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Crystallization of quinohaemoprotein alcohol dehydrogenase from *Comamonas testosteroni*: crystals with unique optical properties

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Quinohaemoprotein alcohol dehydrogenase from *Comamonas testosteroni* is a functional electron-transfer protein containing both a haem *c* and a pyrroloquinoline quinone cofactor. The enzyme has been crystallized at 277 K using polyethylene glycol 6000 as precipitant. The crystals belong to space group *C*2, with unit-cell parameters $a = 98.1$, $b = 74.3$, $c = 92.2$ Å, $\beta = 105.9^\circ$. A native data set with a resolution of 2.44 Å resolution has been collected. The approximate orientation of the haem group with respect to the unit-cell axes has been determined from the optical properties of the crystals.

1. Introduction

Quinoproteins are a class of oxidoreductases that use one of four different quinone cofactors to convert alcohols and amines to the corresponding aldehydes/ketones (Salisbury *et al.*, 1979; Janes *et al.*, 1990; McIntire *et al.*, 1991; Anthony, 1996; Wang *et al.*, 1996). The largest quinoprotein subclass is formed by enzymes which contain the prosthetic group pyrroloquinoline quinone (PQQ; Duine, 1991).

So far, two different classes of PQQ-containing alcohol dehydrogenases (ADHs) have been characterized. The first class includes quinoprotein ethanol dehydrogenases (Q-EDHs) from several *Pseudomonas* species (Groen *et al.*, 1984; Rupp & Gorisch, 1988; Görisch & Rupp, 1989; Toyama *et al.*, 1995) as well as the well characterized quinoprotein methanol dehydrogenases (MDHs) from methylotrophic bacteria (Westerling *et al.*, 1979; Anthony *et al.*, 1994; Anthony, 2000).

The second class of PQQ-dependent alcohol dehydrogenases is that of the quinohaemoprotein alcohol dehydrogenases (QH-ADHs). In addition to PQQ, these enzymes contain a covalently bound haem *c*. Two types of QH-ADH are known. The first type is soluble and monomeric (Groen *et al.*, 1986; Toyama *et al.*, 1995; Shimao *et al.*, 1996; Yasuda *et al.*, 1996; Zarnt *et al.*, 1997; Vangnai & Arp, 2001). Type II QH-ADHs, on the other hand, are membrane-associated and consist of three subunits of about 72, 48, and 15 kDa, respectively (Adachi *et al.*, 1978; Tayama *et al.*, 1989; Matsushita & Adachi, 1993).

Although both classes of ADHs are different in terms of subunit composition, substrate specificity and catalytic properties (Matsushita & Adachi, 1993), the PQQ-binding domain of about 600 residues is conserved (Stoorvogel *et al.*, 1996). Since the structures of several MDHs and Q-EDH are known (Xia *et al.*, 1992, 1996; Blake *et al.*, 1994; Ghosh *et al.*, 1995; Keitel *et al.*, 2000), detailed information is available on how quinoprotein ADHs oxidize their substrates (Oubrie & Dijkstra, 2000; Zheng *et al.*, 2001). In contrast, the reoxidation of the enzymes, *i.e.* the transfer of protons and electrons from reduced PQQ to the protein's exterior and to the physiological electron acceptor, respectively, is less well understood.

To obtain more information on the oxidative half-reactions of quinoprotein ADHs, we have started a structural investigation of the type I QH-ADH from *C. testosteroni* (73 kDa; 677 residues). This enzyme oxidizes primary alcohols (except methanol) and aldehydes to their corresponding aldehydes and acids at a pH optimum of 7.7 (Groen *et al.*, 1986; de Jong *et al.*, 1995). Substrate oxidation is coupled to PQQ reduction, followed by electron transfer from reduced PQQ to haem *c* (Geerlof *et al.*, 1994). The final physiological electron acceptor is azurin (Matsushita *et al.*, 1999). Here, we describe the successful crystallization of QH-ADH, as well as the determination of the orientation of the haem *c* with respect to the unit-cell axes from the optical properties of the crystals.

2. Materials and methods

2.1. Crystallization and data collection

QH-ADH from *C. testosteroni* was routinely purified in the apo form (*i.e.* without PQQ) as described previously (Groen *et al.*, 1986; de Jong *et al.*, 1995). Reconstitution with PQQ was carried out at room temperature in 3 m*M* $CaCl_2$, 100 m*M* Tris–HCl pH 7.5. After recon-

stitution, the protein was dialyzed against various buffer solutions used for crystallization experiments. Alternatively, the enzyme was purified in the holo form when PQQ was supplemented to the growth medium as a vitamin (Groen *et al.*, 1986).

