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Crystallization and preliminary diffraction studies of two quinoprotein alcohol dehydrogenases (ADHs): a soluble monomeric ADH from *Pseudomonas putida* HK5 (ADH-IIB) and a heterotrimeric membrane-bound ADH from *Gluconobacter suboxydans* (ADH-GS)

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Crystals of a soluble monomeric quinocytochrome alcohol dehydrogenase (ADH-IIB) and of a trimeric membrane-associated quinocytochrome alcohol dehydrogenase (ADH-GS) have been obtained. The ADH-IIB crystals are triclinic, with one monomer in the unit cell, and were obtained in the presence of PEG 8000, sodium citrate, HEPES buffer and 2-propanol. X-ray data were collected at 110 K to 1.9 Å resolution ($R_{\rm merge}$ = 6.4%) and the orientation of a methanol dehydrogenase search molecule (from *Methylophilus methylotrophus* W3A1) was obtained by molecular replacement. Preliminary refinement of this model (10.0–3.0 Å resolution, R = 0.37, $R_{\rm free}$ = 0.40) led to tentative identification of the two highest peaks in a native anomalous difference Fourier map as the Fe atom of the heme and a calcium ion interacting with the PQQ prosthetic group. The ADH-GS crystals are tetragonal, displaying six similar lattices, both primitive and centered, and were grown by the sitting-drop method after replacement of Triton X-100 by dodecylmaltoside or octaethylene glycol monododecyl ether in the presence of ammonium sulfate and sodium acetate buffer, with and without PEG 3500 and calcium ion. The best diffraction is obtained at 110 K where the resolution extends to about 4 Å in the a and b directions and about 3 Å in the c direction.

1. Introduction

Many aerobic bacteria produce alcohol dehydrogenases (ADHs) which are independent of pyridine nucleotides and contain non-covalently bound pyrroloquinoline quinone (PQQ) as a redox cofactor. A well known example (Anthony, 1993) found in many methylotrophic bacteria is methanol dehydrogenase (MEDH), an $\alpha_2\beta_2$ heterotetramer of ~60 kDa (α) and ~8 kDa (β) subunits. The larger subunit is an eight-stranded β-propeller and the PQQ is located close to the eightfold pseudo-symmetry axis and is slightly buried in a funnel-like depression (Xia *et al.*, 1996; Ghosh *et al.*, 1995).

In non-methylotrophic bacteria, there are three classes of quinoprotein ADHs (Matsushita *et al.*, 1994). Type I ADH (isolated from *Pseudomonas putida* and from *P. aeruginosa*) is a soluble dimeric enzyme of identical subunits of ~60–65 kDa containing PQQ but no other redox cofactors.

Type II ADHs are monomeric soluble quinohemoproteins of ~70–75 kDa which contain PQQ and covalently bound heme and are found in several strains of *P. putida* (Toyama *et al.*, 1995) and in *Comamonas testosteroni* (Groen *et al.*, 1986). Sequence analysis of the *C. testosteroni* enzyme indicates that the first 60 kDa is homologous to MEDH and the remaining 15 kDa appears to form a *c*-type cytochrome domain. Two forms of type II ADH, ADH-IIB and ADH-IIG, can be isolated from *P. putida* strain HK5 cells grown on 1-butanol or on glycerol, respectively. Their substrate specificities are somewhat different, with ADH-IIB preferring medium chain-length primary and secondary alcohols and ADH-IIG preferring diols or glycerol. ADH-IIB is estimated to have a molecular weight of 69 kDa on the basis of sodium dodecyl sulfate polyacrylamide gel electrophoresis and has been shown to be monomeric (Toyama *et al.*, 1995). Its amino-acid sequence is unknown. *In vitro*, potassium ferricyanide can serve as an artificial electron acceptor for ADH-IIB and is used in a dye-linked assay. An azurin isolated from the same organism has been shown to act as an efficient electron acceptor for ADH-IIB (Matsushita, Yamashita *et al.*, 1999) suggesting that it acts *in vivo* as an electron-transfer mediator in a PQQ-dependent alcohol respiratory chain.

Table 1
Data-collection statistics for ADH-IIB.

Resolution (Å)	Number of reflections	Redundancy	Completeness (%)	$I/\sigma(I)$	R_{merge} (%)
30.0–4.09	5519	4.1	98.7	35.4	3.6
4.09–3.25	5420	4.1	97.2	27.9	4.6
3.25–2.84	5357	3.9	95.7	18.2	7.0
2.84–2.58	5264	3.8	94.5	15.6	8.5
2.58–2.39	5214	3.6	93.6	12.8	9.5
2.39–2.25	5144	3.5	91.5	10.8	11.0
2.25–2.14	5055	3.3	90.5	8.4	12.8
2.14–2.05	4961	3.2	89.1	6.5	15.7
2.05–1.97	4080	3.0	73.1	5.2	18.6
1.97–1.90	2643	2.8	47.3	4.2	21.5
Overall	48657	3.6	87.1	18.2	6.4

Table 2
Crystallization conditions and crystal properties of active ADH-GS.

