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Expression, purification and preliminary crystallographic analysis of human sorbitol dehydrogenase

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Human sorbitol dehydrogenase (SDH) was expressed in *Escherichia coli* BL21 cells and purified using ammonium sulfate precipitation and anion-exchange and dye-affinity chromatography. Purified SDH was crystallized from polyethylene glycol solutions using the hanging-drop vapour-diffusion method. X-ray data were collected to 2.75 Å resolution. The crystals belong to the monoclinic $C2$ space group, with unit-cell parameters $a = 145.9$, $b = 52.3$, $c = 169.0$ Å, $\beta = 101.8^\circ$. This is the first crystallization report of human sorbitol dehydrogenase.

1. Introduction

Sorbitol dehydrogenase (SDH), a member of the medium-chain dehydrogenase/reductase protein family, is the second enzyme of the polyol pathway of glucose metabolism. The polyol pathway is comprised of aldose reductase, which converts glucose to sorbitol, and sorbitol dehydrogenase, which converts sorbitol to fructose strictly using NAD^+ as coenzyme. It is believed that the increased flux of glucose through the polyol pathway during hyperglycaemia contributes to the development of diabetic complications (Kinoshita & Nishimura, 1998; The Diabetes Control and Complication Trials Group, 1993). SDH is a tetramer with a catalytic Zn atom bound in the active site (Jeffery *et al.*, 1984). Human liver SDH has been purified previously and was shown to have a molecular weight of 155 kDa, with subunits of approximately 37 kDa (Maret & Auld, 1988).

Homology-modelling studies have suggested that SDH is structurally homologous to mammalian alcohol dehydrogenases (ADH) with respect to a conserved zinc-binding motif and a hydrophobic substrate-binding pocket (Jeffery *et al.*, 1981; Eklund *et al.*, 1985). Additionally, a model of human SDH in complex with coenzyme and inhibitor, based on the structure of human β_3 alcohol dehydrogenase (26% identity; Hurley *et al.*, 1991), has been published (Darmanin & El-Kabbani, 2001). More recently, the crystal structure of rat SDH has been solved (Johansson *et al.*, 2001) and was found to be similar to the mammalian ADH structure, containing two distinct catalytic and coenzyme-binding domains. However, the zinc coordination in the active site of rat SDH was found to be different from that of mammalian alcohol dehydrogenase. In rat SDH, the coordinating ligands are His69, Cys44, Glu155 and Glu70. On the other hand, the coordinating ligands in alcohol dehydrogenase are His69, Cys44 and Cys155 (using the residue numbering for rat SDH). Glu70 in alcohol dehydrogenase is in close proximity to the Zn atom but is not a ligand (Johansson *et al.*, 2001). Interestingly, the zinc coordination found in rat SDH is similar to that found in the bacterial tetrameric NADP(H)-dependent alcohol dehydrogenase of *Clostridium beijerinckii* (Korkhin *et al.*, 1998).

While the amino-acid sequences of rat and human SDH are similar (82% sequence identity), biochemical and modelling studies have suggested that non-conserved residues may be involved in the binding of both substrate and inhibitor (Darmanin & El-Kabbani, 2001; Höög *et al.*, 1993). These include a substitution at residue 274, which was shown by modelling to bind to both substrate and inhibitor, and substitutions on residues immediately adjacent to those involved in ligand binding (residues 203, 208, 272 and 344). These residues may be responsible for the differences in substrate and inhibitor specificities between the two enzymes. Höög *et al.* (1993) compared the kinetic constants of recombinant rat SDH and human liver SDH with sorbitol, fructose, 2,3-butanediol, ribitol, L-threitol and xylitol as substrates. They found that the enzymes have similar K_M values for most of these substrates, but the k_{cat} values were about tenfold lower for the rat enzyme.

Sorbitol dehydrogenase is expressed almost ubiquitously in all mammalian tissues, including the brain, lens, erythrocytes and liver (O'Brien *et al.*, 1983; Jedziniak *et al.*, 1981; Barretto *et al.*, 1985). The human enzyme has attracted considerable interest owing to its implication in the development of diabetic complications such as cataracts, neuropathy, retinopathy and nephropathy (Obrosova *et al.*, 1999). The determination of the crystal struc-

ture of human SDH will help to elucidate the catalytic mechanism and model the interactions of the enzyme with substrate and inhibitor. In this study, the purification of human sorbitol dehydrogenase, obtained from the expression of the SORD gene, is reported. The first report of the crystallization and preliminary crystallographic analysis of human sorbitol dehydrogenase is also presented. The structure of the human enzyme may facilitate the development of drugs for the treatment of diabetic complications.