Crystallization conditions were screened using the hanging-drop vapour-diffusion method. Equal volumes (3–5 µl) of protein and precipitant solution were mixed and equilibrated against a 1 ml reservoir of precipitant solution.

For X-ray analysis crystals were flash-frozen in a stream of evaporating nitrogen. Diffraction data were collected on an in-house MacScience DIP2030H image plate (Nonius, Delft, The Netherlands) using Cu $K\alpha$ radiation from a Nonius FR591 rotating-anode generator with Franks' mirrors. Data were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and software from the Groningen *BIOMOL* package was used to convert the intensities to structure-factor amplitudes.

2.2. Determination of the orientation of haem *c* in the crystals

A crystal was mounted in a glass capillary and the orientation of the unit cell was determined using a FAST area detector (Nonius, Delft, The Netherlands). The directions of the unit-cell axes were indicated by sticking three pieces of metal wire into the clay that was used for fixing the capillary on the goniometer head. The latter was then transferred to a crystal alignment device (in-house design) mounted on a Leitz SM Lux Pol polarizing microscope. This setup allows the crystal to be rotated about two perpendicular axes, one along the viewing direction of the microscope and another one about the axis of the goniometer head. Both axes are perpendicular to the light source. The relation between crystal colour and crystal orientation was established by visual inspection under plane-polarized light.

3. Results and discussion

3.1. Crystallization

Crystals could be obtained by using a 5–8 mg QH-ADH per millilitre of protein solution and a reservoir solution containing 17–20%(*w*/*v*) polyethylene glycol (PEG) 6000, 0–4 m*M* EDTA, 100 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 5.5–5.7. Single crystals grew at 277 K within one week to approximate dimensions of 0.4 × 0.2 × 0.1 mm. At temperatures above 283 K no crystals grew. EDTA was added to reduce the formation of amorphous precipitate. It was difficult to obtain crystals from reconstituted holo enzyme and these crystals did not appear very regular. In contrast, a higher number of large regular single crystals could be grown from protein that was purified as holo enzyme. The diffraction quality of both types of crystals is comparable, however. No crystals were obtained of the apo enzyme. QH-ADH crystals are stable in a solution containing 20%(*w*/*v*) PEG 6000, 100 m*M* MES pH 5.7. They can be flash-frozen after soaking for 1 min in a cryopreservative consisting of 20%(*v*/*v*) glycerol, 20%(*w*/*v*) PEG 6000, 100 m*M* MES pH 5.7.

3.2. Data collection

A native data set was collected to 2.44 Å resolution. The crystals belong to space group *C*2 and have unit-cell parameters a = 98.1, b = 74.3, c = 92.2 Å, β = 105.9°. Assuming one monomer of 73 kDa per asymmetric unit, the crystal volume per unit mass (V_M) is 2.2 Å^3 Da^{-1} and the solvent content is 44.0%, values which are typical for protein crystals (Matthews, 1968). The presence of only one monomer per asymmetric unit is supported by the absence of peaks in self-Patterson and self-rotation functions. Data-collection statistics are summarized in Table 1.

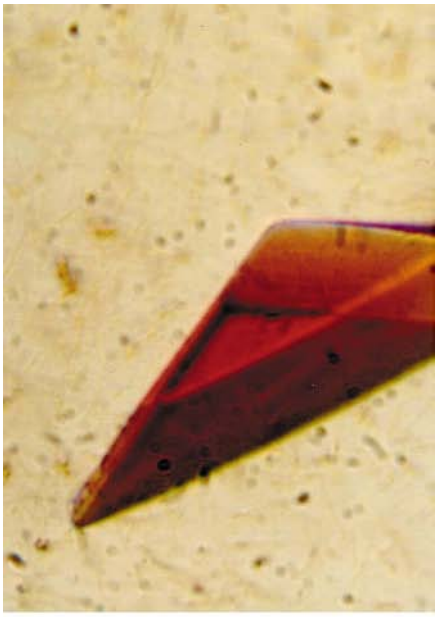
3.3. Haem orientation

Crystals of QH-ADH have special optical properties. When viewed under plane-polarized light the intense orange colour caused by the presence of the haem group disappears completely (Fig. 1). This property was used to derive an approximate orientation of the normal to the haems in the crystals.