All crystallizations were performed using the sitting-drop technique at 277 K.

Crystal form	*A*	*B*
Protein concentration (mg ml^{-1})	6–10	10
pH†	4.7	4.5
Precipitant	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$
Salt	—	$CaCl_2$ (2 m*M*)
Detergent	DM (0.26 m*M*)	$C_{12}E_8$ (0.16 m*M*)
Crystal size (mm)	0.6 × 0.3 × 0.2	0.2 × 0.13 × 0.1
Space group	*I*422	$P42_12$
Unit-cell parameters (Å)		
a	241.6	241.6
c	307.8	307.8
Z (molecules per asymmetric unit)	2	4
V_M (Å^3 Da^{-1})	4.0	4.0
Solvent content (%)	69	69
Resolution (Å)		
Along *a* and *b*	4.0	7.0
Along *c*	3.0	4.0

† 100 m*M* sodium acetate.

Type III ADH is a membrane-bound complex of three subunits which catalyzes the oxidation of ethanol and the subsequent reduction of ubiquinone within the cytoplasmic membrane (Matsushita *et al.*, 1994). The enzyme is found mainly in acetic acid bacteria such as *Gluconobacter suboxydans* and *Acetobacter aceti*. In these organisms, the type III ADH is one of two major protein complexes within the membrane, the other being ubiquinol oxidase, a cytochrome *o*-type terminal oxidase. Type III ADH contains one PQQ and four heme groups and requires the detergent Triton X-100 for solubilization. Subunit I (∼75 kDa) contains one PQQ and one heme *c* per monomer. Subunit III (∼15 kDa) has unknown function, but may act as a molecular chaperonin. Subunit II is a three-heme cytochrome (∼50 kDa) and contains the membrane-binding function of the complex. Dissociation of ADH from *G. suboxydans* (ADH-GS) into its subunits and reconstitution indicates that oxidation of alcohol (ethanol) occurs in subunits I/III, while the ubiquinone reductase activity is localized in subunit II (Matsushita *et al.*, 1996). Growth of *G. suboxydans* can be manipulated to produce either an 'active' or an 'inactive' form of ADH (Matsushita *et al.*, 1995). The former has an approximately tenfold higher ubiquinone-reductase activity than the latter. The 'active' form is isolated in a fully reduced state, whereas the heme in the isolated 'inactive' form of the enzyme is partially oxidized and can be reduced fully with dithionite. The subunit composition and prosthetic group content (one PQQ and four hemes) are identical in the two forms, but the folding of the 'inactive' form appears to be 'looser', as judged by its higher intrinsic tryptophan fluorescence when compared with the 'active' form. The ratio of 'active' to 'inactive' ADH in the organism is controlled by growth conditions, with higher pH and low aeration favoring the 'active' form. The 'inactive' form of ADH has increased ubiquinol-oxidase activity compared to the 'active' form, and this oxidase activity appears to be located at a site different from that of the ubiquinone-reductase activity (Matsushita, Yakushi *et al.*, 1999).

The full sequences of ADH from *G. suboxydans* (Kondo & Horinouchi, 1997) and from *A. aceti* (Inoue *et al.*, 1989) are known and share approximately 60% identity. A potential membrane-binding motif consisting of two short amphipathic helices has been identified on the basis of the sequence of subunit II (Matsushita, 1999). The sequence of the first 600 amino acids of subunit I of *A. aceti* is about 30% identical to that of the large subunit of MEDH from *Methylobacterium extorquens* AM1 (Anderson *et al.*, 1990). A molecular model of the PQQ-binding domain of *A. aceti* ADH has been constructed on the basis of the MEDH structure (Cozier *et al.*, 1995).

2. Experimental

2.1. ADH-IIB

ADH-IIB was isolated and purified as described previously (Toyama *et al.*, 1995). A solution of ADH-IIB (5 mg ml^{-1}) was screened for crystallization conditions using the sparse-matrix sampling technique (Jancarik & Kim, 1991) with Crystal Screens I and II from Hampton Research (USA) and the hanging-drop vapor-diffusion method at room temperature. After optimizing crystal-growth conditions obtained from the screens, tiny needle-like crystals were obtained by sitting-drop vapor diffusion at room temperature with a protein concentration of 7.5 mg ml^{-1} and 22% PEG 8000, 200 m*M* sodium citrate, 100 m*M* HEPES–HCl pH 7.5 and 6% 2-propanol. A tiny red crystal was crushed for use as seeds and transferred using a hair to fresh drops set up as above. Single crystals grew to an approximate size of 0.5 × 0.4 × 0.1 mm in 3–7 d.