2. Experimental

2.1. Expression and purification

The coding region of human sorbitol dehydrogenase (SORD) cDNA previously isolated from liver cDNA library (Iwata *et al.*, 1995) was inserted into prokaryotic expression vector [pET23(+) Novagen, Madison, WI, USA] and transformed in *Escherichia coli* BL21 (DE3) (Novagen). The SORD expression was triggered by an addition of 1 m*M* IPTG and incubated at 310 K for 4 h. The bacteria were collected by centrifugation and the pellet was resuspended in 10 m*M* sodium phosphate buffer pH 7.4 containing 5 m*M* β-mercaptoethanol and sonicated to release the protein into the supernatant. The supernatant containing the SDH was collected by centrifugation. Ammonium sulfate fractionation of the supernatant was carried out at 30, 50 and 70% saturation, with SDH precipitating out at both the 30 and 50% saturation. These pellets were resuspended in 10 m*M* sodium phosphate buffer pH 7.4 and dialyzed in the same buffer to remove any salt present in solution. The dialyzed samples were loaded onto a Q-Sepharose column (Amersham-Pharmacia) and eluted with a stepwise sodium chloride gradient. The fractions that eluted out at 0.1 *M* salt concentration were collected and concentrated using an Omega 10 kDa membrane (Pall) and dialyzed in 20 m*M* sodium phosphate buffer pH 6.5 containing 2 m*M* magnesium chloride. The dialyzed sample was loaded onto a Green 18 dye-affinity column and the bound SDH was again eluted with a salt gradient. Peak samples were assayed for activity and the purity of SDH was checked by 12.5% SDS–PAGE. A single band corresponding to approximately 37 kDa was obtained from the 0.3 *M* salt peak (Fig. 1). The purification protocol is shown in Table 1. The fractions that showed co-purification of the 37 kDa band and SDH activity were pooled. The combined fraction was then subjected to three concentration–dilution cycles at 3200*g* with milli-Q water, using a 10 kDa Ultrafree-4 centrifugal unit (Millipore) in a Megafuge 1.0 R centrifuge (Heraeus) to reduce the salt concentration in the sample. The enzyme was finally concentrated to 13 mg ml^{-1} for crystallization.

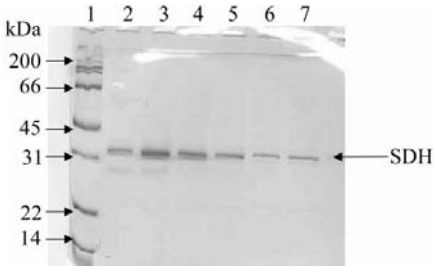

Figure 1
SDS–PAGE showing purified fractions of human SDH. Lane 1 shows standard molecular-weight markers and lanes 2–7 show purified enzyme from consecutive collection fractions.

Table 1
Purification of human sorbitol dehydrogenase.

Sample	Activity (mU ml^{-1})	Protein concentration (mg ml^{-1})	Specific activity (mU mg^{-1})	Overall purification†
Crude extract	ND‡	336.84	ND‡	—
Ammonium sulfate fraction	1.93×10^{-2}	57.19	3.34×10^{-4}	—
Q-column	3.21×10^{-3}	2.38	1.35×10^{-3}	Fourfold
Green column	1.61×10^{-3}	0.211	7.62×10^{-3}	23-fold

† Based on the specific activity of each subsequent step compared with that of the ammonium sulfate fraction (since the crude extract data was not available). ‡ ND, not determined: the initial reaction was too rapid to be followed by the conventional UV–Vis technique.

2.2. Enzyme assays and protein measurements

Sorbitol dehydrogenase activity was determined on a Shimadzu UV–Vis spectrophotometer (model UV160A) by following the increase in absorbance of NADH at 340 nm (ε = 6220 M^{-1} cm^{-1}). Each 1 ml assay sample contained 42 m*M* glycine buffer pH 9.9, 9.9 m*M* D-sorbitol and 0.5 m*M* β-NAD$^+$. The reaction commenced on the addition of the enzyme. One milliunit (mU) of activity is defined as the amount of enzyme needed to oxidize one millimole of substrate per minute under initial velocity conditions at room temperature (293 K).