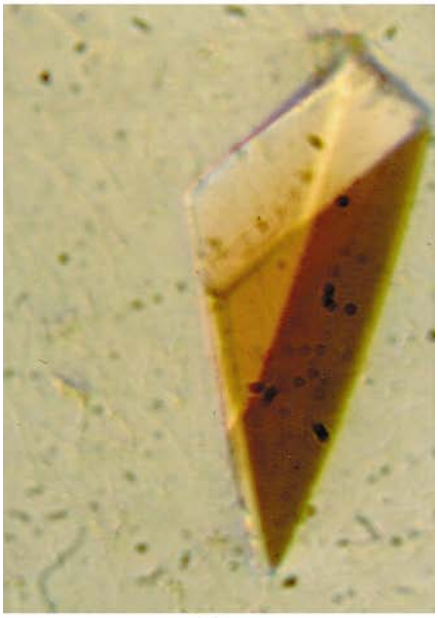
The colour of haemoproteins is mainly caused by $\pi\rightarrow\pi^*$ electronic transitions in the conjugated ring system of the porphyrin ring (Campbell & Raymond, 1984). Since the transition dipole moments of these transitions lie in the plane of the porphyrin, light will only be absorbed if the electric field component oscillates in the plane of the haem. Consequently, a crystal viewed under plane-polarized light will appear colourless if the planes of all haems are perpendicular to the plane of polarization.

In the QH-ADH crystals with *C*2 symmetry and one molecule in the asymmetric unit, the four haems in the unit cell have only two different orientations, related by an 180° rotation about the *b* axis. These crystals can become completely colourless only when both orientations are simultaneously oriented perpendicular to the plane of polarization. From symmetry considerations it can be derived that this is only possible if the *b* axis is either perpendicular to or parallel with the plane of the haems.

These two possibilities can be discriminated by observing the change in crystal colour while looking along and rotating



(*a*)



(*b*)

Figure 1
Illustration of the optical properties of QH-ADH crystals when viewed under plane-polarized light: (*a*) a crystal in an arbitrary orientation, (*b*) the same crystal in a special orientation. In this orientation, the crystal appears colourless because all haems in the crystal are simultaneously perpendicular to the plane of polarization.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.48–2.44 Å).

Space group	*C*2
Unit-cell parameters (Å, °)	a = 98.1, b = 74.3, c = 92.2, β = 105.9
V_M (Å^3 Da^{-1})	2.2
Solvent content (%)	44.0
Resolution (Å)	2.44
No. of measured reflections	84155
No. of unique reflections	23377
Redundancy	3.6
Completeness (%)	99.0 (91.7)
$R_{sym}(I)$† (%)	7.9 (28.2)
Data with $I > 3\sigma(I)$ (%)	84.3

† $R_{sym}(I) = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

about the *b* axis. If the *b* axis is perpendicular to the plane of the haem, the light running parallel to the *b* axis will be absorbed in all orientations of the crystal, which will therefore always appear orange. If, however, the *b* axis is parallel with the plane of the haem, the crystal colour will alternate from intensely orange to fully colourless.

Following this procedure, it was observed that the colour alternated from intensely orange to completely colourless, showing that the haem rings are oriented parallel to the *b* axis. The angles between the planes of the haem and the *a* and *c* axes were determined by marking the orientations in which the crystal was colourless with respect to the known directions of the *a* and *c* axes. For three crystals, the angles between the *c* axis and the haem plane were measured to be 4, 7 and 9°. Fig. 2 shows the orientation of the haem with respect to the unit-cell axes.

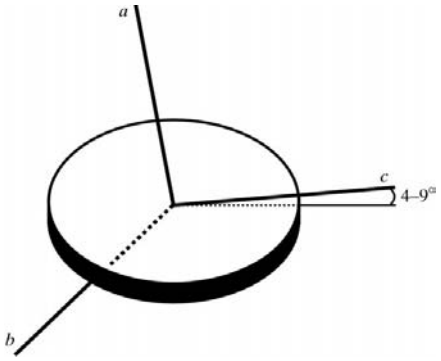


Figure 2
Approximate orientation of the planes of haem *c* with respect to the unit-cell axes in crystals of QH-ADH. The haem is depicted as a flat disc.

It should be noted that only the orientation of the plane of the haem with respect to the unit cell can be determined using this method, since a rotation about the normal to the haem or turning the haem upside-down does not effect the absorption of light significantly.

We are currently trying to solve the structure of QH-ADH by molecular replacement using the coordinates of the structures of MDH and Q-EDH. Since these proteins do not contain a haem-binding domain, this domain will have to be located independently. It is possible that knowledge of the orientation of the haem in the crystals will assist in this process.

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