First publ. in: Flavins and flavoproteins 1999 : proceedings of the Thirteenth International Symposium, Konstanz, Germany, August 29 - September 4, 1999, pp. 567-570

Very High Resolution Crystal Structure of D-Amino Acid Oxidase. Insights into the Reaction Mechanisms and Mode of Ligand Binding

S. Umhau, K. Diederichs, W. Welte, S. Ghisla
Department of Molecular Biology, University of Konstanz, Germany
L. Pollegioni, G. Molla, D. Porrini, M.S. Pilone
Department of Structural and Functional Biology, University of Insubria, via J.H. Dunant 3, 21100 Varese, Italy

Introduction

D-amino acid oxidase (DAAO) is the prototype of flavin containing oxidases and has accordingly been studied intensively since its discovery by Krebs in 1935 (1). Recently structural information on DAAO from pig kidney was independently reported by two groups (2, 3). Here we present the crystal structures of oxidised DAAO from the yeast *Rhodotorula gracilis* (RgDAAO) in complex with various ligands and substrate analogs: Anthranilate at 1.9 Å resolution, L-lactate an enantiomeric counterpart of D-amino acid substrates at 1.46 Å resolution and D-CF_3-alanine a quasi substrate at 1.74 Å resolution. The structure of the reduced RgDAAO complexed with the substrate D-alanine, showing a partial presence (20 % occupancy) of a dioxygen species was refined at 1.2 Å resolution.

Results and Discussion

Three-dimensional structure of DAAO

A chimeric form of RgDAAO (with six additional residues at the N-terminus) was expressed in *E.coli* and purified to homogenity. Crystals were grown by vapor diffusion with the hanging-drop method. The crystals belong to the space group I422 with one molecule per asymmetric unit and cell axes of a = b = 120 Å and c = 136 Å. Phases were obtained by molecular replacement of the pig kidney DAAO model (PDB code 1aa8). The RgDAAO complexes were formed upon soaking with an appropriate (20 mM - 40 mM) amount of the corresponding compound in the cryo-protection solution (except for the L-lactate that was present in the nature crystals). The four structures are identical in their overall shape. Therefore we have selected the structure at the highest resolution for the ribbon presentation in Figure 1. In all cases clear electron density was found for 361 of the 368 amino acids. At the N-terminus two additional amino acids, that are part of the hexapeptide not present in native RgDAAO, were identified, and were found to play an important role in crystal formation by contributing to crystal contacts. Several intermolecular hydrogen bridgesenlarge the crystal contact area. At the C-terminus four residues, containing the SKL tripeptide targeting signal to the PAS8-like receptor (4), are not visible, underlining the importance of flexibility of this region for targeting to the peroxisome.Also in a long surface exposed loop near the C-terminus considered to be

important for dimerisation, no defined electron density for four amino acids is observable.
We propose a mode of dimerisation different to that of pkDAAO, an elongated elypsoid with „head to head" contacts. RgDAAO yields a spherical dimer that makes „head to tail" interaction of two monomers.This is in agreement with four experimental evidences: a) dynamic light scattering and b) gel filtration chromatography, both suggesting the presence of a spherical molecule in solution; c) the buried surface area in the dimer interaction mode shown in Figure 1 (3049 Å^2) is maximal; d) the presence of a long 21 amino acid loop connecting two β-sheets near the N-terminus, which contributes largely to the contact area. This loop (not conserved in other DAAOs) can be cleaved off by limited proteolysis resulting in a monomeric molecule. The FAD cofactor is bound in an extended conformation interacting via several hydrogen bonds with the core of the protein. As in other flavoproteins, both the βαβ-motif near the phosphatidyl group and the GXGXXG consensus sequence are also present.

Figure 1: Structure of the proposed dimeric form of RgDAAO.

