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Crystallization and preliminary X-ray analysis of glucose dehydrogenase from *Bacillus megaterium* IWG3

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Glucose dehydrogenase from *Bacillus megaterium* IWG3 has been crystallized in the presence of NAD^+ using the hanging-drop vapour-diffusion method with PEG 2000 as the precipitant. Crystals belong to space group $C2$ and have unit-cell parameters a = 120.8 (1), b = 66.7 (1), c = 119.6 (1) Å, β = 93.25 (3)° with standard deviations in parentheses. Assumption of four subunits in the asymmetric unit gave the most probable Matthews coefficient V_M of 2.1 $Å^3$ Da^{-1} (solvent content 41.7% by volume). X-ray diffraction data were collected to 1.7 Å on a synchrotron-radiation source.

1. Introduction

Glucose dehydrogense (GlcDH; E.C. 1.1.1.47) catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone with NAD^+ or $NADP^+$ acting as a coenzyme (Pauly & Pfleiderer, 1975). GlcDH from *B. megaterium* is a tetrameric enzyme (M_r = 112 800) of four identical subunits (Jany *et al*., 1984; Makino *et al.*, 1989). The enzyme is inactivated in alkaline solution because of the dissociation of the tetramer into inactive monomers, which reassociate into the fully active tetramer reversibly when the pH is lowered to 6.5 (Pauly & Pfleiderer, 1977; Maurer & Pfleiderer, 1985; Maurer & Pfleiderer, 1987). The addition of 3 *M* NaCl prevents the alkaline dissociation, thereby stabilizing the tetramer structure (Maurer & Pfleiderer, 1985).

GlcDH belongs to the short-chain dehydrogenase/reductase (SDR) family (Jörnvall *et al.*, 1995). Currently, more than 1000 DNA sequences of SDRs can be found in the sequence database (Jörnvall *et al.*, 1999) and more than 50 different enzymes belonging to the family have been characterized (Jörnvall *et al.*, 1995). The SDR family is highly divergent in terms of sequence, with a typical pairwise sequence identity of 15–30%. The enzymes act on wide variety of substrates such as alcohols, sugars, steroids and aromatic compounds. The active form of the SDR enzyme is either a tetramer or a dimer, where each subunit typically consists of 250 amino-acid residues. The SDR enzymes have a typical N-terminal coenzyme-binding sequence G*xxx*G*x*G and an active-site sequence pattern Y*xxx*K (Jörnvall *et al.*, 1995).

About ten three-dimensional structures of the SDR family have been solved. In spite of their rather low sequence identities, all of them share a common subunit fold and intersubunit contacts (Ghosh *et al.*, 1991; Tanaka *et al.*, 1996; Benach *et al.*, 1998). Accordingly, the overall structure of GlcDH is inferred to resemble those of the tetrameric short-chain enzymes of known structure. To our knowledge, as a remarkable feature different from other SDRs, only GlcDH shows a reversible dissociation–association of subunits under moderate conditions. For the purpose of investigating this interesting characteristic, we cloned the GlcDH gene from *B. megaterium* IWG3 and isolated several stability-increasing mutant enzymes (Makino *et al.*, 1989). Subsequently, we constructed an overexpression and purification system for wild-type and mutant enzymes in *Escherichia coli* and characterized them biochemically (Nagao *et al.*, 1989). This shows that at least three amino-acid residues, Glu96, Gln252 and Tyr253, are responsible for the stabilization or destabilization of intersubunit interactions and/or tertiary structure of the protomer (Nagao *et al.*, 1989). Knowledge of the three-dimensional structure of the enzyme is indispensable for further understanding of the stability and subunit interactions of GlcDH. Pal *et al.* (1987) reported the crystallization of GlcDH from *B. megaterium* M1286. Although the amino-acid sequence identity is 82.8% between these two GlcDHs, the K_m value of GlcDH from *B. megaterium* M1286 for NAD^+ is about tenfold higher than that of GlcDH from *B. megaterium* IWG3. There has been no report of the crystal structure of this GlcDH and the authors have informed us that they have suspended their structure analysis (personal communication). Since our crystals were excellent in quality and suitable for high-resolution analysis, we started crystal structure analysis of our enzyme.

Table 1
Crystal data and intensity statistics.

Values in brackets refer to the last resolution shell (1.76–1.70 Å).

Space group	*C*2
Unit-cell parameters† (Å,°)	*a* = 120.8 (1)
	b = 66.7 (1)
	c = 119.6 (1)
	β = 93.25 (3)
V_M (Å^3 Da^{-1})	2.1
Number of subunits per asymmetric unit	4
Temperature (K)	290
Wavelength (Å)	1.0000
Resolution range (Å)	100.0–1.7
No. of unique reflections	94821
Completeness (%)	91.1 [77.3]
R_{merge}‡	0.033 [0.166]
$I/\sigma(I)$	29.2 [11.2]
Redundancy	3.77 [3.65]

† Standard deviations in parentheses. ‡ $R_{merge} = \sum_{hkl}\sum_i |I(hkl)_i - \langle I(hkl)\rangle| / \sum_{hkl} I(hkl)$, where $I(hkl)_i$ is the ith measurement of the intensity of reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity of reflection hkl.

In the present work, we report the crystallization of GlcDH in the presence of NAD^+ and present a preliminary crystallographic analysis of the crystals obtained.

2. Materials and methods

2.1. Crystallization

Cloning and expression of the GlcDH gene in *E. coli* has been described previously (Makino *et al.*, 1989). Overexpression and purification of GlcDH from *E. coli* cells were performed according to the method previously described (Nagao *et al.*, 1989).

