Crystallization and Preliminary X-Ray Analysis of the Flavoenzyme Vanillyl-Alcohol Oxidase From Penicillium simplicissimum

Mattevi, Andrea; Fraaije, Marco; Coda, Alessandro; Berkel, Willem J.H. van

Published in:
Proteins

DOI:
10.1002/(SICI)1097-0134(199704)27:4<601::AID-PROT12>3.0.CO;2-O

PROTEINS: Structure, Function, and Genetics 27:601–603 (1997)

Crystallization and Preliminary X-Ray Analysis of the Flavoenzyme Vanillyl-Alcohol Oxidase From *Penicillium simplicissimum*

Andrea Mattevi,[1]* Marco W. Fraaije,[2] Alessandro Coda,[1] and Willem J.H. van Berkel[2]
[1]*Department of Genetics and Microbiology, University of Pavia, 27100 Pavia, Italy*
[2]*Department of Biochemistry, Agricultural University, NL-6703 HA Wageningen, The Netherlands*

ABSTRACT **Vanillyl-alcohol oxidase catalyses the oxidation of several 4-hydroxybenzyl alcohols by using 8-α-(N^3-histidyl)-FAD as a covalently bound prosthetic group. Crystals of vanillyl-alcohol oxidase from *Penicillium simplicissimum* have been grown by using the vapor diffusion technique. The space group was found to be I4, with cell dimensions $a = b = 140.5$ Å, $c = 132.9$ Å. Diffraction data have been recorded to 3.2 Å resolution by using a laboratory source and to 2.5 Å resolution on flash freezing the crystal at the ELETTRA Synchrotron X-ray diffraction beam line. Proteins 27: 601–603, 1997.** © **1997 Wiley-Liss, Inc.**

Key words: FAD; catalysis; X-ray crystallography; molecular symmetry

INTRODUCTION

The fungus *Penicillium simplicissimum* (ATCC 90172) can use a range of aromatic compounds as a sole source of carbon. This organism when grown on veratryl alcohol induces an intracellular H_2O_2-generating oxidase catalyzing the oxidation of vanillyl alcohol to vanillin.[1] This enzyme has been characterized and shown to possess a broad substrate specificity (Fig. 1), thereby being able to catalyze not only the oxidation of various 4-hydroxybenzyl alcohols but also the oxidative deamination of 4-hydroxybenzylamines and the oxidative demethylation of 4-methoxymethyl phenols. On this basis, it has been suggested that vanillyl alcohol may not be the real physiological substrate of the enzyme as further indicated by the fact that this compound appears not to be an intermediate in the cellular degradation of veratryl alcohol.[2]

Vanillyl-alcohol oxidase (VAO) is a homooctamer of ~0.5 MDa. Electron micrographs have revealed that the quaternary structure of the octamer is similar to that of methanol oxidase from methyltrophic yeasts.[3] Each enzyme subunit contains an 8-α-(N^3-histidyl)-FAD molecule as a covalently bound prosthetic group. A preliminary characterization of the catalytic properties suggests that the reaction proceeds via a direct hydride transfer from the substrate to the flavin with concomitant formation of a *p*-quinone methide intermediate.[4] This and other mechanistic data, such as the lack of reactivity toward sulfite, indicate that VAO represents a novel type of FAD-dependent enzyme, unrelated to the well-characterized class of flavoprotein oxidases.[5] In this respect, the covalent attachment of the flavin to a histidine side chain is a feature of particular interest. Five types of covalent bonds between the flavin and proteins have been revealed.[6] However, in all cases the mechanism and the functional role of the covalent flavinylation process remain unresolved problems.

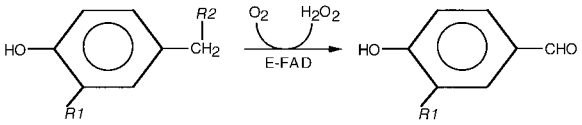
Understanding the flavinylation mechanism is particularly relevant for the biotechnological exploitation of flavoproteins, which has been thus far limited because under stress conditions the FAD cofactor tends to dissociate resulting in the unstable apoenzyme form. This problem could be overcome by designing semisynthetic flavoenzymes in which the covalent linkage of the cofactor to the protein leads to higher stability and prevents FAD dissociation. Such an approach has been successfully used in the case of D-amino acid oxidase and it is likely to be applicable to other flavoenzymes.[7]

In the framework of a project devoted to the structural characterization of FAD-dependent oxidases,[8] we have undertaken the X-ray analysis of VAO. Knowledge of the structure will provide the basis for the understanding of the novel mechanistic properties of this enzyme and will shed light into the structural and functional role of the covalent attachment of the FAD cofactor.

