First publ. in: Flavins and flavoproteins 2002 : proceedings of the fourteenth international symposium, St. John's College, University of Cambridge, UK, July 14 - 18, 2002, pp. 299-304

Studies on the Elimination Reaction of *Rhodotorula gracilis* D–Amino Acid Oxidase with β–Chloro–D–alanine

Gianluca Molla, Mirella S. Pilone, Loredano Pollegioni
Department of Structural and Functional Biology, University of Insubria, via J.H. Dunant 3, 21100 Varese (Italy)
Sandro Ghisla
Fachbereich Biology, University of Konstanz, D–78547 Konstanz (Germany)

Introduction

D–amino acid oxidase (EC 1.4.3.3, DAAO) catalyses the oxidative deamination of the D–isomers of amino acids to yield the corresponding α–keto acids, ammonia and hydrogen peroxide (for a review see ref. 1). The enzyme from pig kidney (pkDAAO) has been demonstrated to catalyse a second, intriguing reaction, the α,β–elimination of halide from β–halogenated amino acids such as β–chloro–D–alanine (2). Two different products are formed:

chloropyruvate, arising from the normal oxidation reaction (eq. 1)
$EFl_{ox} + CH_2Cl\text{-}CH(NH_3^+)\text{-}COO^- \rightarrow EFl_{red} + \mathbf{CH_2Cl\text{-}C(=NH_2^+)\text{-}COO^-}$
$\rightarrow \mathbf{CH_2Cl\text{-}C(=O)\text{-}COO^-} + NH_4^+$

pyruvate and chloride from the elimination reaction (eq. 2)
$CH_2Cl\text{-}CH(NH_3^+)\text{-}COO^- \rightarrow CH_2{=}C(\text{-}NH_3^+)\text{-}COO^- + \mathbf{Cl^-} \rightarrow \mathbf{CH_3\text{-}C(=O)\text{-}COO^-} + NH_4^+$

This led to the reasonable conclusion that catalysis involves abstraction of the amino acid α–H as a proton via the so–called "carbanion mechanism", a process that requires an active site base. On the other hand, the work on pkDAAO reconstituted with the artificial flavin 5–deazaFAD (3) favored a hydride mechanism proceeding via transfer of the substrate αC–H to the flavin N(5). More recently the three–dimensional structure of pkDAAO (4,5) and of the enzyme from the yeast *Rhodotorula gracilis* (RgDAAO) has been resolved (6). The high resolution of this latter structure in complex with various substrates/ligands shows that the orientation of the flavin cofactor and of the substrate is in favor of a hydride mechanism. The following experimental results also support this conclusion: a linear energy correlation carried out with *T. variabilis* DAAO and *para*–substituted phenylglycines show that little or no charge develops in the transition state and that dehydrogenation proceeds via concerted rupture of the two involved bonds (7); the effects of pH, solvent isotope effects and primary isotope replacement on D–Ala and D–Asn dehydrogenation by RgDAAO are compatible with the absence of functional groups essential to acid/base catalysis at the active center of DAAO (8); all active site residues of RgDAAO carrying functional groups capable of base catalysis (namely Y223, Y238, R285 and

S335) have been mutated. In all cases activity is retained demonstrating that none of these is functional as an active site base (see Boselli et al., this Symposium). In order to shed light on the mechanism of α,β–elimination reaction we have (re)investigated it using RgDAAO, the properties of which were promising on new insights.

Materials and Methods

Determination the composition and the amount of reaction products

Total keto acid production was determined by reaction with 2,4–dinitrophenylhydrazine as the respective 2,4–dinitrophenylhydrazone (DNP) derivative (method A). This method was used for the estimation of the pyruvic to chloropyruvic acid proportion produced on the basis of the different absorbance values at 445 and 535 nm of the corresponding DNP–derivatives (2). The amount of pyruvic acid was determined by reaction with thiosemicarbazide and based on the large (140–fold) difference in the extinction coefficient at 285 nm between pyruvate and chloro pyruvate derivatives (method B). The amount of chloride eliminated was determined spectrophotometrically by reaction with $Hg(CNS)_2$ and $Fe_2(SO_4)_3$ (method C).

