Journal of Structural Biology 170 (2010) 32–40

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Structural characterization of the organic solvent-stable cholesterol oxidase from *Chromobacterium* sp. DS-1

Martin Sagermann [a,b,*], Akashi Ohtaki [a,c], Keith Newton [a], Noriyuki Doukyu [d]

[a] Department of Chemistry and Biochemistry, University of California, Santa Barbara, CA 93106-9510, USA
[b] Interdepartmental Program in BioMolecular Science and Engineering, University of California, Santa Barbara, CA 93106-9510, USA
[c] Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan
[d] Bio-Nano Electronic Research Center, Toyo University, 2100, Kujirai, Kawagoe Saitama 350-8585, Japan

ARTICLE INFO

Article history:
Received 11 September 2009
Received in revised form 19 January 2010
Accepted 21 January 2010
Available online 25 January 2010

Keywords:
Cholesterol
Oxidase
Organic solvent-stability
Oxygen channel

ABSTRACT

Cholesterol oxidase is of significant commercial interest as it is widely used as a biosensor for the detection of cholesterol in clinical samples, blood serum and food. Increased stability of this enzyme with regards to temperature and different solvent conditions are of great importance to the reliability and versatility of its applications. We here report the crystal structure of the cholesterol oxidase of *Chromobacterium* sp. DS-1 (CHOLOX). In contrast to other previously characterized cholesterol oxidases, this enzyme retains high activity in organic solvents and detergents at temperatures above 85 °C despite its mesophilic origin. With the availability of one other homologous oxidase of known three-dimensional structure, a detailed comparison of its sequence and structure was performed to elucidate the mechanisms of stabilization. In contrast to factors that typically contribute to the stability of thermophilic proteins, the structure of CHOLOX exhibits a larger overall cavity volume, less charged residues and less salt bridge interactions. Moreover, the vast majority of residue substitutions were found on or near the protein's solvent exposed surface. We propose that the engineering of enhanced stability may also be accomplished through selective engineering of the protein periphery rather than by redesigning its entire core.

1. Introduction

Human arteriosclerosis, hyperlipemia as well as obesity are diseases that have been associated with an imbalance of cholesterol. Accurate determinations of this lipid is thus of fundamental importance to the diagnosis and prevention of diseases that result from unhealthy concentrations of this compound. Today, the majority of cholesterol quantitations are performed by a protein-based biosensor consisting of multiple enzymes including cholesterol oxidase. With the exception of glucose oxidase, this enzyme has become one of the most widely used biosensors in clinical applications (MacLachlan et al., 2000). This enzyme, which is derived from a prokaryotic organism, is uniquely capable of translating the amount of sample cholesterol into a readable signal, H_2O_2, for many optical or electronic devices (MacLachlan et al., 2000).

In order to enhance the accuracy of the measurements, prior isolations of the lipid are frequently required. For this purpose, organic solvents are used for the solubilization and extraction of lipidic compounds from biological membranes. Moreover, organic solvents are also utilized frequently to facilitate chemical modifications and for the immobilization of sensor proteins onto polymer matrices and electrodes (Arya et al., 2009; MacLachlan et al., 2000; Wu and Choi, 2003). Unfortunately, however, many of these commonly used solvents denature proteins, limiting their applicability dramatically (Doukyu and Aono, 2001). Strong interactions of the solvents with the protein and the withdrawal of water molecules from the protein can often lead to disintegration of structure and function.

Based on their wide ranging and unique thermal stabilities, the search for suitable enzymes has mainly focused on thermophilic organisms as their stabilities appear attractive for many medical and industrial applications (Egorova and Antranikian, 2005).

Recently, we have identified a new cholesterol oxidase from *Chromobacterium* sp. DS-1 (CHOLOX) (Doukyu and Aono, 2001). This enzyme belongs to the type II cholesterol oxidases, which have the FAD cofactor covalently attached to the protein. In contrast to oxidases from other organisms, this enzyme still functions in most organic solvents and detergents, as well as at high temperatures. While most of the commercially available enzymes loose their activity upon a 30 min incubation at 50–75 °C, remarkably, this enzyme retained 80% of its activity after incubation at 85 °C, i.e. hyperthermal conditions, rendering this enzyme as the most stable to date (Doukyu et al., 2008, 2009). Until now, only one other homologous structure of this enzyme class from *Brevibacteri-*

* Corresponding author. Address: Department of Chemistry and Biochemistry, University of California, Santa Barbara, CA 93106-9510, USA. Fax: +1 805 893 4120.
E-mail address: Sagermann@chem.ucsb.edu (M. Sagermann).

1047-8477/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jsb.2010.01.012

um sterolicum has been determined (PDBid:1I19, (Coulombe et al., 2001), (here also referred to as BCO)). In contrast to CHOLOX, this enzyme, which is also derived from a mesophilic prokaryote, is only moderately stable to about 50 °C as judged by loss-of-activity measurements at different temperatures (data on systematic melting studies with respect to the enzyme's structure were not available). Furthermore, it is also rapidly inactivated by organic solvents such as isopropanol or detergents like Triton X-405, for example (Doukyu, 2009).

In order to understand the differences of its physical properties in comparison to its mesophilic counterpart BCO, we have determined the crystal structure to 1.54 Å resolution. In this study, we take inventory of the respective amino acid compositions, cavity volumes, number of interactions and distributions of electrostatic potentials and compare it to other thermophilic/solvent stable enzymes. Furthermore, we characterize the structural distribution of residues that are unique (non-conserved) to CHOLOX but not to BCO as they are most likely to contribute to the superior stability of the enzyme. These residues will be referred to as "new" residues in the text.

