Structure of NADP⁺-bound 7 β -hydroxysteroid dehydrogenase reveals two cofactor-binding modes

Rui Wang, Jiaquan Wu, David Kin Jin, Yali Chen, Zhijia Lv, Qian Chen, Qiwei Miao, Xiaoyu Huo and Feng Wang*

Wuxi Biotus Biosciences Co. Ltd, A5, 6 Dongsheng West Road, 214437 Jiangyin, Jiangsu, People's Republic of China.

*Correspondence e-mail: fwang@biotus.cn

Received 14 November 2016

Accepted 21 March 2017

Edited by W. N. Hunter, University of Dundee, Scotland

Keywords: crystal structure; NADP⁺-bound 7 β -HSDH; rational protein engineering.

PDB reference: 7 β -hydroxysteroid dehydrogenase, 5gt9

Supporting information: this article has supporting information at journals.iucr.org/f

In mammals, bile acids/salts and their glycine and taurine conjugates are effectively recycled through enterohepatic circulation. 7 β -Hydroxysteroid dehydrogenases (7 β -HSDHs; EC 1.1.1.201), including that from the intestinal microbe *Collinsella aerofaciens*, catalyse the NADPH-dependent reversible oxidation of secondary bile-acid products to avoid potential toxicity. Here, the first structure of NADP⁺ bound to dimeric 7 β -HSDH is presented. In one active site, NADP⁺ adopts a conventional binding mode similar to that displayed in related enzyme structures. However, in the other active site a unique binding mode is observed in which the orientation of the nicotinamide is different. Since 7 β -HSDH has become an attractive target owing to the wide and important pharmaceutical use of its product ursodeoxycholic acid, this work provides a more detailed template to support rational protein engineering to improve the enzymatic activities of this useful biocatalyst, further improving the yield of ursodeoxycholic acid and its other applications.

1. Introduction

In mammals, bile acids/salts and their glycine and taurine conjugates are recycled through enterohepatic circulation in the body (Ridlon *et al.*, 2006). During this process, these bile acids/salts and conjugates undergo a variety of reactions induced by the intestinal microflora. These reactions include the hydrolysis of sulfate esters and the oxidation and dehydroxylation of ring hydroxyl groups (Magee *et al.*, 2000). The 7-dehydroxylation of bile acids is a key step in these reactions, reducing the potential toxicity of the secondary bile-acid products (Coleman *et al.*, 1994). High concentrations of secondary bile-acid products in the blood and bile have been associated with the pathogenesis of cholesterol gallstone disease and colon cancer (McGarr *et al.*, 2005). In this respect, 7 β -hydroxysteroid dehydrogenase (7 β -HSDH; EC 1.1.1.201) from the intestinal microbe *Collinsella aerofaciens* (Liu *et al.*, 2011), which catalyses the NAD(P)H-dependent reversible oxidation of the hydroxyl group in the β -configuration at the C-7 position of bile acids to avoid this potential toxicity, has attracted great attention as a biocatalyst for the transformations of steroid substrates (Savino *et al.*, 2016).

Many years ago, Carrea and coworkers used 7 β -HSDH to synthesize a cholic acid derivate (Carrea *et al.*, 1992). Since then, with the wide use of ursodeoxycholic acid, an FDA-approved cholic acid derivate drug used to treat cholesterol gallstones and as an alternative to surgical intervention, the synthetic exploitation of 7 β -HSDH has attracted great attention, and many researchers from academia and industry have devoted many resources to improving the enzymatic efficiency of 7 β -HSDH in producing ursodeoxycholic acid (Crosignani *et*

Table 1

Macromolecule-production information.

