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Crystallization and preliminary X-ray analysis of glucose dehydrogenase from *Haloferax mediterranei*

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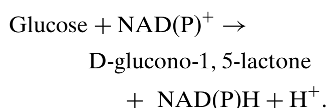
Glucose dehydrogenase (E.C 1.1.1.47; GlcDH) from *Haloferax mediterranei* has been overexpressed in *Escherichia coli*, solubilized by the addition of 8 *M* urea and refolded by rapid dilution. The protein has been purified by conventional techniques and crystallized by the hanging-drop vapour-diffusion method using sodium citrate as the precipitant. Two crystal forms representing the free enzyme and the binary complex with $NADP^+$ grow under these conditions. Crystals of form I diffract to beyond 3.5 Å resolution and belong to the hexagonal space group $P622$, with unit-cell parameters $a = b = 89.1$, $c = 214.6$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. Crystals of form II diffract to greater than 2.0 Å and belong to the orthorhombic space group $I222$ or $I2_12_12_1$, with unit-cell parameters $a = 61.8$, $b = 110.9$, $c = 151.7$ Å, $\alpha = \beta = \gamma = 90°$. Calculated values for V_M and consideration of the packing for both crystal forms suggests that the asymmetric units in both crystal forms contain a monomer.

1. Introduction

Archaea are recognized as a separate branch of organisms that are adapted to survive under conditions of extremes of temperature, pH or salinity (Woese *et al.*, 1990). Halophilic Archaea are specialized to function under high-salt conditions (Eisenberg *et al.*, 1992) and grow optimally in solutions containing 2.5–5.2 *M* NaCl; they are unable to grow at salinities below 2 *M* (Kamekura, 1998). Halophilic organisms have been found growing in natural salt lakes in which the concentration of salt can exceed 3 *M* (Galinski & Trüper, 1994). In order to survive, halophilic organisms must maintain a positive turgor pressure and therefore commonly accumulate inorganic ions to osmotically balance the high environmental salt concentrations.

Novel pathways of sugar metabolism have been found in Archaea. In *H. mediterranei*, a modified Entner–Doudoroff pathway is used for the catabolism of glucose (Danson, 1989). The first step of this pathway is performed by the enzyme glucose dehydrogenase (GlcDH), which catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone using NAD^+ or $NADP^+$ as a coenzyme,



$$\text{Glucose} + \text{NAD(P)}^+ \rightarrow \text{D-glucono-1, 5-lactone} + \text{NAD(P)H} + \text{H}^+.$$

H. mediterranei GlcDH has recently been cloned, sequenced and characterized and shown to function as a zinc-dependent homodimeric enzyme of subunit molecular weight 39 kDa with dual cofactor specificity, but with a marked preference for $NADP^+$ (Bonete *et al.*, 1996). Sequence comparisons have shown that the *H. mediterranei* GlcDH shares considerable sequence similarity with the homologues from other Archaea, including those from *Sulfolobus solfataricus* and *Thermoplasma acidophilium*, to which it shows 39 and 41% identity, respectively (John *et al.*, 1994). Analysis of the sequences of these enzymes and the structure determination of *T. acidophilium* GlcDH have established that they are members of the zinc-dependent medium-chain dehydrogenase superfamily (MDR; Edwards *et al.*, 1996) with highly conserved residues Cys46, Cys174 and His67 (*T. acidophilium* numbering) playing crucial roles in the activity of this enzyme (John *et al.*, 1994).

Previous structural studies of proteins adapted to high salt concentrations have revealed that a common feature is that their molecular surfaces are negatively charged (Dym *et al.*, 1995). Other comparative analyses have highlighted a significant reduction in exposed hydrophobic surface owing to a loss of exposed lysine residues (Britton *et al.*, 1998). However, there are currently only a limited number of halophilic proteins in the protein Data Bank. Thus, in order to enhance our understanding of the molecular factors controlling salt tolerance, we have initiated a structural program on *H. mediterranei* GlcDH. This will permit a detailed comparison to be made with the *T. acidophilium* structure as a contribution to this area. In this paper, we report the crystallization and preliminary X-ray analysis of GlcDH crystals from *H. mediterranei*.