Time resolved stopped–flow spectrophotometry

The steady state and pre–steady state kinetics of the reaction with β–Cl–D–alanine was studied using a thermostated BioLogic SFM–300 stopped–flow spectrophotometer equipped with a J&M diode–array detector at 25 °C. For reductive half–reaction experiments, enzyme and substrate solutions were made anaerobic in tonometers by 10 cycles of evacuation and equilibration with pure argon (8). Enzyme monitored turnover experiments were performed with air–equilibrated solutions (8).

Results

The products from the reaction of RgDAAO with β–chloro–D–alanine depend from the O_2 concentration

When 20 mM β–Cl–D–alanine was incubated with 0.45 μM RgDAAO and 0.14 μM catalase in 20 mM sodium pyrophosphate buffer, pH 8.5, in the presence of 100 % O_2, the production of keto acid was evident. Analysis of the reaction products using method A indicates that only ≈8 % of the keto acid produced is chloropyruvate (see Fig. 1C). The amount of chloride produced (determined using method C) corresponds to the pyruvate production. The total keto acid production finishes after 5 min–10 min of reaction mainly due to inactivation of RgDAAO (less than 40 % of the enzyme activity is recovered after 1 h of incubation). At pH 6.5 and under similar experimental conditions, the overall production of keto acid is 3–fold higher indicating that the reaction of DAAO with β–Cl–D–alanine is pH dependent. At pH 8.5 and 21 % O_2 (continuous air saturation), keto acid production continues for more than 120 min (enzyme stability is higher under these conditions): more that 50 % of the initial β–Cl–D–alanine is converted to yield pyruvic acid, while no significant amounts of

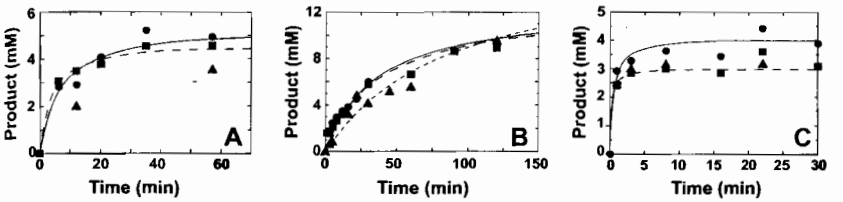


Figure 1: Time courses of keto acid production and effect of O_2 concentration. The incubations contained 20 mM β–Cl–D–alanine and 0.45 μM RgDAAO at pH 8.5 in the absence of oxygen (A), in the presence of 21 % O_2 (B) and 100 % O_2 (C). Total keto acid (•,——), pyruvic acid (■, – – –), and chloride (▲, - - -) concentrations.

chloropyruvate is detected. Analogously, the enzymatic product obtained under anaerobic conditions is pyruvate (see Fig. 1A). Interestingly, similar results have been obtained using the Y238F and the Y223F RgDAAO mutants (not shown).
These data indicate that RgDAAO catalyses the α,β–elimination of chloride from β–Cl–D–alanine. At all oxygen concentrations used the elimination reaction is the favorite reaction: chloropyruvate production (the product of the oxidation reaction, see eq. 1) is observed only at 100 % O_2 and represents only 8 % of the total keto acid formed. The partition between elimination and oxidation reaction of β–Cl–D–alanine is thus significantly different between RgDAAO and pkDAAO. With this latter enzyme, at $[O_2] > 21$ % the oxidation reaction is favored (at 100 % O_2 saturation about 90 % of the keto acid produced is chloropyruvate) (2).

Reaction of β–chloro–D–alanine with the reduced form of RgDAAO
In preliminary experiments, the reduced form of RgDAAO, obtained by anaerobic addition of a 2–fold excess of D–alanine, was incubated with 20 mM β–Cl–D–alanine. Although the spectrum of the reduced flavin was unaltered during the incubation time, the keto acid assay using method A indicated formation of 13 mM pyruvate upon 180 min incubation. This interesting result requires further investigation.