Source organism	<i>C. aerofaciens</i>
DNA source	Synthetic
Forward primer	GAAGTTCTGTTCCAGGGGCCCATGAATCTGCGTG- AAAAATATG
Reverse primer	TTTGTAGCAGCCGGATCTCAATCGCGATAAAG- CTGCCCAT
Cloning vector	pET-28a
Expression vector	pET-28a
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the protein produced	MGSSHHHHHHENLYFQGMSPILGYWKIKGLVQPT- RLLEYLEEKYEELHYERDEGDKWRNKKFELG- LEFPNLPYYIDGDKLTQSMATIRYIADKHN- LGGCPKERAIEISMLEGAVLDIRYGVSRISY- DFETLKVDFLSKLPEMLKMFEDRLCHKTYLNG- DHTVHPDFMLYDALDVLYMDPMCLDAFPKLV- CFKKRIEAIPIQIDKYLKSSKYIAWPLQGWQAT- FGGDDHPPKSDLEVLFGQPMNLREKYGEWGLI- LGATEGVGKAFCEKIAAGGMNVVMVGRREEKL- NVLAGEIRETYGVETKVVRADFSQPGAAETVF- AATEGLDMGFMYSVACLHSFGKIQDTPWEKHE- AMINNVVTFLLKCFHHYMRIFAQDRGAVINV- SSMTGISSSPWNGYQAGKAFILKMTAEVACE- CEGTGVDVEVITLGTTLTPSLLSNLPGGPQGE- AVMKIALTPEECVDEAFELGKELSVIAGQRN- KDSVHDWKANHTEDEYIRYMGSFYRD

al., 1996; Monti *et al.*, 2009; Eggert *et al.*, 2014). Recently, the first structure of 7 β -HSDH in an apo form was reported (Savino *et al.*, 2016). It provided much important structural information on the conserved NADP(H)-binding site, and a theory was proposed that some specific elements in the substrate-binding pocket were related to the distinct stereoselectivity of the enzyme. In order to expand our understanding to structural knowledge of 7 β -HSDH with the cofactor NAD(P)H bound, we solved the 1.70 Å resolution structure of dimeric 7 β -HSDH from *C. aerofaciens* complexed with NADP⁺. Surprisingly, we observed that one NADP⁺ cofactor adopts a mode of binding similar to that observed in other members of this enzyme family. However, a second cofactor adopts a mode of binding that differs in the orientation of the nicotinamide, and we hypothesize that this is an intermediate mode of binding. This new structure provides a more detailed template that may support the rational protein engineering of 7 β -HSDH to improve the enzymatic activities of this useful biocatalyst, further improving the yield of ursodeoxycholic acid and its other applications.

2. Materials and methods

2.1. Macromolecule production and isothermal titration calorimetry (ITC)

We generated the recombinant plasmid pET-28a-7 β -HSDH, encoding 7 β -HSDH from *C. aerofaciens* ATCC25986 with 6 $\times$ His and glutathione *S*-transferase (GST) tags. In order to facilitate the removal of the 6 $\times$ His tag and GST tag, a *Tobacco etch virus* nuclear-inclusion-a endopeptidase (TEV protease) enzymatic site and a PreScission enzymatic site were also introduced followed by the tags. The fusion protein 6 $\times$ His-TEV-GST-PreScission-7 β -HSDH was expressed in *Escherichia coli* BL21 (DE3) cells in LB medium. To obtain

purified 7 β -HSDH, *E. coli* BL21 (DE3) cells containing the recombinant plasmid that had been induced with 0.5 mM IPTG at 288 K for about 16 h were collected by centrifugation. The pellet was resuspended in buffer consisting of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT and disrupted in a homogenizer (Microfluidics, USA). The supernatant was obtained by centrifuging the cell lysate at 40 000g and 277 K for 1 h. The 6 $\times$ His-GST tag was removed with PreScission protease (in a 1:40 ratio) by incubation at 277 K overnight. The protein was recovered from the flowthrough of a HisTrap HP column (GE Healthcare, USA). Ion-exchange column chromatography (Mono Q and Mono S; GE Healthcare, USA) was used for further purification. The high-purity fraction was then harvested using size-exclusion chromatography (Superdex 75; GE Healthcare, USA) and concentrated for crystallization. Macromolecule-production information is summarized in Table 1. The protein concentration was measured by the NanoDrop method (Desjardins *et al.*, 2009) using an extinction coefficient of 33 720 M⁻¹ cm⁻¹ at 280 nm (Wilkins *et al.*, 1999).

