X-ray diffraction analysis of flavin-dependent oxidase from thermophilic fungus *Chaetomium thermophilum*

Aleš Kravic[1], Leona Švecová[1,2], Petr Kolenko[1,2], Lars Henrik Østergaard[3], Jan Dohnálek[2],

[1] *Faculty of Nuclear Sciences and Physical Engineering, Czech Technical University in Prague, Břehová 7, 115 19, Praha 1, Czech Republic*

[2] *Institute of Biotechnology of the Czech Academy of Sciences, Biocev, Průmyslová 595, 252 50, Vestec, Czech Republic*

[3] *Novozymes A/S, Biologiens Vej 2, 2800 Kgs. Lyngby, Denmark*

kraviale@fjfi.cvut.cz

Abstract

FAD-dependent oxidoreductase from thermophilic filamentous *Chaetomium thermophilum* (*Cht*FDO) is monomeric thermostable glycoprotein, that can find application in industries, where enzyme activity at higher temperatures is required. Here we present a study of reduction processes in *Cht*FDO caused by the absorbed X-ray dose. Our data show that the reduction of FAD occurs rapidly and experiments will have to be realized at synchrotron radiation sources.

Keywords: FAD-dependent oxidoreductase, X-ray diffraction, protein crystallography

Introduction

Most of oxidoreductases use covalently or non-covalently bound small molecule e.g. flavin adenine dinucleotide (FAD) for catalyzing the redox reactions. During redox reaction, the FAD molecule transforms between the reduced and oxidized state, which is associated with its conformational change and ability to absorb at visible light.

FAD-dependent oxidoreductase (*Cht*FDO) belongs to glucose-methanol-choline (GMC) oxidoreductase family. It is expected that *Cht*FDO catalyzes two-electron oxidation of primary and secondary alcohols accompanied by two-electron reduction of molecular oxygen to hydrogen peroxide [2]. Preliminary X-ray studies suggest that FAD of the enzyme may be always observed in the reduced state. It is assumed that FAD of this enzyme must be observable in its oxidized state (as already proved spectrophotometrically but never observed in structure). [3].

In this paper, we tried to investigate the progressive reduction process from oxidized to reduced state of FAD in dependence on X-ray dose. The diffraction experiment was performed using an intensive in-house X-ray source.

Materials and methods

The recombinant enzyme provided by Novozymes A/S was transfered to a buffer containing 25 mM Tris-HCl pH 7.5 and 100 mM NaCl at a concentration of 9.2 mg/ml. Crystallization

of *Cht*FDO was performed at 20 °C by the vapor diffusion method in a hanging drop arrangement with 1:1 volume ratio of protein to reservoir, respectively. Protein crystals were grown in crystallization condition 0.15 M magnesium formate, 0.1 M sodium acetate pH 5.5, 18 % w/v PEG 5000.

Diffraction data were measured at 100 K on a single crystal diffractometer D8 Venture (Bruker) using an X-ray source with liquid anode MetalJet D2 (Excillum) and a CPAD detector Photon II at the Institute of Biotechnology of the Czech Academy of Sciences, Vestec. Data collection parameters on this diffractometer are listed in Table 1.

The *Cht*FDO crystal was irradiated for 240 minutes. Obtained diffraction data were processed with XDS [4], divided into 6 sectors with equal exposure times and scaled with the AIMLESS [5] program. All sectors were processed with a diffraction limit of 2.6 Å. Statistical values of processed diffraction data from the first and fifth sector are shown in Table 2. These two sectors meet the criteria for data quality (overall completeness over 90 % and $CC_{1/2}$ in the shell with the highest resolution over 0.3). Next, the model structure (PDB ID: 6ZE2 [5]) with removed FAD cofactor and waters was refined against data from sectors 1 and 5 using the REFMAC5 [6] software.