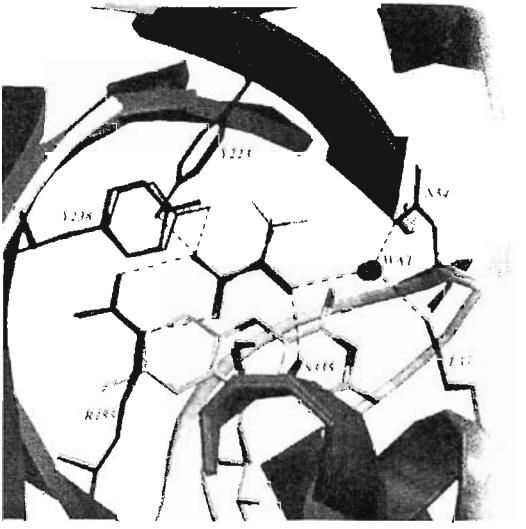

Figure 2: Close up of the active site

The active site
An overlay of active sites with the ligands D-alanine, D-CF_3-alanine and L-lactate is depicted in Figure 2, wherein the isoalloxazine rings are superimposed. In all cases the mode of ligand binding is identical: a tight network of H-bonds is responsible for the mode of substrate binding. It is somewhat surprising, that L-lactate is bound in the same way as the D-enantiomers, with the only exception of the orientation of the αC-H which points away from the cofactor. From this orientation it is clear that only for

the D-enatiomers the αC-H can be transferred to the flavin. In the case of D-CF_3-alanine, which is not a substrate of DAAO, we assume that the electronic, inductive effects of the fluorine substituents alter the redox potential of the amino acid to such an extent that dehydrogenation does not occur.

The funnel for substrate uptake

The structure of the RgDAAO-anthranilate complex shows the existence of a funnel, which allows substrate motion into the active site, as illustrated by the surface map shown in Figure 3. Actually two anthranilate molecules are found in complex with RgDAAO. One is located in the active site and the second is placed at the entrance of the active site, interacting with Tyr238. In contrast to the superimposed structures in Figure 2, Tyr238 does not interact with the carboxylate group of the anthranilate molecule bound inside the active site. From the two conformation of Tyr238 it is evident that RgDAAO has what can be called an open and closed form. In the complex with anthranilate RgDAAO is in its open form. In the three other structures the closed form results from a small conformational change of Tyr238, that than interacts with the carboxyl group of the substrate. A loop acting as a lid as proposed in pkDAAO is not present.

Figure 3: Detail of the funnel formed for substrate uptake.

A dioxygen species is bound in contact with the flavin
The very high resolution of the D-alanine dataset allows the identification of a biatomic species with approx. 20 % occupancy, located on the flavin plane. In Figure 4 the orientation with the electron density is shown. From the distance to the flavin and the way it is located it must be concluded, that it is not covalenty bound to the flavin itself. In agreement with this conclusion, the planarity of the alloxazine ring is not distorted. The electron density of not mobile hydrogens with defined conformation can be observed (Figure 4). In contrast to this, the hydrogen at the N5 position of the reduced flavin is not seen, because of its higher mobility due to pyramidal inversion.

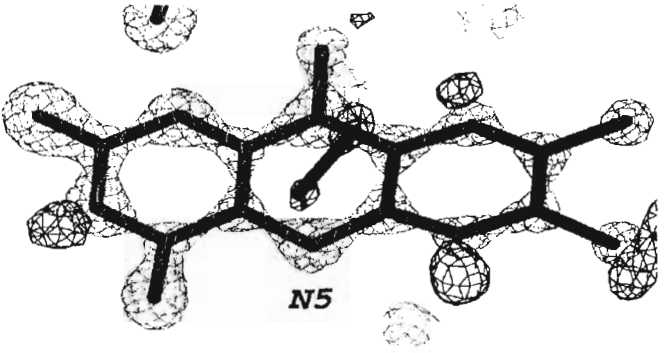

Figure 4: High resolution electron density for hydrogens and dioxygen species.

References

1. Krebs H.S. (1935): Metabolism of amino-acids. III Deamination of amino-acids. Biochem. J. **29**, 1620–1625.

2. Mattevi A., Vanoni M.A., Todone F., Rizzi M., Teplyakov A., Coda A., Bolongesi M. Curti B. (1996) Crystal structure of D-amino acid oxidase: a case of active site mirror-image convergent evolution with flavocytochrome b2. Proc. *Natl. Acad. Sci. U.S.A.* **93,** 7496-7501

3. Miura R., Setoyama C., Nishina Y., Shiga K., Miyutani H., Miyuhara I. and Hirotsu K. (1997) Structural and mechanistic studies on D-amino acid oxidase. Substrate complex: Implications of the crystal structure of enzyme. Substrate analog complex. J. Biochem. **122**, 825-833

4. Subramani, S. Components involved in peroxisome import, biogenesis, proliferation, turnover and movement. *Physiol. Rev.* **78 (1)** 171-188 (1998)