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Structural characterization of the thermostable *Bradyrhizobium japonicum* d-sorbitol dehydrogenase

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Bradyrhizobium japonicum* sorbitol dehydrogenase is NADH-dependent and is active at elevated temperatures. The best substrate is d-glucitol (a synonym for d-sorbitol), although l-glucitol is also accepted, giving it particular potential in industrial applications. Crystallization led to a hexagonal crystal form, with crystals diffracting to 2.9 Å resolution. In attempts to phase the data, a molecular-replacement solution based upon PDB entry 4nbu (33% identical in sequence to the target) was found. The solution contained one molecule in the asymmetric unit, but a tetramer similar to that found in other short-chain dehydrogenases, including the search model, could be reconstructed by applying crystallographic symmetry operations. The active site contains electron density consistent with d-glucitol and phosphate, but there was not clear evidence for the binding of NADH. In a search for the features that determine the thermostability of the enzyme, the $T_m$ for the orthologue from *Rhodobacter sphaeroides*, for which the structure was already known, was also determined, and this enzyme proved to be considerably less thermostable. A continuous β-sheet is formed between two monomers in the tetramer of the *B. japonicum* enzyme, a feature not generally shared by short-chain dehydrogenases, and which may contribute to thermostability, as may an increased Pro/Gly ratio.

1. Introduction

Rare sugars have been proposed to have a broad potential for application, but low availability often hinders systematic studies (Beerens et al., 2012). They are known as sugar replacements (Lu et al., 2008; Baek et al., 2010), as therapeutics in cancer proliferation, as anti-inflammatory agents and as immunosuppressants (Lim & Oh, 2011). The so-called Izumoring (Granström et al., 2004) shows in detail that all polyols and rare sugars can be produced from natural sources by the use of oxidoreductases, epimerases and isomerases, but the corresponding enzymes often lack sufficient turnover numbers and product yields, generating a constant demand for better catalysts.

Among the rare sugars, d-sorbose is expected to be a building block for industrially interesting compounds in addition to its use as a low-calorie sweetener (Huwig et al., 1996). The subject of this study, *Bradyrhizobium japonicum* d-sorbitol dehydrogenase (*BjSDH*), was investigated within a project to find stable enzyme variants, specifically for use in
the construction of an electro-enzymatic flow-cell device for the production of rare sugars with electrochemical cofactor regeneration (Wang et al., 2012). B/SDH has been shown to preferentially catalyse the oxidation of D-glucitol to D-fructose, but additionally to catalyse the oxidation of L-glucitol to D-sorbitose, and offers interesting application potential with respect to its apparent $K_m$ value and its temperature stability (Gauer et al., 2014).

B/SDH is part of the superfamily of Zn-independent short-chain dehydrogenases (SDRs; Kallberg et al., 2010), to which about 25% of all dehydrogenases belong. This enzyme belongs to the classic subfamily of SDRs, as it includes the cofactor-binding motif $\text{TGxxx}[\text{AG}]xG$ and the active-site residues YxxK (Kavanagh et al., 2008). There are many structurally determined homologues of the sorbitol dehydrogenases, but the closest in sequence to B/SDH are only 33–35% identical in sequence. Most of them exist in a tetrameric form in solution (Philipsen et al., 2005). The pyridine nucleotides NAD(H) and NADP(H) always act as soluble cofactors, i.e. they are not covalently bound to the enzyme (Wang et al., 2012).

2. Materials and methods

2.1. Macromolecule production

B/SDH had previously been cloned from B. japonicum USDA110 chromosomal DNA and expressed in Escherichia coli. Briefly, the gene was cloned into pET-24a (+) from chromosomal DNA and an N-terminal His$_6$ tag was included (Table 1). The gene construct was expressed in E. coli BL21-Gold (DE3) and after an initial step of heat treatment at 50°C (Gauer et al., 2014) the protein was purified for structural studies by metal-affinity chromatography.

Rhodobacter sphaeroides sorbitol dehydrogenase (RsSDH; Philipsen et al., 2005) was produced for thermostability studies in a similar way. A His-tagged construct, N-His$_6$-RsSDH, containing the corresponding gene in the expression vector pET-24a (+) (Novagen), was expressed in E. coli BL21-Gold (DE3). The crude extract was applied onto a HiTrap FF Crude column (GE Healthcare, column volume 5 ml) equilibrated with binding buffer (20 mM Na$_2$HPO$_4$, 2 mM NaCl, 20 mM imidazole pH 7.4). After washing the column with two column volumes of binding buffer to eliminate unbound material, His-tagged dehydrogenase was eluted with elution buffer (20 mM Na$_2$HPO$_4$, 500 mM NaCl, 500 mM imidazole pH 7.2). Fractions containing the enzyme were pooled and loaded onto a Desalting XK 26/20 G25 column (GE Healthcare), which was equilibrated with 20 mM Tris–HCl buffer pH 7. The enzyme was eluted using the same buffer.