Stopped–flow experiments
The steady state reaction of RgDAAO at a starting 0.253 mM O_2 and using different β–Cl–D–alanine concentrations was investigated in the pH range 6–9. The spectral changes following the mixing resemble those previously observed for RgDAAO and D–alanine (7,8) (see Fig. 2). A parallel line pattern in the Lineweaver–Burk plots was obtained at all pH values used (Fig. 2, inset), with k_{cat} values ranging from 0.3 s^{-1} to

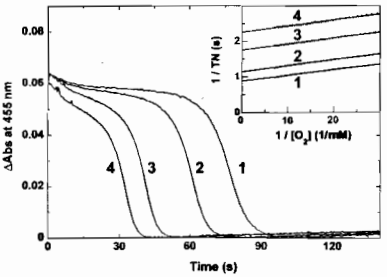


Figure 2: Turnover of RgDAAO with β–Cl–D–alanine at pH 8.0. The 455 nm–traces were obtained using the stopped–flow instrument by mixing 8 μM oxidized enzyme with (1) 1.25 mM; (2) 2.5 mM, (3) 5 mM and (4) 10 mM β–Cl–D–alanine at 25 °C and 0.253 mM $[O_2]$. Inset: Lineweaver–Burk plot of the same data as in the main graph.

4 s^{-1} (a figure at least 100–fold lower than the corresponding values obtained using D– alanine as substrate) (8). The spectral changes at 340 nm observed during the time course of reaction indicate that the production of keto acids is also pH dependent (and small at pH ≥ 7). The reaction of RgDAAO with β–Cl–D–alanine was also investigated under anaerobic conditions. The rate of conversion of oxidized enzyme into the reduced form was again pH dependent: the rate of enzyme reduction increases with pH (Fig. 3). A significant difference induced by pH regards the absorbance

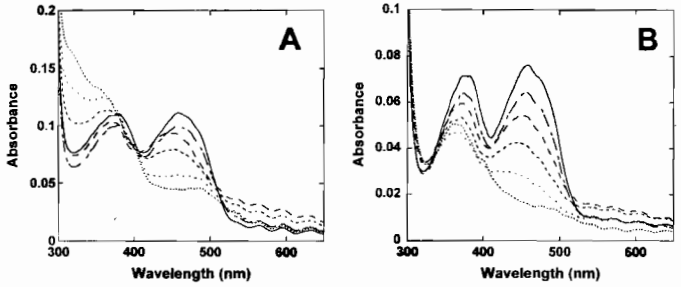
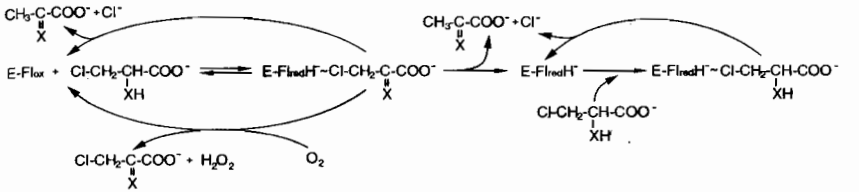


Figure 3: Spectral courses of the anaerobic reduction of 9 μM RgDAAO with 5 mM β–Cl–D–alanine at pH 6.0 (A) and with 0.5 mM β–Cl–D–alanine at pH 9.0 (B). The spectra were recorded at 25 °C using the stopped flow instrument at (A): (——) 10 ms, (— —) 30 ms, (– – –) 5 s, (- - -) 70 s, (•••) 190 s and (…) 400 s after mixing; (B): (——) 10 ms, (— —) 20 ms, (– – –) 50 ms, (- - -) 190 ms, (•••) 790 ms and (…) 10 s after mixing.



Scheme 1: Possible modes of α,β–elimination of Cl^- from β–Cl–D–alanine. The scheme shows partitioning at the level of reduced flavin. An elimination occurring at the locus of oxidized enzyme as discussed earlier (9), and requiring a reversal of flavin reduction (see upper left branch), also would be compatible with the results.

changes at 340 nm that reflect keto acid formation: production of keto acid was observed only at pH 6 and 7 (see different spectral courses at pH 6 and 9 reported in Fig. 3).