Data collection was performed with an R-AXIS IV image-plate system mounted on a Rigaku RU-200 rotating-anode X-ray generator operating at 50 kV and 100 mA. The crystal (0.4 × 0.3 × 0.1 mm) used for the diffraction studies at 110 K was soaked with 15% glycerol for 15 min and diffracted to 1.8 Å resolution. The rotation range was 2° per frame, the crystal-to-distance distance was 160 mm and the exposure time was 30 min. Autoindexing with *DENZO* (Otwinowski, 1993) gave a triclinic cell, space group *P*1, with unit-cell parameters a = 54.8, b = 57.4, c = 67.5 Å, α = 89.6, β = 69.4, γ = 68.4°. Data frames were processed with *DENZO*/*SCALEPACK* (Otwinowski, 1993) giving an R_{sym} of 5%, where $R_{sym} = \sum|I - \langle I\rangle|/\sum I$. The data are 87% complete to 1.9 Å resolution (Table 1). Assuming a calculated molecular weight of 69 kDa and one molecule per asymmetric unit, the volume per asymmetric unit, V_m, is 2.74 Å^3 Da^{-1}, which lies in the normal range for globular proteins (Matthews, 1968) and corresponds to a solvent content of 55%.

2.2. ADH-GS

ADH-GS was prepared as described previously (Matsushita *et al.*, 1995). Crystals of both the active and inactive forms of ADH-GS have been grown by sitting-drop vapor diffusion at 277 K using 5–15 mg ml^{-1} protein concentration. The detergent used during enzyme purification, Triton X-100, was replaced either by *n*-dodecyl-β-D-maltoside (DM) or by octaethylene glycol monododecyl ether ($C_{12}E_8$) using a hydroxylapatite mini-column prior to crystallization. The crystallization drops contained 10 mg ml^{-1} protein, 100 m*M* sodium acetate buffer pH ∼4.5, 0.34 m*M* DM or 0.16 mM $C_{12}E_8$ and either ∼150 m*M* ammonium sulfate/6% PEG 3350 or ∼1.3 *M* ammonium sulfate only; 2 m*M* Ca^{2+} was

Table 3
Crystallization conditions and crystal properties of inactive ADH-GS.

All crystallizations were performed using the sitting-drop technique at 277 K.

Crystal form	*A*	*B*	*C*	*D*
Protein concentration (mg ml^{-1})	10	10–15	7–10	7–10
pH†	4.3	4.5	4.5	4.5
Precipitant	$(NH_4)_2SO_4$ (1.2 *M*)	PEG 3350 (6%)	PEG 3350 (6%)	PEG 3350 (6%)
Salt	—	$(NH_4)_2SO_4$ (150 m*M*)	$(NH_4)_2SO_4$ (150 m*M*), $CaCl_2$ (2 m*M*)	$(NH_4)_2SO_4$ (150 m*M*)
Detergent	DM (0.34 m*M*)	DM (0.34 m*M*)	DM (0.26 m*M*)	DM (0.26 m*M*)
Other	—	—	—	Sorbitol (100 m*M*)
Crystal size (mm)	0.5 × 0.2 × 0.1	0.8 × 0.2 × 0.1	0.8 × 0.2 × 0.1	0.8 × 0.2 × 0.1
Space group	*I*422	$P42_12$	*I*422	$P42_12$
Unit-cell parameters (Å)				
a	240.5	243.0	253.4	245.4
c	309.4	306.8	302.0	307.5
Z (molecules per asymmetric unit)	2	4	2	4
V_M (Å^3 Da^{-1})	4.0	4.0	4.3	4.1
Solvent content (%)	69	69	72	70
Resolution (Å)				
Along *a* and *b*	5.0	5.0	5.0	5.0
Along *c*	3.5	3.5	3.5	3.5

† 100 m*M* sodium acetate.

Table 4
Data-collection statistics for ADH-GS.