2.2. Crystallization and data collection

The tagged B/SDH was purified to homogeneity as judged by SDS–PAGE. B/SDH was dialyzed three times against 133 mM Tris–HCl pH 8.5 and concentrated to 21 mg ml$^{-1}$ using ultrafiltration cellulose membranes (Amicon). Final concentrations of 100 mM D-glucitol and 0.4 mM NAD$^+$ were added to the protein solution, which was subsequently spun for 10 min at 9500 RCF to remove higher molecular-weight aggregates. The supernatant was used to set up MRC 2-well sitting-drop trays with an Oryx 8 liquid-handling robot (Douglas Instruments). Initially, 0.3 μl drops (with protein solution:reservoir solution ratios of 1:1 and 3:1) were set up in Index HT (from Hampton Research), PACT and JCSG+ (both from Molecular Dimensions) screens and revealed crystalline material in 1.4 M Na$_2$HPO$_4$/K$_2$HPO$_4$ pH 5.6 (Index HT condition B5). 2 μl sitting drops were then set up manually in

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Table 1

<table>
<thead>
<tr>
<th>Source organism</th>
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<td>Expression host</td>
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Table 2

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Refinement

| Final $R_{	ext{free}}$ (%) | 27.00 |
| Final $R_{	ext{free}}$ (%) | 29.51 |
| No. of non-H atoms | Total | 1817 |
|                     | Protein | 1800 |
|                     | Ligand | 17 |
| R.m.s. deviations |
| Bonds (Å) | 0.004 |
| Angles (°) | 0.765 |
| Average B factors (Å$^2$) |
| Overall | 68.05 |
| Protein | 68.10 |
| Ligand | 63.11 |
| Ramachandran plot |
| Favoured regions (%) | 88.6 |
| Additionally allowed (%) | 9.3 |
| Outliers (%) | 2.1 |


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a 1:1 ratio at 10 mg ml⁻¹ protein concentration, varying the precipitant concentration. Microcrystals were obtained in 1.8 M NaH₂PO₄/K₂HPO₄ pH 5.6 and were used to streak-seed protein solution and 1.4 M NaH₂PO₄/K₂HPO₄ pH 5.6 in a 1:1 ratio. Disc-shaped crystals grew within six months. They were cryoprotected with 26.1% glycerol in reservoir solution, mounted in nylon loops and flash-cooled in liquid nitrogen.

Data were collected on ESRF beamline ID29 (de Sanctis et al., 2012) at a wavelength of 0.9 Å and a temperature of 100 K. A total of 250° of data were collected over 500 images. Processing and scaling were performed with XDS (Kabsch, 2010) using XDSAPP (Krug et al., 2012). The data could be processed in the hexagonal space groups P6₂22 or P6₄22. Because of visible radiation damage, only the first 100° of data were used as the final data set, but because of the high symmetry a complete and redundant data set was obtained nonetheless (Table 2). A resolution limit of 2.90 Å was chosen according to the criterion of a CC₁/₂ value (Karplus & Diederichs, 2012) of around 50% in the outer resolution shell.

A criterion of I/σ(I) > 2 in the outer resolution shell would result in a resolution limit of 3.15 Å.

2.3. Structure determination and refinement

Using BLASTP to perform a search of the Protein Data Bank with the BjSDH sequence identified the closest relative to be FabG from Bacillus sp. strain SG-1 (PDB entry 4nbu; Javidpour et al., 2014), with 33% sequence identity to BjSDH. Using the A monomer, a model for molecular replacement was sculpted using phenix.sculptor with the BjSDH sequence as input (Bunkóczi & Read, 2011). The model was loaded into PyMOL (v.1.8; Schrödinger) together with PDB entries 2jap and 3a1l (the second and third hits in the BLASTP search) and then pruned manually by removing the most variable regions (identified visually as residues 36–48, 191–208 and 238–242; Bj/SDH numbering). The resulting coordinates were used as a search model in molecular replacement with phenix.automr (McCoy et al., 2007), resulting in a solution in