Interestingly, the absorbance changes did not follow the classic behavior observed using D–alanine as substrate (biphasic decay). This observation indicates that a different kinetic pattern is operative during the reductive half–reaction with β–Cl–D–alanine compared to that with D–alanine.

Discussion

Our preliminary investigation of the reaction of RgDAAO with β–Cl–D–alanine indicates that, even in the absence of active site groups acting as acid/base catalysts, yeast DAAO catalyses the α,β–elimination of halide from β–halogenated amino acids, as well as the oxidation reaction with the same substrate. Thus, both pkDAAO and RgDAAO catalyze the same basic reactions. A further analogy exists with the elimination reaction catalysed by L–lactate oxidase with β–chlorolactate where a competition between oxidation and elimination reactions also was observed (9). A common feature of the three enzymes is the dependence of the two reactions from oxygen. As already pointed out earlier (9), this can best be rationalised if partitioning occurs at the locus of reduced enzyme flavin, the only species in the systems capable to react with oxygen (Scheme 1). A further important point is the preliminary observation of the ability of RgDAAO to catalyse elimination starting from its reduced state. Although this point needs further investigation, there are also further important differences between RgDAAO and the other systems: competition between the oxidation and α,β–elimination reaction is observed only at 100 % O_2 saturation suggesting a different ratio of the partitioning steps. We have attempted to describe the various reactions in Scheme 1, a simplification and adaptation of a previously presented one (9).

Acknowledgements
We are indebted to dr. Fabio Battaini and Grazia Bernasconi for technical help in performing these experiments.

References

1. Curti B., Ronchi S., Pilone Simonetta M.: D– and L–amino acid oxidases. In: Muller F. (Ed.): Chemistry and Biochemistry of Flavoenzymes. CRC Press, Boca Raton. (1992), pp. 69–94.

2. Walsh C.T., Schonbrunn A., Abeles R. (1971): Studies on the mechanism of action of D–amino acid oxidase. Evidence for removal of substrate α–hydrogen as a proton. J. Biol. Chem. **248**, 6855–6866.

3. Hersh L.B., Schuman Jorns M. (1975): Use of 5–deazaFAD to study hydrogen transfer in the D–amino acid oxidase reaction. J. Biol. Chem. **250**, 8728–8735.

4. Mattevi A., Vanoni M.A., Todone F., et al (1996): Crystal structure of D–amino acid oxidase: a case of active site mirror–image convergent evolution with flavocytochrome b_2. Proc. Natl. Acad. Sci. U.S.A. **93**, 7496–7501.

5. Mizutani H., Miyahara I., Hirotsu K. (1996): Three–dimensional structure of porcine kidney D–amino acid oxidase at 3 Å resolution. J. Biochem. (Tokyo) **120**, 14–17.

6. Umhau S., Pollegioni L., Molla G., et al (2000): The X–ray structure of D–amino acid oxidase at 1.2 Å resolution identifies the chemical mechanism of flavin–dependent substrate dehydrogenation. Proc. Natl. Acad. Sci. U.S.A. **97**, 12463–12468.

7. Pollegioni L., Blodig W., Ghisla S. (1997): On the mechanism of D–amino acid oxidase. Structure/linear free energy correlations and deuterium kinetic isotope effects using *p*–substituted phenylglycines. J. Biol. Chem. **272**, 4924–4934.

8. Harris C.M., Pollegioni L., Ghisla S. (2001): pH and kinetic isotope effect in D–amino acid oxidase catalysis. Eur. J. Biochem. **268**, 5504–5520.

9. Massey V., Ghisla S., Ballou D. P., et al.: Singer T. P. (Ed.): Rapid reaction studies on dehydrogenation and elimination reactions of D–amino acid oxidase and lactate 2–monooxygenase. In Flavins and Flavoproteins. Elsevier Publ. Comp., Amsterdam. (1976), pp. 199–212.