Resolution (Å)	Number of reflections	Redundancy	Completeness (%)	$I/\sigma(I)$	R_{merge} (%)
30.0–6.66	8397	5.9	98.2	29.7	5.2
6.66–5.29	8087	5.3	97.8	12.9	11.2
5.29–4.63	8002	5.0	97.4	9.8	13.6
4.63–4.21	7893	4.5	96.7	7.2	16.0
4.21–3.90	7779	3.9	95.5	4.4	22.3
3.90–3.67	7692	3.4	94.6	3.2	27.3
3.67–3.49	7625	3.1	94.0	2.7	31.9
3.49–3.34	7363	2.8	90.8	2.3	36.7
3.34–3.21	5829	2.4	71.8	2.0	38.8
3.21–3.10	4575	2.1	56.9	1.9	41.3
Overall	73242	4.0	89.5	6.3	13.0

sometimes included as an additive. The crystals grow as prisms of approximate dimensions 0.6 × 0.2 × 0.1 mm and are tetragonal; the space group is either *I*422 or $P42_12$ (depending on growth conditions) with two or four complexes per asymmetric unit, respectively, and have ∼70% solvent content. For active ADH-GS (Table 2), the two space groups have the same unit-cell parameters, $a = 242.6$, $c = 307.8$ Å, while for the inactive form several lattices have been observed (Table 3), with *a* ranging from 240.5 to 253.4 Å and *c* ranging from 302.0 to 309.4 Å. In most cases, the crystals were frozen using 20% glycerol as a cryoprotectant and diffract anisotropically. In typical cases, the diffraction extends to 5.0 Å in the *a* and *b* directions and to 3.5 Å in the *c* direction and in the best case (under home-laboratory conditions) to 4.0 Å along *a* and *b* and to 3.0 Å along *c* (Tables 2 and 3).

Data were collected from the best crystal of active ADH-GS (form *A*, Table 2) using the R-AXIS IV image-plate system and RU-200 X-ray generator. With this crystal, we obtained 90% completeness to 3 Å resolution with an overall R_{sym} of 13% and $\langle I/\sigma \rangle \simeq 6$, where $\langle I/\sigma \rangle$ is the signal-to-noise ratio; in the highest resolution shell, the data were 57% complete with R_{sym} = 41% and $\langle I/\sigma \rangle \simeq 2$. The data-collection statistics are shown in Table 4.

3. Results and discussion

3.1. ADH-IIB

The data set from native ADH-IIB was analyzed by molecular replacement (MR) with *AMoRe* (Navaza, 1994), using MEDH from *M. methylotrophus* W3A1 (Xia *et al.*, 1996) as a search molecule. A single peak was found in the rotation function which was three times higher than the next highest peak. Calculation of a translation function was not necessary as the origin of the triclinic cell is arbitrary. The present *R* factor after refinement of the search model in the 10.0–3.0 Å resolution range using *X-PLOR* (Brünger, 1992) is 0.37 (R_{free} = 0.40), where $R = \sum |F_o - F_c| / \sum |F_o|$.

An anomalous difference electron-density map has been calculated for native ADH-IIB using the MR phases. The map showed two prominent peaks. The higher peak (14σ) is ∼20 Å from the PQQ position in the MEDH search molecule and we attribute this peak to iron. The lower peak (9σ) is close to the position of a calcium ion in MEDH which bridges the PQQ and the protein side chains and we attribute this peak to calcium.

About 35 soaking experiments have been carried out with about 20 different heavy-atom reagents, and changes in structure-factor amplitudes of 4–20% have been observed. The unit-cell parameters are nearly unchanged from the native for all derivatives tested, except for the β angle which changes by up to 3°; the larger changes in β are correlated with the larger change in structure factors.

The MR phases have been used to analyze the heavy-atom derivatives. Two compounds, $K_3UO_2F_5$ and CH_3HgCl, gave 12–15% changes in the structure-factor amplitudes and a change of approximately 0.4% in the β angle. Anomalous difference Fourier maps indicated two uranium-binding sites at 11σ and 10σ (separated by about 6 Å) and one mercury-binding site at 10σ. The heavy-atom parameters were refined using *SHARP* (La Fortelle & Bricogne, 1997) and the multiple isomorphous replacement (MIR) phases were refined by solvent flattening.

The MIR map shows electron density for the cytochrome domain as well as for the MEDH-like domain. There is planar electron density centered on the Fe atom which could accommodate a heme group. There is also planar density near the calcium site which could accommodate the PQQ. The planes of the two prosthetic groups are roughly perpendicular and the closest distance from the Fe atom to the PQQ is estimated to be approximately 20 Å. The MIR map is noisy, making interpretation difficult. However, using the MEDH model as a guide, we can follow the density in the MIR map and recognize some differences in the loops from the search model. In three areas (∼25–30 residues each), the MIR density is weak and difficult to follow, but the remainder of the large β-propeller domain could be interpreted.

3.2. ADH-GS

A search for heavy-atom derivatives has been initiated by soaking small crystals with heavy-atom reagents and recording data to about 6–8 Å resolution. So far, we have tested four compounds under one or two sets of conditions. One of these, K_2PtCl_4, appears to give significant changes (∼23%) in the structure factors compared with the other compounds (∼15%). We are also attempting to improve the diffraction quality of the ADH-GS crystals by employing other detergents and crystallization conditions.

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