ITC was employed to obtain the thermodynamic parameters of the interaction between NADP⁺ and 7 β -HSDH using a MicroCal iTC200 (Malvern, England). β -Nicotinamide adenine dinucleotide phosphate sodium salt was dissolved in the same buffer as 7 β -HSDH (50 mM Tris-HCl buffer containing 150 mM NaCl pH 7.5). The sample cell contained 23 μ M 7 β -HSDH (300 rev min⁻¹ stirring speed with an initial delay of 60 s) and 1 mM NADP⁺ was titrated in. The cell temperature was kept at 298 K and 10 μ cal s⁻¹ of reference power was applied to maintain a flat baseline. A total of 20 injections of 1 mM NADP⁺ were performed, where the initial injection was of 0.4 μ l and the remaining 19 injections were 2 μ l each. Each injection took place over a period of 0.8 s with an interval of 150 s and a filter time of 5 s. An equivalent control experiment to subtract the heat of dilution of the NADP⁺ in buffer was performed (*i.e.* the same concentration of NADP⁺ was injected into the buffer solution, keeping the same experimental parameters). The results obtained were plotted using the MicroCal *Origin* 6.0 software.

2.2. Crystallization

Random microseed matrix screening (rMMS; D'Arcy *et al.*, 2014) was performed at 293 K by the sitting-drop vapour-diffusion method. 200 nl protein solution was mixed with 180 nl reservoir solution plus 20 nl seeding solution and was equilibrated against 15 μ l reservoir solution. The seeding solution was prepared by crushing a large fresh crystal of lysozyme (Hampton Research) and crystallization was set up with about 60 μ l of well solution [0.6 M sodium chloride, 0.1 M sodium acetate trihydrate pH 4.5, 25%(v/v) glycerol]. Commercial crystallization kits from Hampton Research and Qiagen were used for crystal screening. Suitable crystals of the complex were observed in the condition 0.1 M sodium malonate pH 7.0, 12%(w/v) polyethylene glycol (PEG) 3350. The crystal was mounted for diffraction using a cryoprotection solution consisting of 0.1 M sodium malonate pH 7.0,

Table 2
Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	Swissci SD-3
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	19.6
Buffer composition of protein solution	20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 1% (v/v) DMSO, 0.1 mg ml ⁻¹ NADP ⁺
Composition of reservoir solution	0.1 M sodium malonate pH 7.0, 12% (w/v) PEG 3350
Volume and ratio of drop	200 nl protein, 200 nl reservoir
Volume of reservoir (μl)	15

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL17U1, SSRF
Wavelength (Å)	0.97915
Temperature (K)	100
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	249.77
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	1
Space group	<i>P</i> ₄ ₁ ₂ ₁ ₂
<i>a</i> , <i>b</i> , <i>c</i> (Å)	72.73, 72.73, 171.31
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.74
Resolution range (Å)	49.26–1.70 (1.79–1.70)
Total No. of reflections	331 630 (38 606)
No. of unique reflections	51 175 (7164)
Completeness (%)	99.4 (97.5)
Multiplicity	6.5 (5.4)
$\langle I/\sigma(I) \rangle$	13.7 (4.3)
<i>R</i> _{merge} (%)	8.3 (33.8)
<i>R</i> _{r.i.m.} [†] (%)	9.0 (37.4)
Overall <i>B</i> factor from Wilson plot (Å ²)	17.134

[†] *R*_{r.i.m.}, the precision-indicating merging *R* factor, is *R*_{merge} adjusted by a factor of $[1/(N-1)]^{1/2}$, where *N* is the number of times a given reflection is observed.

12% (w/v) PEG 3350, 25% (v/v) glycerol. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

Data were obtained using an ADSC Q315r detector (Area Detector Systems Corporation, USA) on BL17U1 at Shanghai Synchrotron Radiation Facility (SSRF). Since the crystallization condition contained cryoprotectants, the crystal was flash-cooled in liquid nitrogen and directly mounted for X-ray diffraction. The diffraction data were collected at 100 K and were processed with *iMosflm* (Battye *et al.*, 2011). The crystal diffracted to 1.7 Å resolution and belonged to the tetragonal space group *P*₄₁₂₁₂, as checked with *POINTLESS* (Evans, 2011), with unit-cell parameters *a* = *b* = 72.73, *c* = 171.31 Å. A total of 331 630 reflections were integrated to a resolution of 1.70 Å and were merged to obtain 51 175 unique reflections with an overall *R*_{meas} of 0.090 and a completeness of 99.04%. Data-collection statistics are summarized in Table 3.

2.4. Structure solution and refinement

The contents of the unit cell were analysed using the Matthews coefficient (Matthews, 1968). The Matthews

Table 4
Structure refinement.