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2. Experimental

The gene coding *H. mediterranei* GlcDH (ATCC 33500) was cloned in the expression vector pET3a (Novagen) and used to transform the *E. coli* strain BL21(DE3). The transformed cells were grown at 310 K to an OD_{600} of 0.5–1.0 in Luria–Bertani (LB) medium containing 50 µg ml^{-1} ampicillin. Induction of expression was achieved by the addition of IPTG to a final concentration of 0.4 m*M* and the cells were harvested by centrifugation after 3 h and then frozen. Examination of the cell extract showed that the overexpressed enzyme is present in the insoluble fraction.

The cells were resuspended in 20 m*M* Tris–HCl pH 7.4 containing 2 *M* NaCl and 1 m*M* EDTA (buffer *A*), lysozyme to a final concentration of 100 µg ml^{-1} and 0.1%(*v*/*v*) Triton X-100 and incubated at 303 K for 60 min. Cell disruption was achieved by sonication for 2 × 20 s in a Soniprep-200 sonicator with amplitude 16–18 µm. Debris was removed by centrifugation at 14 000*g* for 10 min and the insoluble pellet containing the enzyme was washed with buffer *A* and centrifuged at 10 000*g* for 5 min. This step was then repeated. The resultant pellet was then resuspended in 20 m*M* Tris–HCl pH 8.0 containing 8 *M* urea, 2 m*M* EDTA and 50 m*M* DTT and incubated at 310 K for 30 min.

A 40-fold rapid dilution with buffer *A* was then performed and $(NH_4)_2SO_4$ added to a final concentration of 2.5 *M*. The solution was centrifuged at 27 000*g* for 30 min. The supernatant was then loaded onto a DEAE-cellulose column equilibrated in 50 m*M* phosphate buffer pH 6.6 containing 2.5 *M* $(NH_4)_2SO_4$ and 1 m*M* EDTA. The GlcDH protein was eluted with 50 m*M* phosphate buffer pH 7.4 containing 2 *M* NaCl and 1 m*M* EDTA (Pire *et al.*, 2001) and prior to crystallization, protein samples were concentrated to approximately 25 mg ml^{-1} using a Vivaspin concentrator (30 000 Da MW cutoff; Viva Science). For crystallization of the binary complex of GlcDH with $NADP^+$, the cofactor was added after protein concentration to a final concentration of 1 m*M*. Crystals were grown using the standard hanging-drop vapour-diffusion technique by mixing small volumes (5–10 µl) of both the free enzyme and its complex with $NADP^+$ with an equal volume of a precipitant solution of sodium citrate over the concentration range 1.4–1.6 *M* in 100 m*M* HEPES buffer pH 7.0–8.0 and allowing the mixture to equilibrate by vapour diffusion with reservoirs of precipitant solution at 290 K. After approximately one week, crystals with a hexagonal bipyramid morphology (form I) with maximum dimensions of 0.25 × 0.40 × 0.25 mm were obtained for the free enzyme and crystals with a cubic morphology (form II) with maximum dimensions of 0.6 × 0.6 × 0.4 mm were obtained for the binary complex of the enzyme with $NADP^+$.

Form I and II crystals were mounted in X-ray-transparent capillaries. Preliminary data sets were collected by the rotation method with 1° rotations per frame using a MAR345 detector with double-mirror focused Cu $K\alpha$ X-rays produced by a Rigaku RU-200 rotating-anode generator. Data were processed and analysed using the *HKL* suite of programs (Otwinowski & Minor, 1997) and subsequently handled using *CCP*4 (Collaborative Computational Project, Number 4, 1994).

Table 1
X-ray data-collection statistics for the two crystal forms of glucose dehydrogenase.

Values in parentheses refer to the highest resolution shell (form I, 3.38–3.3 Å; form II, 2.05–2.0 Å).