Prior to crystallization, GlcDH was dialyzed against 50 m*M* (or 40 m*M*) sodium phosphate buffer pH 6.0 and its concentration was adjusted to 10 mg ml^{-1}. The protein concentration was determined by use of a molar absorption coefficient of ε_{280} = 132 000 M^{-1} cm^{-1} (Nakamura *et al.*, 1986).

Crystallization was performed by the hanging-drop vapour-diffusion method with the microseeding technique (McPherson, 1982). Falcon 3047 multi-well plates or their equivalents were used. A drop was prepared by mixing 5 µl of the protein solution in 50 m*M* sodium phosphate pH 6.0 and 5 µl of the reservoir solution *A* [30%(*w*/*v*) PEG 6000, 4 m*M* NAD^+, 50 m*M* sodium phosphate pH 6.0]. The drop was then equilibrated against 1.5 ml of reservoir solution *A*. Minute crystals appeared within two weeks at 277 K. Improved crystals were prepared with the microseeding technique as follows. 5 µl of 10 mg ml^{-1} protein solution in 40 m*M* sodium phosphate pH 6.0 and 5 µl of the reservoir solution *B* [28%(*w*/*v*) PEG 2000, 4 m*M* NAD^+, 40 m*M* sodium phosphate pH 6.0] were mixed and equilibrated against 1.5 ml of reservoir solution *B* at 294 K. A seed suspension was prepared by crushing the crystals obtained above in solution *B* and was diluted several times with solution *B*. 3 d after preparing the drop for crystallization, a seed suspension was added to the drop with a fine glass rod. Rod-shaped crystals grew to maximum dimensions of 0.25 × 0.25 × 3.0 mm in two weeks (Fig. 1). Since the relative humidity of the crystallization room was found to influence the reproducibility of the crystallization, it was kept at more than 55% during handling of crystallization drops in this study, securing the reproducibility of our crystallization.

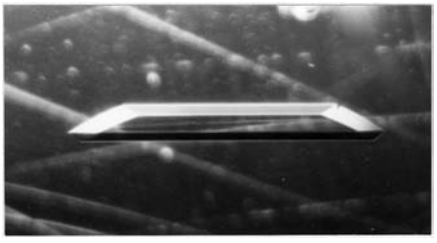

Figure 1
Crystal of glucose dehydrogenase with dimensions of 0.25 × 0.25 × 3.0 mm.

2.2. Data collection and processing

Prior to X-ray data collection, crystals were transferred from the hanging drops into the 'stock solution' containing 32%(*w*/*v*) PEG 2000, 4 m*M* NAD^+ and 40 m*M* sodium phosphate pH 6.0.

A data set for structure analysis was collected using synchrotron radiation at beamline BL-18B of the Photon Factory operated at 2.5 GeV at the High Energy Accelerator Research Organization, Tsukuba, Japan. Reflections were recorded on 400 × 800 mm imaging plates mounted on a screenless Weissenberg camera for macromolecular crystals (Sakabe, 1991) with a cylindrical cassette of 430 mm radius at 290 K. The X-ray beam was monochromated to 1.0000 Å with a Si(111) monochromator and collimated with a 0.2 mm diameter aperture. 100 oscillation photographs were recorded with an oscillation range of 1.8° and an exposure time of 72 s per frame on the Weissenberg camera and their latent images were digitized with a Rigaku SOR-DS48 scanner. The X-ray images were processed with the program *DENZO* and scaled with the program *SCALEPACK* (Otwinowski & Minor, 1997). The space group of the GlcDH crystals was determined to be monoclinic *C*2. Since *DENZO* did not evaluate the standard deviations of the unit-cell parameters, we estimated them by calculating standard deviations of unit-cell parameters produced for each frame by *DENZO*.

3. Results and discussion

Glucose dehydrogenase was crystallized in the presence of NAD^+ in two steps with the microseeding technique. In the present study, we noticed the effect of the humidity of the crystallization room on the crystallization during handling of crystallization drops. Whenever crystallization was carried out at a relative humidity of less than 55% at 294 K, the protein aggregated in drops within a day. Low humidity in the crystallization room increases the evaporation rate of water and hence might cause protein aggregation. The time lag between the preparation of a drop and seeding is also important. If seed suspension was added immediately after preparing a drop, no crystals were obtained because of the dissolution of seed crystals. Seed crystals must be added to the drop after the concentrations of protein and precipitant in the drop have risen high enough through water evaporation from the drop to the reservoir solution. Crystals belong to the monoclinic space group *C*2 and the unit-cell parameters (with standard deviations in parentheses) are *a* = 120.8 (1), *b* = 66.7 (1), *c* = 119.6 (1) Å, β = 93.25 (3)°. High-resolution data to 1.7 Å were collected at 290 K using the synchrotron-radiation source at the Photon Factory. Crystals did not show any sign of decay during data collection. Detailed data-collection statistics for the synchrotron data are given in Table 1. 357 354 measured reflections reduced to 94 821 unique reflections with an overall R_{merge} of 3.3%. This represented 91.9% completeness at 1.7 Å resolution. The outermost shell of resolution between 1.76 and 1.70 Å was 77.3% complete with an R_{merge} of 16.6%.

Assuming four subunits in the asymmetric unit, the Matthews parameter (Matthews, 1968) V_M = 2.1 Å^3 Da^{-1} (41.7% solvent content by volume) is well within the allowed range for crystalline proteins.

Despite their low sequence identities, the three-dimensional structures of SDR enzymes turn out to be very similar. This promising fact had lead to our undertaking the structure analysis of GlcDH by molecular replacement using SDRs of known structure as search models; the structure analysis is in progress.

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