Resolution range (Å)	44.92–1.70
Completeness (%)	99.21
No. of reflections, working set	48 520
No. of reflections, test set	2554
Final <i>R</i> _{cryst}	0.1655
Final <i>R</i> _{free}	0.2012
Cruickshank DPI [†] (Å)	0.104
No. of non-H atoms	
Protein	4049
Ligand	96
Water	203
Total	4348
R.m.s. deviations	
Bonds (Å)	0.0179
Angles (°)	1.8089
Average <i>B</i> factors (Å ²)	
Protein	16.718
Ligand	17.826
Water	25.259
Ramachandran plot	
Favoured regions (%)	94.38
Additionally allowed (%)	5.23
Outliers (%)	0.39

[†] Diffraction-component precision indicator (Cruickshank, 1999).

coefficient of this complex was 1.96 Å³ Da⁻¹, corresponding to the presence of two subunits of 7β-HSDH in the asymmetric unit. The structure was determined by molecular replacement using *Phaser* (McCoy *et al.*, 2007) with the monomer from PDB entry 5fyd as a search model (Savino *et al.*, 2016). Regions that were less defined or where the electron density was ambiguous were deleted from the model. The models were completed and refined by iterative cycles of manual building using *Coot* (Emsley *et al.*, 2010). Subsequent stages of refinement were carried out with *REFMAC5* (Murshudov *et al.*, 2011) within the *CCP4* suite (Winn *et al.*, 2011; Collaborative Computational Project, Number 4, 1994) and manual improvement in *Coot*. During refinement, the two polypeptides in the asymmetric unit were treated independently. The quality of the structure was evaluated using *MolProbity* (Chen *et al.*, 2010) and *PROCHECK* (Laskowski *et al.*, 1993). Coordinates and structure factors have been deposited in the Protein Data Bank with accession code 5gt9. All structural representations were generated using *PyMOL* (DeLano, 2002). Structure-solution and refinement statistics are summarized in Table 4.

3. Results and discussion

7β-HSDH was purified to homogeneity as assessed by analytic size-exclusion chromatography, SDS-PAGE and LC-MS (Agilent 6224 TOF LC/MS, USA; Figs. 1*a*, 1*b* and 1*c*). Size-exclusion chromatography identified that the enzyme forms a dimer in solution. ITC was used to obtain the thermodynamic parameters of the NADP⁺–7β-HSDH interaction. Using a one-site model, we calculated that NADP⁺ binds to 7β-HSDH with a dissociation constant *K*_d of approximately 22 μM, and the ITC titrations revealed a close to 1:1 binding stoichiometry (Fig. 1*d*).

The structure of the 7β -HSDH-NADP⁺ complex contains two monomers in each asymmetric unit, referred to here as 'complex A' and 'complex B', with each monomer binding one molecule of NADP⁺ (Fig. 2*a*). Like most members of the short-chain dehydrogenases/reductases (SDR) family, seven β -strands constitute the enzyme core and 11 α -helices wrap around the core to make a typical Rossmann fold, with NADP⁺ binding in a bowl-like enzymatic centre in complex A and complex B (Bennett *et al.*, 1997). A PDB BLAST search using the DALI server (Holm & Rosenström, 2010) shows that the whole structure of the 7β -HSDH-NADP⁺ complex

resembles those of many dehydrogenases and the most similar structure is, of course, the apo form of 7β -HSDH (Savino *et al.*, 2016). Surprisingly, structural alignment of the 7β -HSDH-NADP⁺ complex and apo 7β -HSDH by the DALI server gives a Z-score of 42.8 with a root-mean-square deviation of 1.5 Å for complex A and a Z-score of 42.1 with a root-mean-square deviation of 1.3 Å for complex B (Holm & Rosenström, 2010).

Although structural superposition of complex A and complex B reveals that the whole backbone of the structure is conserved, details of the interactions between 7β -HSDH and NADP⁺ show that the insertion of NADP⁺ gives rise to several

Figure 1
Purification of the 7β -HSDH protein. (a, b) Analytic size-exclusion column chromatography of the purified 7β -HSDH protein. The absorbance of the eluate was monitored at 280 nm. The protein markers used to produce the standard curve in (b) were purchased from GE Healthcare and were used according to the manufacturer's protocol. (c) Coomassie-stained 12% SDS-PAGE showing the purity of 7β -HSDH. Lane 1, molecular-weight markers; lane 2, 5 μg 7β -HSDH; lane 3, 10 μg 7β -HSDH; lane 4, 20 μg 7β -HSDH. (d) LC-MS shows the calculated molecular weight of the purified 7β -HSDH protein. (e) ITC profiles for the titration of 7β -HSDH with NADP⁺.