	Form I	Form II
Resolution range (Å)	15–3.3	15–2.0
Wavelength (Å)	1.54	1.54
Space group	*P*622	*I*222 or $I2_12_12_1$
Unit-cell parameters (Å)		
a	89.1	61.8
b	89.1	110.9
c	214.6	151.7
Unique reflections	79132	156861
Completeness (%)	100 (100)	98.2 (95.8)
Multiplicity	9.84 (4.07)	4.8 (2.42)
$I/\sigma(I)$	21.0 (4.62)	16.0 (3.05)
R_{merge}†	0.08 (0.40)	0.06 (0.30)

† $R_{merge} = \sum |I - \langle I \rangle| / \sum I$, where *I* is the integrated intensity of a given reflection.

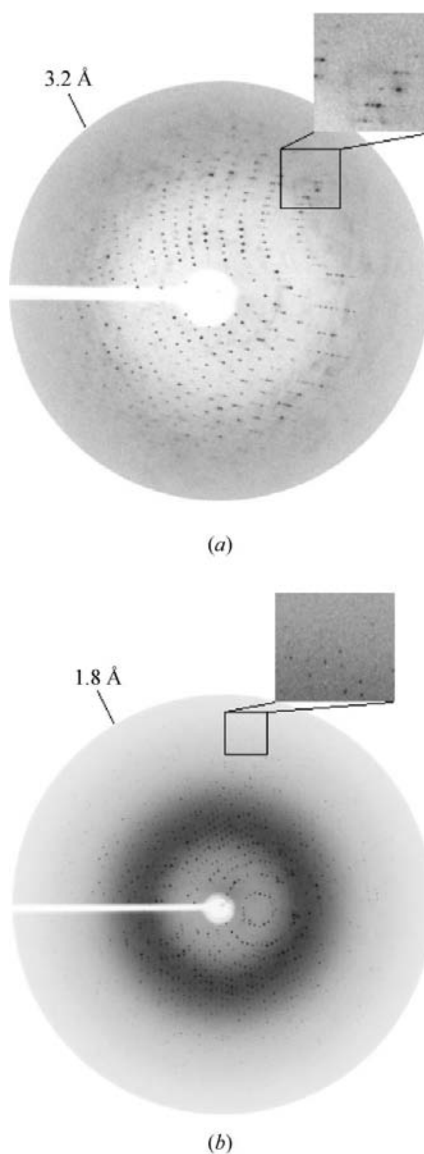

Figure 1
X-ray diffraction images recorded at room temperature on a MAR345 image plate of the form I (*a*) and form II (*b*) GlcDH crystals. The diffraction limit to the edge of the images is 3.2 and 1.8 Å, respectively.

3. Results and discussion

Analysis of the diffraction pattern of the form I crystals (Fig. 1*a*) showed that they belong to a hexagonal lattice (PG622), with unit-cell parameters $a = b = 89.1$, $c = 214.6$ Å, $\alpha = \beta = 90$, $\gamma = 120°$ and a unit-cell volume of 1.47×10^6 Å^3. Given the GlcDH subunit molecular weight of 39 kDa, the V_M for a monomer in the asymmetric unit is 3.1 Å^3 Da^{-1}, which lies within the normal range for proteins (Matthews, 1977). The form II crystals diffract to better than 2.0 Å resolution (Fig. 1*b*) and belong to one of the special pair of space groups *I*222 or $I2_12_12_1$, with unit-cell parameters $a = 61.8$, $b = 110.9$, $c = 151.7$ Å, $\alpha = \beta = \gamma = 90°$ and a unit-cell volume of 1.05×10^6 Å^3. As with the form I crystal, the asymmetric unit also appears to contain a monomer (V_M = 3.3 Å^3 Da^{-1}). Data-collection statistics for both crystal forms are given in Table 1.

A full structure determination is now under way and, given the high-resolution analysis possible with the form II crystals, we will be able to carry out a detailed comparison with the *T. acidophilium* enzyme as a contribution towards understanding the molecular basis of the salt tolerance of this enzyme.

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