2. Materials and methods

2.1. Purification

Purification of the enzyme was performed as previously reported (Doukyu and Aono, 1998). Briefly, a 25 mL overnight culture of CHOLOX–plasmid bearing BL21 *Escherichia coli* cells were grown in 1 L shaker flasks in the presence of 1 μM ampicillin. 1 μM IPTG was added at an OD_{600} of 0.6. Subsequently, induction was carried out for 3 h before harvest with a centrifuge (Beckmann Rotor JA10, 4000 rpm for 30 min). The resultant cell pellet was either stored at −75 °C or lysed directly using the French-Press operated at 1200 PSI. The cell homogenate was submitted to a high-speed centrifugation spin (Beckman Rotor JA20, at 12,000 rpm). The supernatant was collected (typically approximately 50 mL) and incubated immediately in a water bath at 80 °C for approximately 45 min to denature soluble *E. coli* proteins. The homogenate was then submitted to another high-speed centrifugation and the resultant supernatant was loaded onto a DEAE–Sepharose column (Merck). The mostly pure enzyme was collected in the flow-through fraction, concentrated and dialyzed against Standard Buffer (50 mM Tris, pH 8.2, 100 mM NaCl and 1 mM EDTA).

2.2. Crystallization

Prior to crystallizations, the protein solution was concentrated to approximately 3 mg/mL. Initial crystallizations were carried out with commercially available screens from Hampton (Crystal Screens I + II) and Jena Bioscience GmbH (JB Screen HTS II). Optimal conditions for crystal growth were identified with 500 mM ammonium sulfate, 1.0 M lithium sulfate and 10 mM sodium citrate. Crystals were readily identified by their yellow color.

2.3. Data collection and processing

Data collections were performed at the Stanford Synchrotron Radiation Laboratory, beamline 7-1. For this purpose, crystals were incubated with the addition of 5% sucrose into the mother liquor for one hour prior to flash-freezing in liquid nitrogen. Data integration, reduction and scaling were carried out with the programs XDS, and XSCALE (Kabsch, 1993). The structure was determined by molecular replacement using the program AMoRe (CCP4, 1994; Trapani and Navaza, 2008) using the structure PDBid:1I19 as a search model. Subsequently the structure was refined using the programs CNS (Brunger et al., 1998) and REFMAC5 (CCP4, 1994; Murshudov et al., 1997). A random-selected invariant number of reflections (4.6%) was maintained as a test set throughout the refinement calculations (Adams et al., 1997). The final statistics on data collection, reduction and refinement are shown in Table 1. Superpositions and structural comparisons were carried out with the program DALI (Holm et al., 2008). Electrostatic surface potentials were calculated with the program APBS (Baker et al., 2001) and rendered and displayed with the program VMD (Humphrey et al., 1996). The number of salt bridges was also determined with this program. Volume and cavity calculations were performed with the program VOIDOO using a 1.4 Å probe radius and default parameters (Doukyu and Aono, 2001; Kleywegt and Jones, 1994). Packing efficiencies as defined by Pattabiraman et al. (1995) and Voss and Gerstein (2005) were calculated using the program OS or the Voronoi Packing Efficiency Calculator.

3. Results

3.1. Overall structure

The structure of CHOLOX (residues 1–540) closely resembled the structure of BCO (residues 57–613) (Coulombe et al., 2001), which is reflected by the pronounced sequence identity of 44.9%. Both peptide backbones superimpose with ca. 1 Å rmsd accuracy (Fig. 1). As it is expected for class II enzymes, the prosthetic group, FAD, was covalently bound to His63 *via* the C8 methyl of the flavin group. The most pronounced structural differences between the two proteins were observed in loop regions. In comparison to BCO, a structural sequence alignment with the program DALI exhibited three small insertions (totaling four residues, i.e. at positions 181–182, 314 and 492), and only one 3-residue deletion,

Table 1
Crystallographic data and refinement statistics.

Data set	CHOLOX
X-ray source	BL7-1
Cell dimensions (Å)	
a	62.22
b	90.38
c	124.19
Resolution (high res. bin)	40–1.54 (1.62–154)
Wavelength (Å)	0.97949
Space group	$P2_12_12_1$
Number of reflections (total/unique)	96,731 (12,447)
Number of reflections (observed)	351,568 (44,270)
R-merge	0.046 (0.347)
$I/\mathrm{Sig}(I)$	21.42 (4.34)
Completeness	93.3 (86.1)
Redundancy	3.63 (3.56)
Refinement	
R_{work}/R_{free}	0.176/0.196
Number of atoms	
Protein	4300
Water	406
Ligands	76
rmsd	
Bond lengths	0.023
Bond angles	2.1

$R_{sym} = \sum|I - \langle I\rangle|/\sum\langle I\rangle$; where I is the observed intensity and $\langle I\rangle$ is the statistically weighted average intensity of multiple symmetry related observation.
R-factors: $R = \sum||F_{calc}| - |F_{obs}||/\sum|F_{obs}|$; where F_{calc} and F_{obs} are the calculated and observed structure factors, respectively. The $R_{(free)}$ was calculated using the same formula with 4.6% of the observed reflections.
rmsd, root-mean-square deviation from ideal geometry.
Coordinates were deposited at the Brookhaven Bank with the Accession No. PDBid = 3JS8.