differences in the cofactor-binding pocket in the two complexes. One difference is that the nicotinamide group of NADP⁺ in complex *A* pushes helix 8 out to expand the binding site. At the same time, the nearby helix 9 adjusts its position. The middle two phosphate groups appear to push helix 2 out. Another conformational difference appears to arise from the adenine, which also pushes helix 3 out to enlarge the cofactor-binding pocket in complex *A* (Figs. 2*b*, 2*c*, 2*d* and 2*e*). Helix 10 and helix 11 from complex *B* act as a lid to close the enzymatic centre (Fig. 2*a*). The results of the ITC experiment fit a one-site model much better than a two-site mode; this might mean that the difference is difficult to detect using ITC or comes from the crystal packing.

Structural alignment of the 7 β -HSDH–NADP⁺ complex with NAD⁺-bound 7 α -HSDH proved interesting. The typical complex *B* of 7 β -HSDH shows a similar structural arrangement to 7 α -HSDH, with the cofactors NADP⁺ and NAD⁺ in almost the same orientation, with a root-mean-square deviation of 0.296 Å over 214 C α atoms (Fig. 3). The Arg pair (Arg40–Arg41), Thr17, Glu18 and Phe67 of 7 β -HSDH are perfectly located to make hydrogen bonds to the adenosine phosphate, as predicted by Savino *et al.* (2016); also, Val20, Val91, Lys160, Thr189, Thr191 and Ser193 make hydrogen bonds to the nicotinamide group of NADP⁺. In NAD⁺-bound 7 α -HSDH, only Asp42 and Ile69 make hydrogen bonds to the adenosine, while Ile23, Asn95, Lys163, Ile192 and Thr194

Figure 2
Structure of the 7 β -HSDH–NADP⁺ protein complex. (a) The structure of 7 β -HSDH bound to NADP⁺ in cartoon representation. The unique 7 β -HSDH-bound NADP⁺ is shown in olive, while the normal 7 β -HSDH is in green. The unique and normal NADP⁺ molecules are shown in stick representation, with C and O atoms depicted in green and red, respectively, for the unique NADP⁺ and in yellow and red, respectively, for the normal NADP⁺. (b) OMIT map (3.0 σ) demonstrating that the unique NADP⁺ binds to the 7 β -HSDH cofactor-binding pocket. (c) OMIT map (3.0 σ) demonstrating that the normal NADP⁺ binds to the 7 β -HSDH cofactor-binding pocket. (d) The alignment of two NADP⁺ from complex A (unique) and complex B (normal). (e) Alignment of the unique 7 β -HSDH–NADP⁺ structure (olive) with apo 7 β -HSDH (PDB entry 5fyd; wheat) shown as separate cylindrical representations. The unique 7 β -HSDH–NADP⁺ has the same colour as in (a).

Figure 3

Structural interaction details of the cofactors NADP⁺ and NAD⁺ with 7β-HSDH and 7α-HSDH. (a) Superimposition of the unique 7β-HSDH–NADP⁺ and the normal 7β-HSDH–NADP⁺ complexes shown as line representations. (b) Superimposition of the unique 7β-HSDH–NADP⁺, the normal 7β-HSDH–NADP⁺ and the 7α-HSDH–NAD⁺ (PDB entry 1fmc; Tanaka *et al.*, 1996) complexes shown as line representations. Hydrogen-bond interactions are indicated by black arrows. 7α-HSDH is shown in red and NAD⁺ is shown in stick representation, with C and O atoms depicted in light magenta and red, respectively.

make hydrogen bonds to the nicotinamide. In complex A of 7β-HSDH there is a large bend with about 90° torsion of the nicotinamide on comparing the molecular location of NAD⁺. In the alignment of 214 C^α atoms with a root-mean-square deviation of 0.296 Å, although there is only minor translation of the adenosine phosphate, the nicotinamide of NADP⁺ is rotated about 90° to push helix 8 of 7β-HSDH out to expand the bowl. In the interactions that stabilize NADP⁺, we found the predicted arginine pair (Arg40–Arg41), Glu18 and Phe67 remain to stabilize the adenosine phosphate, while Glu18, Val20 and Gly21 orient the nicotinamide with the help of Ser240 from another molecule of 7β-HSDH.