Figure 1
(a) Cartoon representation of the monomer of BjSDH, coloured according to secondary structure (red, helices; yellow, β-strands; green, loop regions). The seven β-strands βA–βG form a parallel β-sheet surrounded by α-helices. A longer insert between βF and αG contains a small helix–turn–helix motif serving as a ‘lid’ to close the active site. Glucitol, phosphate and the residues involved in catalysis are shown in stick representation. (b) Cartoon representation of the tetramer of BjSDH. The seven-stranded β-sheet can be seen as it continues between monomers (teal/green and pink/yellow). (c) The active-site catalytic residues with mFobs – DFc difference OMIT density at +3σ shown in green. The map is from before ligands were included in the model. (d) The residual mFobs – DFc electron-density map shown in green at +3σ after the inclusion of glucitol and phosphate in the model.
space group \textit{P}6\textsubscript{2}22 with a final LLG of 135.4 and a TFZ of 14.3 (a value of >8 indicates a clear solution). After one round of refinement with \textit{phenix.refine} (Afonine et al., 2012) initial \(R_{\text{work}}\) and \(R_{\text{free}}\) values of 43 and 48\%, respectively, were obtained, further indicating a correct solution. Several rounds of refinement and careful manual model building using \textit{Coot} (Emsley et al., 2010) gave a final model (Fig. 1) including residues 4–242, one molecule of glucitol and one phosphate ion, with a final \(R_{\text{work}}\) and \(R_{\text{free}}\) of 27.0 and 29.5\%, respectively (see Table 2 for further statistics). Glucitol and one phosphate ion were included in the model based on the difference density at 3\(\sigma\) in a refinement map (shown in Fig. 1c). No water molecules have been included in the model owing to the low resolution. Five residues (Cys80, Ile105, Arg131, Leu199 and Ser207) were flagged as Ramachandran outliers in the PDB validation report. The model was checked against a composite OMIT map calculated with \textit{phenix.composite_omit_map} using standard parameters.

2.4. Structure analysis and representation

The \textit{DALI} server (Holm & Rosenström, 2010) was used to identify the closest structural relatives to the \textit{Bj}SDH structure. Each chain in a PDB file is included in the server database, so the final list was filtered for identical chains to obtain the top five most similar structures. The quaternary assembly of monomers was analysed with \textit{Proteins, Interfaces, Structures and Assemblies (PISA)} through \textit{qPISA} (Krissinel, 2015) from the CCP4 suite of programs (Winn et al., 2011). \textit{THESEUS} was used to superpose structures (Theobald & Steindel, 2012). \textit{PyMOL} was used to generate the representations of the analysed structures and \textit{Inkscape} (http://inkscape.org; v.0.48) was used to annotate and arrange the figures as well as to draw the structural diagram.

2.5. Circular-dichroism measurements

A J-815 CD spectrometer (Jasco) equipped with a Jasco CDF-426S/15 temperature-control unit was used to record CD spectra and denaturation curves for \textit{Rs}SDH. Owing to some precipitation, all samples were centrifuged (10 min, 9500 RFC) before use. The clear supernatant was used for CD measurements. Spectra were recorded in water and diluted buffer (\textit{Rs}SDH at 1 mM Tris–HCl pH 7.0). Quartz glass far-UV and near-UV CD cuvettes (Hellma) with light paths of 1 mm were used. The denaturation curves were recorded at a single wavelength at 222 nm. The temperature range was 4–60°C. The peak in the second numerical derivation was used to estimate \(T_m\) after fitting of the sigmoidal curves in \textit{Excel} (Microsoft).

3. Results and discussion

3.1. Crystal structure

The \textit{Bj}SDH structure folds in the Rossmann fold (Rao & Rossmann, 1973), which is typical for NADH-dependent enzymes and the SDR family (Fig. 1a). The monomer of \textit{Bj}SDH is composed of a central parallel \(\beta\)-sheet surrounded by three \(\alpha\)-helices on each side. At the top of the \(\beta\)-sheet a small helix–turn–helix motif is located between the two final \(\beta\)-strands \(\beta F\) and \(\beta G\) (see Fig. 1a). The conserved catalytic
residues are located at the beginning of αE (Asn112), just after βE (Ser140) and at the beginning of αF (Tyr153 and Lys157). The glucitol is primarily bound by the helix–turn–helix motif, which acts as a lid on top of the structure.

As B/SDH was crystallized with both NAD⁺ and glucitol, the active site was expected to include both of these ligands. After carefully modelling the glucitol molecule into the density corresponding to its most likely location (Fig. 1c), residual electron density was still visible, some of which could be modelled as a phosphate ion. However, the remaining density does not warrant the modelling of a full NAD molecule. Residual electron density in the active site after the modelling of glucitol and phosphate is shown in Fig. 1(d).