Table 1: Data collection parameters

Wavelength [nm]	0.13418
Temperature [K]	100
Crystal-detector distance [mm]	80
Exposure time per frame [s]	2
Oscillation angle/range [°]	0.1/696.5

Table 2: Statistical values of processed diffraction data of the first and fifth sectors. The values in parentheses correspond to the values in the shell with the highest resolution

	Sector 1	Sector 5
No. of images	1 - 1200	4801 - 6000
Exposure time start-end [min]	40	200
Resolution range [Å]	50.00 – 2.59 (2.71 – 2.59)	50.00 – 2.59 (2.72 – 2.59)
Space group	$P\ 22_12_1$	$P\ 22_12_1$
Unit-cell parameters [Å, °]	$a = 47.07$; $b = 110.30$ $c = 116.65$; $\alpha = 90.00$ $\beta = 90.00$; $\gamma = 90.00$	$a = 46.52$; $b = 109.49$ $c = 116.04$; $\alpha = 90.00$ $\beta = 90.00$; $\gamma = 90.00$
No. observed reflections	63 818 (2 512)	73 563 (4 891)
No. unique reflections	17 438 (1 448)	17 206 (1 723)
Completeness [%]	91.8 (64.1)	90.4 (75.6)
Average multiplicity	3.7 (1.7)	4.3 (2.8)
$CC_{1/2}$	0.933 (0.387)	0.933 (0.351)
Average I/σ_I	3.3 (0.8)	2.9 (0.8)
R_{meas}	0.383 (0.828)	0.438 (1.061)
R_{merge}	0.334 (0.638)	0.389 (0.892)

Results and Discussion

The progressive reduction process of FAD in *Cht*FDO was studied depending on the dose of X-rays. Sectors 1 and 5 were selected for structure refinement to observe the maximum differences between the electron densities of initial (anticipated as oxidized) and final state.

A comparison of data from sectors 1 and 5 led us to the fact that the current measurement was not sufficiently optimized to observe the progressive reduction of FAD, although we tested the best possible optimization of data selection (sector classification, low exposure time, choice of resolution range). In the first sector we can already see a comparable angle of bending with the other observed states from previous X-ray studies, i.e. no planar conformation was observed. This outcome very likely corresponds to a reduced state of FAD (Figure 1).

From these results it follows that the observation of the progressive reduction process in *Cht*FDO under these experimental conditions has not been possible yet. We think that the reason for this outcome is the long exposure of crystal in the first sector. It is possible that sufficient irradiation of the crystal with X-rays leads to a change of state [7]. A possible step in the future is to perform a similar experiment using a better source of X-rays and a better detector, which will allow us to very accurately limit the small doses of X-rays to achieve observation of the progressive reduction process.

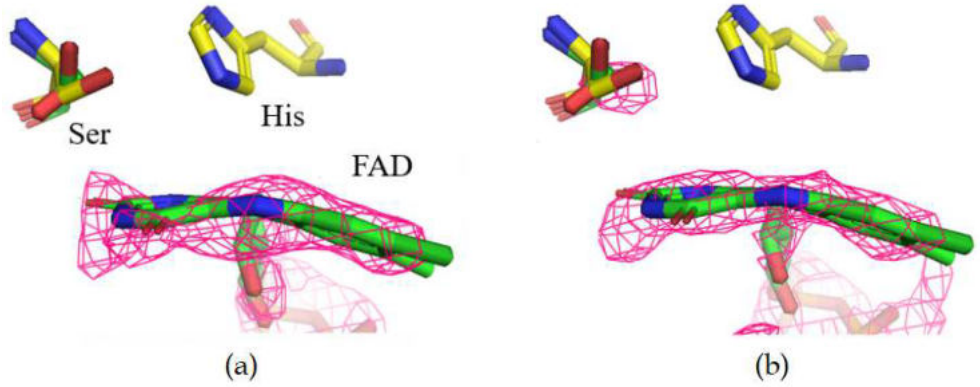

Figure 1: Maps of difference electron mF_O - DF_C densities in the active site at different exposure times at the level of 3 σ shown as hot pink contour. Model (PDB ID: 6ZE2) with removed cofactor FAD was refined against data from individual sectors. Residues of the refined structure are shown in yellow, residues and cofactor from model structure (PDB ID: 6ZE2) are shown in green. Difference density obtained by refining the structure against data from (a) Sector 1 (total exposure time 40 minutes) (b) Sector 5 (total exposure time 200 minutes)

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