of a group with higher accessibility to water on the free enzyme [16].

When the $V_{\max}/K_m$ profile in Figure 1 is compared with the pK_1 profile in Figure 3, it is clear that the pK_1 observed in the $V_{\max}/K_m$ profile reflects the ionization of a group responsible for catalysis. There is no sign of a pK of 6.5–8 in the pK_1 profile and thus the protonation state of this group is not important for binding. This is somewhat surprising since this group appears to be one involved in the abstraction of the proton from the substrate and thus one would expect it to have some effect on binding. It is possible that D-aspartate does not bind exactly in the same manner as D-alanine does.

The pK_1 from the $V_{\max}$ profile in Figure 1 is displaced outward by about 1.7 pH units from the value seen in the corresponding $V_{\max}/K_m$ profile, which presumably represents the true pK value of the enzyme taking into account that D-alanine is not a sticky substrate for DAAO from *Rhodotorula gracilis* [7]. This displacement suggests that binding of the substrate favours the release of the corresponding proton.

With respect to the identity of the group responsible for pK_2 , results are more difficult to interpret. Nevertheless it could be ascribed to the amino group of the substrate provided that no pK_2 was observed in $V_{\max}/K_m$ profile when correction for the ionization of the substrate was performed. Addition of 20 %

DMSO resulted in a decrease in K_m , suggesting a tighter binding of the substrate to the enzyme and supporting an ionic interaction between enzyme and substrate and the outward shift in pK_2 seen in the $V_{\max}$ profile. Nevertheless, when a non-pH-dependent slow step follows the catalytic reaction and is rate limiting (e.g. the release of a product), the pK values are displaced outwards in the $V_{\max}$ profile to the point where the catalytic reaction becomes rate limiting. This phenomenon does not affect the $V_{\max}/K_m$ profile which reflects steps only through the release of the first product; thus, the release of the product (pyruvic acid) as rate limiting in the presence of solvent cannot be ruled out. Since both $V_{\max}$ and $V_{\max}/K_m$ decrease above pK_2 , D-alanine binds to the enzyme both in the protonated and unprotonated form, although the enzyme prefers to bind the former [12]. Values of ΔH_{ion} point to an amino group but the results of solvent perturbation studies do not allow definitive identification of the ionizing group, provided that pK_2 from $V_{\max}/K_m$ profiles does not change significantly by addition of 20 % DMSO, either in neutral or cationic acid buffers. This result could be consistent with the increase in catalytic efficiency in the presence of 20 % DMSO at basic pH values.

The inhibitor binding (Figure 3) is prevented when a group with a pK of 8.4 is deprotonated. Since this pK value does not correspond to D-aspartate it must represent a group on the enzyme. As this pK is not also seen in the $V_{\max}/K_m$ profile, it could represent a group in the enzyme involved in the binding of the β -carboxyl group of the inhibitor. To date, DAAOs from different sources are known to bind a large variety of aliphatic and aromatic carboxylic acids [17]. Early work [18] had shown a pK_1 around 8.6 for the free enzyme, pointing to a basic group in the enzyme with which the inhibitor combined through its own carboxylic group. This basic group could be different from the one that interacts with the α -carboxylic group of the substrate and whose nature could not be inferred from the studies presented here probably because its pK value is out of the pH range used in our experiments.

The present work was supported by an ANTIBIOTICOS S.A. research contract and by the Comisión Interministerial de Ciencia y Tecnología through a PETRI Project (Spain).

REFERENCES

- Curti, B., Ronchi, S. and Pilone, M. S. (1992) in *Chemistry and Biochemistry of Flavoenzymes*, (Müller, F., ed.), pp. 69–94, CRC Press, Boca Raton
- Faotto, L., Pollegioni, L., Ceciliani, F., Ronchi, S. and Pilone, M. S. (1995) *Biotechnol. Lett.* **17**, 193–198
- Gadda, G., Negri, A. and Pilone, M. S. (1994) *J. Biol. Chem.* **269**, 17809–17814
- Ramón, F., de la Mata, I., Iannaccone, S., Castillón, M. P. and Acebal, C. (1995) *J. Biochem.* **118**, 911–916
- Swenson, R. P., Williams, Jr., C. H. and Massey, V. (1984) *J. Biol. Chem.* **259**, 5585–5590
- Lindquist, Y. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., ed.), pp. 367–387, CRC Press, Boca Raton
- Pollegioni, L., Langkav, B., Tischer, W., Ghisla, S. and Pilone, M. S. (1993) *J. Biol. Chem.* **268**, 13850–13857
- Massey, V. and Gibson, Q. H. (1964) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **23**, 18–29
- Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol.* **45**, 273–387
- Segel, I. M. (1975) in *Enzyme Kinetics*, pp. 210–214, Wiley-Interscience, New York
- Denu, J. M. and Fitzpatrick, P. F. (1992) *Biochemistry* **31**, 8207–8215
- Cleland, W. W. (1982) *Methods Enzymol.* **87**, 309–405
- Inagami, T. and Sturtevant, J. M. (1960) *Biochim. Biophys. Acta* **38**, 64–79
- Koeppel, R. E. and Stroud, R. M. (1976) *Biochemistry* **15**, 3450–3458
- Hunkapiller, M. W., Smallcombe, S. H., Whitacker, D. R. and Richards, J. H. (1973) *Biochemistry* **12**, 4732–4743
- Parsons, S. M. and Raftery, M. A. (1972) *Biochemistry* **11**, 1630–1633
- Fonda, M. L. and Anderson, B. M. (1968) *J. Biol. Chem.* **243**, 1931–1935
- Dixon, M. and Kleppe, K. (1965) *Biochim. Biophys. Acta* **96**, 383–389