3.2. Most similar structures (DALI)

To identify the closest structural relatives to B/SDH, the DALI server (Holm & Rosenström, 2010) was used, and the top hits were for clavulanic acid dehydrogenase (MacKenzie et al., 2007) from Streptomyces clavuligerus (sequence identity 33%), with a Z-score range from 34.2 to 33.9 in the chains from PDB entries 2jah and 2jap based on 236 aligned Cα positions out of 245 with an r.m.s.d. of 1.4 Å. The second hit revealed a high similarity to a putative blue fluorescent protein from Vibrio vulnificus (Kao et al., 2011; sequence identity 31%), with a Z-score range from 32.5 to 31.3 in the chains from PDB entry 3p19 based on 234 aligned Cα positions out of 239 with an r.m.s.d. of 1.7 Å for the best hit. The third hit was a human putative dehydrogenase (PDB entry 1xg5; Structural Genomics Consortium, unpublished work), with a sequence identity of 28%, a Z-score from 31.5 to 30.5 and an r.m.s.d. of 2.0 Å based on 235 aligned Cα positions out of 257. A serine dehydrogenase from E. coli was the fourth hit (Yamazawa et al., 2011), with a sequence identity of 26% and a Z-score between 31.3 and 31.0 over six chains in PDB entry 3asv, giving an r.m.s.d. of 2.1 Å based on 234 aligned Cα positions out of 248. The model used for molecular replacement (Javidpour et al., 2014) is the fifth hit on the DALI list (sequence identity 29%), with a Z-score range from 30.9 to 30.7 in the chains from PDB entry 4nbu based on 237 aligned Cα positions out of 242 with an r.m.s.d. of 2.2 Å for the best hit.

Evidently the list of closest relatives represents a large sequence diversity of enzymes based on the Rossmann fold that use either NADH or NADPH as the cofactor. Fig. 2(a) shows the five most closely related structures, coloured from blue (low r.m.s.d.) to red (high r.m.s.d.), with non-aligned residues coloured grey. As can be seen from this figure, the helix–turn–helix motif in the loop between βF and αG is the most diverse part of the structure, with the rest of the Rossmann fold very well structurally conserved (at the Cα level).

3.3. Quaternary structure

Even though the asymmetric unit is limited to one monomer chain, B/SDH is a tetrameric enzyme in the crystal, with the tetramer formed by crystallographic symmetry. This is evident, for example, from PISA analysis of the structure (Krisinel, 2015). According to PISA, the tetrameric assembly has a calculated free energy of dissociation of 31.2 kcal mol⁻¹, with a total buried surface area of 13 000 Å² (3233 Å² per monomer). The tetrameric quaternary organization formed in the crystal (222 point-group symmetry) is the quaternary organization also found in the five best DALI hits above. In B/SDH the primary interactions between monomers are hydrogen bonds between the β-sheet from the Rossmann fold (between monomers A and B and monomers C and D in Fig. 1b), which effectively form one continuous sheet, and interactions between helices in two monomers to form a shared four-helix bundle (between monomers A and C and monomers B and D in Fig. 1b). The C-terminal part of B/SDH, starting with Pro237 at the end of βG, is tightly coordinated by arginines 168, 169 and 231 from a symmetry-related monomer (in pairs A/B and C/D; see Fig. 1b), which also coordinate the C-terminal carboxyl group of Leu242.

Previously, characterization of B/SDH by native PAGE and gel filtration showed molecular masses of 77.7 and 83.2 kDa (Gauer et al., 2014). Given the monomer molecular mass of 26.9 kDa, this would suggest a trimer in solution, but taken together with the tetrameric state in the crystal resembling the quaternary structure of sequence-related enzymes, a tetrameric solution structure seems more likely. Some ambiguity in the solution structure was also found for a previously structurally characterized bacterial sorbitol dehydrogenase from R. sphaeroides (Philippsen et al., 2005), which was reported to be dimeric by sedimentation-centrifugation analysis but forms a tetramer in the crystal.