EXPERIMENTAL PROCEDURES

VAO was isolated from *P. simplicissimum* cells and purified as described.[1,4] Crystals of the protein were grown by the hanging drop method. The drops were formed by mixing equal volumes (5 µl) of the protein and the reservoir solutions where the protein solution consisted of 15 mg enzyme/ml in 50 mM sodium phosphate buffer (pH 7.0) and the reservoir solution

*Correspondence to: Dr. Andrea Mattevi, Department of Genetics and Microbiology, University of Pavia, via Abbiategrasso 207, 27100 Pavia, Italy.
Received 1 October 1996; Accepted 10 October 1996



Vanillyl alcohol: *R1*= -OCH_3, *R2*= -OH
Vanillylamine: *R1*= -OCH_3, *R2*= -NH_2
4-(methoxymethyl) phenol: *R1*= -H, *R2*= -OCH_3

Fig. 1. The reaction catalyzed by VAO.

contained 100 mM sodium acetate buffer (pH 4.6), 0.02% w/v NaN_3, and 6% w/v polyethylene glycol (PEG) 4000. The best crystals were obtained by conducting the crystallization experiments at 20°C.

For data collection at room temperature, the crystals were mounted in glass capillaries with a stabilizing solution containing 20% w/v PEG 4000 in the same buffer as the reservoir used for crystallization. Diffraction data were collected on a Rigaku RU-200 X-ray generator by using the R-axis II imaging plate system as a detector. The intensities were evaluated by using the program MOSFLM (A.G.W. Leslie) and internally scaled by using the CCP4 package.[9]

A low temperature data set was collected at the ELETTRA Synchrotron facility (Trieste, Italy). For the low temperature experiments, the crystals were exposed for a few seconds to a solution containing 30% v/v PEG 400, 20% w/v PEG 4000, and 100 mM sodium acetate (pH 4.6) then mounted on a thread loop to be shock-freezed at 100 K under a stream of nitrogen. A 90-mm diameter MAR Research Imaging plate scanner (Hamburg, Germany) was used as a detector with monochromatized radiation (λ = 1.0 Å). The data were integrated, scaled, and merged by using the program DENZO.[10]

RESULTS

The crystals of VAO reproducibly grow in 1-2 days, reaching a maximum size of 0.4 × 0.4 × 0.2 mm. Their space group was determined to be I4 with cell dimensions $a = b$ = 140.5 Å, c = 132.9 Å. A native data set was collected at room temperature by using a laboratory source. A total of 91,356 intensities were evaluated and merged into a unique set of 24,192 independent reflections with a R_{sym} value of 11.1% and a completeness of 97.3% up to 3.2 Å resolution.

The crystals are only moderately stable under the X-ray beam and therefore cryocooling experiments were performed to prevent radiation damage. A data set was collected at 100 K by using PEG 400 as the cryoprotectant. The experiment was conducted with the ELETTRA Synchrotron X-ray diffraction beam line (Trieste, Italy). The 330,030 intensities measured at low temperature were merged into a set of 38,274 independent reflections with a R_{merge} value of 9.3% and completeness of 98.4% up to 2.5 Å resolution. In the highest resolution shell (2.60–2.50 Å), the R_{sym} is 21.0% with 67.9% of the measured intensities greater than 3σ(I) and a completeness of 97.9% (Table I). With flash-freezing, the unit cell dimensions shrunk to $a = b$ = 130.2 Å, c = 133.0 Å.

TABLE I. Intensity Statistics of Data Collection of VAO at ELETTRA

	Completeness (%)		
Resolution (Å)	$I > 0\sigma(I)$	$I > 3\sigma(I)$	R_{sym}* (%)
99–5.2	97.4	93.3	6.7
5.2–4.1	93.9	92.5	7.3
4.1–3.6	96.9	93.4	8.8
3.6–3.3	98.5	92.1	10.4
3.3–3.0	99.2	88.8	13.7
3.0–2.8	98.9	80.8	14.2
2.8–2.7	98.5	76.1	15.9
2.7–2.6	98.3	71.5	18.0
2.6–2.5	97.9	67.9	21.0
Overall	98.4	82.0	9.3

N of measured reflections, 330,030; N of independent reflections, 38,274.
*$R_{sym} = \Sigma_h\Sigma_i (I_{ih} - \langle I_h \rangle)/\Sigma_h\Sigma_i I_{ih}$, where $\langle I_h \rangle$ is the mean intensity of the i observations of reflection h.