Protein concentrations were routinely determined by using Coomassie Blue dye according to the method of Bradford (1976) or by measurement of the absorbance at 280 nm ($\varepsilon_{280\text{nm}}$ at 1 mg ml^{-1} = 0.57; Maret & Auld, 1988).

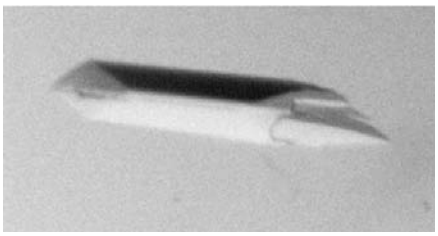

Figure 2
A crystal of human sorbitol dehydrogenase. The crystal in this photograph has dimensions of 0.3 × 0.06 × 0.06 mm.

2.3. Crystallization and X-ray data collection

Crystals of human sorbitol dehydrogenase were grown at 295 K by the vapour-diffusion method (McPherson, 1985). The enzyme and cofactor NAD$^+$, at a respective molar ratio of 1:6, were incubated at 277 K for 2 h. Each hanging drop consisted of 2.5 µl SDH holoenzyme, 2 µl well buffer (0.1 *M* Tris pH 8.6, 0.2 *M* sodium acetate and 10% PEG 3350) and 0.5 µl 30%(*v*/*v*) MPD (2-methyl-2,4-pentanediol). Crystals grew within one week to maximum dimensions of 0.3 × 0.06 × 0.06 mm (Fig. 2). The crystals were picked up with a nylon loop and flash-cooled at 100 K in a stream of gaseous nitrogen. A diffraction data set from one flash-cooled crystal was recorded at 100 K on a MAR345 image plate mounted on a Rigaku RU-300 rotating-anode X-ray generator operated at 50 kV and 90 mA. Each frame was recorded with a 1800 s exposure and 0.5° oscillation around φ. The crystal-to-detector distance was set to 250 mm so that the spots were well resolved. The data was processed and scaled using the *HKL* software package (Otwinowski & Minor, 1997).

3. Results

A near-complete set of data was collected from a single crystal to a resolution of 2.75 Å (Table 2 shows the data-collection statistics). Sorbitol dehydrogenase crystallized in the monoclinic *C*2 space group, with unit-cell parameters a = 145.9, b = 52.3, c = 169.0 Å, β = 101.8°. The Matthews coefficient was

Table 2
X-ray data-collection statistics.

Values in parentheses refer to the highest resolution shell, 2.85–2.75 Å.

Space group	*C*2
Unit-cell parameters	
a (Å)	145.9
b (Å)	52.3
c (Å)	169.0
β (°)	101.8
Resolution (Å)	20–2.75
Observed reflections	96908
Unique reflections	32335 (2865)
$R_{\rm merge}$† (%)	6.7 (16.2)
Completeness (%)	98.3 (89.2)
$I/\sigma(I)$	12.7 (5.2)
Average redundancy	3.0 (1.8)

† $R_{\rm merge} = (\sum |I_i - \langle I \rangle| / \sum I_i)/100$, where I_i is an individual intensity observation, $\langle I \rangle$ is the mean intensity for that reflection and the summation is over all reflections.

calculated to be 2.036 Å^3 Da^{-1} assuming two molecules (MW = 37 000 Da) of SDH to be present in the asymmetric unit (the remaining two molecules of the tetramer may be generated by crystallographic symmetry) and the estimated solvent content was 39.7% (Matthews, 1968).

Following the methodology described in the structure determination of rat SDH (Johansson *et al.*, 2001), the molecular-replacement method utilizing the coordinates of *C. beijerinckii* ADH (PDB code 1ped) as the starting model is currently being applied to solve the crystal structure of human SDH (atomic coordinates for rat SDH have yet to be deposited in the PDB). There is 22% sequence identity between *C. beijerinckii* ADH and human SDH. This will be the first crystal structure determined for human SDH. The structure will help to elucidate the catalytic mechanism and accurately model the interactions of the enzyme with substrate and inhibitor. Additionally, the comparisons between the crystal structures of human SDH, rat SDH and mammalian alcohol dehydrogenase will reveal important information about the different structural features between their active sites that are responsible for the differences in their substrate and inhibitor specificities. This information may be useful in the development of specific drugs for the treatment of diabetic complications.

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