A dimeric constitution has been reported by analytical ultracentrifugation (Kao et al., 2011) for the blue fluorescent protein from V. vulnificus, one of the DALI hits above, where mutations in αE and αF could disrupt the dimer, indicating that this shared four-helix bundle was the interface for the observed dimer. For B/SDH the interaction through βG is more pronounced, suggesting that this particular enzyme is a tetramer in solution.
3.4. Thermostability

BjSDH has previously been reported (Gauer et al., 2014) to have a $T_m$ of 62°C as measured by CD at 222 nm. Furthermore, it was shown that the enzyme activity was stable for more than 1 h at 55°C. This thermostability is favourable for its use as an industrial enzyme. In order to have another enzyme for detailed comparison, RsSDH, a previously structurally characterized sorbitol dehydrogenase (Philippsen et al., 2005), was here characterized in terms of melting temperature by CD spectroscopy, as performed for BjSDH in Gauer et al. (2014), and was found to have a $T_m$ of 47°C (Fig. 3). Over the years, many structural determinants of thermostability in proteins have been identified, for example an increased number of hydrogen bonds/salt bridges, increased rigidity and increased secondary-structure content, to name a few, forming the overall view that a number of stabilization strategies exist (Petsko, 2001). In order to find the structural source of the added thermostability, the sequences and structures of RsSDH and BjSDH were compared with respect to some of the known thermostabilization features.

We found that the BjSDH sequence contains roughly twice as many proline residues (13 in BjSDH versus 6 in RsSDH), but only half the number of glycine residues (15 in BjSDH versus 27 in RsSDH), which could explain some of the stability differences. See Fig. 2(b) for a comparison of the positions of prolines in BjSDH and RsSDH.

As mentioned in §3.3, in the BjSDH structure the parallel β-sheets of two monomers (in pairs) interact with each other to effectively form one continuous intermolecular β-sheet. The sheets from each monomer are arranged in an antiparallel fashion with respect to each other and form a total of 22 hydrogen bonds. Corresponding interactions are not found in RsSDH; thus, the formation of this continuous sheet in BjSDH might contribute to the tetramer stability. On the other hand, the number of intramolecular hydrogen bonds and salt bridges is comparable between BjSDH and RsSDH.

Since the Pro/Gly content seems to be one of the most likely determinants of thermostability, this factor was also analysed for the top five DALI hits, and BjSDH was found to have the highest Pro/Gly ratio of 0.87 among the analyzed proteins, compared with ratios between 0.26 and 0.6 (for the blue fluorescent protein).

3.5. Specificity and mechanism

B. japonicum SDH showed a 90% conversion of l-glucitol to d-sorbose, which makes it interesting for the conversion of sugar alcohols (Gauer et al., 2014). To the best of our knowledge, only a few other l-glucitol-acting dehydrogenases have been structurally characterized, and none with comparable properties. The new thermostable BjSDH structure is an important step towards the understanding and engineering of the substrate specificity and thermostability of the sorbitol dehydrogenases.

The amino acids building the catalytic tetrad are conserved throughout the SDR family. A structural comparison of the active site of BjSDH with those of galactitol dehydrogenase (GatDH; PDB entry 2wdz; Carius et al., 2010) and sorbitol dehydrogenase from R. sphaeroides (PDB entry 1k2w; Philippsen et al., 2005) shows that the conserved amino acids forming the catalytic tetrad (Asn112, Ser140, Tyr153 and Lys157 in BjSDH) superpose well (Fig. 4). The substrate in BjSDH is stabilized via the side chain of Glu150, Ser140 and the carbonyl groups of Pro183 and Gly184, leading to a 90° orientation compared with the pentanediol bound to GatDH (Fig. 4). The most significant differences are in the loop between βE and αF and the helix-turn-helix motif between the βF and αG loop, containing the prolines Pro195 and Pro196, which closes the active site to the environment in this structure. Despite the addition of NAD, only a phosphate group is observable in the density of the BjSDH structure. The position of this phosphate is closest to the phosphate adjacent to the nicotinamide group in the GatDH structure (Fig. 4).

If NAD or NADH bound to BjSDH exactly in the way it does to GatDH, the distance between the nicotinamide ring and the substrate as oriented in the described structure would be too short (Fig. 4). As the bound phosphate is slightly shifted in BjSDH compared with the corresponding phosphate of the NAD bound in the compared dehydrogenase structures, this could indicate a slightly different position of the cofactor in BjSDH. A reorientation of the substrate in the presence of the cofactor or flexibility of the NAD might be also possible: the latter has been described, for example, for the sheep liver cytosolic aldehyde dehydrogenase (Moore et al., 1998).

However, the limited resolution of the structure and the lack of bound NAD do not allow us to establish with confidence the structural features that allow this enzyme to accept l-glucitol to some extent as a substrate. A higher resolution structure with cofactor bound would definitely be desirable.

Acknowledgements

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Figure 4
Active site of BjSDH compared with the structure of GatDH (Carius et al., 2010; PDB entry 2wdz; pink).
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References