In summary, our work presents the first structure of NADP⁺ bound to dimeric 7β-hydroxysteroid dehydrogenase. In one

active site, NADP⁺ adopts a standard conformation similar to that observed in other SDR-family members. In the other site, the cofactor adopts a different mode of binding with respect to the orientation of the nicotinamide. Since 7β-HSDH has become an attractive enzyme owing to the wide and important pharmaceutical use of its product ursodeoxycholic acid, our work provides a more detailed template for rational protein engineering to improve the enzymatic activities of this useful biocatalyst, further improving the yield of ursodeoxycholic acid and its other applications.

Acknowledgements

The authors gratefully acknowledge financial support from the China Torch Program (grant No. 2015GH581522) and from the Jiangsu Innovation Fund for Technology-Based Firms of China (grant No. BC2013062).

References

- Battye, T. G. G., Kontogiannis, L., Johnson, O., Powell, H. R. & Leslie, A. G. W. (2011). *Acta Cryst.* **D67**, 271–281.
- Bennett, M. J., Albert, R. H., Jez, J. M., Ma, H., Penning, T. M. & Lewis, M. (1997). *Structure*, **5**, 799–812.
- Carrea, G., Pilotti, A., Riva, S., Canzi, E. & Ferrari, A. (1992). *Biotechnol. Lett.* **14**, 1131–1135.
- Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. & Richardson, D. C. (2010). *Acta Cryst.* **D66**, 12–21.
- Coleman, J. P., Hudson, L. L. & Adams, M. J. (1994). *J. Bacteriol.* **176**, 4865–4874.
- Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* **D50**, 760–763.
- Crosignani, A., Setchell, K. D. R., Invernizzi, P., Larghi, A., Rodrigues, C. M. P. & Podda, M. (1996). *Clin. Pharmacokinet.* **30**, 333–358.
- Cruickshank, D. W. J. (1999). *Acta Cryst.* **D55**, 583–601.
- D'Arcy, A., Bergfors, T., Cowan-Jacob, S. W. & Marsh, M. (2014). *Acta Cryst.* **F70**, 1117–1126.
- DeLano, W. L. (2002). *PyMOL*. <http://www.pymol.org>.
- Desjardins, P., Hansen, J. B. & Allen, M. (2009). *J. Vis. Exp.*, e1610.
- Eggert, T., Bakonyi, D. & Hummel, W. (2014). *J. Biotechnol.* **191**, 11–21.
- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
- Evans, P. R. (2011). *Acta Cryst.* **D67**, 282–292.
- Holm, L. & Rosenström, P. (2010). *Nucleic Acids Res.* **38**, W545–W549.
- Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). *J. Appl. Cryst.* **26**, 283–291.
- Liu, L., Aigner, A. & Schmid, R. D. (2011). *Appl. Microbiol. Biotechnol.* **90**, 127–135.
- Magee, E. A., Richardson, C. J., Hughes, R. & Cummings, J. H. (2000). *Am. J. Clin. Nutr.* **72**, 1488–1494.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- McGarr, S. E., Ridlon, J. M. & Hylemon, P. B. (2005). *J. Clin. Gastroenterol.* **39**, 98–109.
- Monti, D., Ferrandi, E. F., Zanellato, I., Hua, L., Polentini, F., Carrea, G. & Riva, S. (2009). *Adv. Synth. Catal.* **351**, 1303–1311.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.

- Ridlon, J. M., Kang, D.-J. & Hylemon, P. B. (2006). *J. Lipid Res.* **47**, 241–259.
- Savino, S., Ferrandi, E. E., Forneris, F., Roviola, S., Riva, S., Monti, D. & Mattevi, A. (2016). *Proteins*, **84**, 859–865.
- Tanaka, N., Nonaka, T., Tanabe, T., Yoshimoto, T., Tsuru, D. & Mitsui, Y. (1996). *Biochemistry*, **35**, 7715–7730.
- Wilkins, M. R., Gasteiger, E., Bairoch, A., Sanchez, J. C., Williams, K. L., Appel, R. D. & Hochstrasser, D. F. (1999). *Methods Mol. Biol.* **112**, 531–552.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.