A self-rotation function was calculated by means of the program GLRF[11] by using the low temperature data in the 8.0-4.0 Å resolution range. A peak 5.5σ above the mean was obtained at κ = 180°, Φ = 0°, ψ = 39°,[12] suggesting that the asymmetric unit contains a dimer of subunits. Particularly, the noncrystallographic twofold axis appears to be perpendicular to the *z*-axis, indicating that the enzyme octamer has 422 symmetry with the molecular fourfold axis being coincident with the crystallographic *c*-axis. This interpretation of the self-rotation function is confirmed by the fact that assuming a dimer (130,000 Da) in the asymmetric unit, the V_m value is 2.5 $Å^3$/Da, implying a solvent content of 49%, which is in the range of values normally found in protein crystals.[13] Moreover, it has been shown that urea induces dissociation of VAO into active dimers,[3] suggesting that the enzyme octamer is formed by a tetramer of dimers.

We are conducting the structure determination of VAO by means of the multiple isomorphous replacement technique, and a search for suitable heavy atom derivatives is underway.

ACKNOWLEDGMENTS

The supervision of Dr. A. Savoia and colleagues during data collection with the ELETTRA Synchrotron is most gratefully acknowledged. This research was supported by a grant from the Consiglio Nazionale delle Ricerche (Contract 9502989CT14) to A.M.

REFERENCES

1. De Jong, E., van Berkel, W.J.H., van der Zwan, R.P., de Bont, J.A.M. Purification and characterization of flavin-

dependent vanillyl-alcohol oxidase from *Penicillium simplicissimum*. Eur. J. Biochem. 208:651–657, 1992.
2. De Jong, E., Beuling, E.E., van der Zwan, R.P., de Bont, J.A.M. Degradation of veratryl alcohol by *Penicillium simplicissimum*. Appl. Microbiol. Biotechnol. 34:33–41, 1990.
3. van Berkel, W.J.H., Fraajie, M.W., de Jong, E., de Bont, J.A.M. Vanillyl-alcohol oxidase from *Penicillium simplicissimum*: a novel flavoprotein containing 8-α-(N^3-histidyl)-FAD. In: Flavin and Flavoproteins—1993. Yagi, K. (ed.), New York, W. de Gruyter, 1994:799–802.
4. Fraaije, M.W., Veeger, C., van Berkel, W.J.H. Substrate specificity of flavin-dependent vanillyl-alcohol oxidase from *Penicillium simplicissimum*. Eur. J. Biochem. 234:271–277, 1995.
5. Ghisla, S., Massey, V. Mechanisms of flavoprotein-catalyzed reactions. Eur. J. Biochem. 181:1–17, 1989.
6. Kim, J., Fuller, J.H., Kuusk, V., Cunane, L., Chen, Z., Mathews, F.S., McIntire, W.S. The cytochrome subunit is necessary for covalent FAD attachment to the flavoprotein subunit of *p*-cresol methylhydroxylase. J. Biol. Chem. 270:31202–31209, 1995.
7. Stocker, A., Hecht, H.J., Buckmann, A.F. Synthesis, characterization, and preliminary crystallographic data of N6-(6-carboamylhexyl)-FAD-D-amino acid oxidase: a semisynthetic oxidase. Eur. J. Biochem. 238:519–528, 1996.
8. Mattevi, A., Vanoni, M.A., Todone, F., Teplyakov, A., Coda, A., Bolognesi, M., Curti, B. Crystal structure of D-amino acid oxidase: a case of mirror image convergent evolution with flavocytochrome b2. Proc. Natl. Acad. Sci. USA 93: 7496–7501, 1996.
9. Collaborative Computational Project Number 4. The CCP4 suite: programs for protein crystallography. Acta Cryst. D50:760–767, 1994.
10. Otwinoski, Z., Minor, W. DENZO: A Film Processing Program for Macromolecular Crystallography. New Haven CT: Yale University, 1993.
11. Tong, L., Rossmann, M.G. The locked rotation function. Acta Crystallogr. A46:783–792, 1990.
12. Rossmann, M.G., Blow, D.M. The detection of sub-units within the crystallographic asymmetric unit. Acta Crystallogr. 15:24–31, 1962.
13. Matthews, B.W. Solvent content of protein crystals. J. Mol. Biol. 33:491–497, 1968.