The N253F mutant structure of trehalose synthase from *Deinococcus radiodurans* reveals an open active-site topology

Sih-Yao Chow,^a Yung-Lin Wang,^a Yu-Chiao Hsieh,^a Guan-Chiun Lee^b and Shwu-Huey Liaw^{a,c,*}

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^aDepartment of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, No. 155, Sec. 2, Linong Street, Taipei 11221, Taiwan, ^bDepartment of Life Science, National Taiwan Normal University, No. 162, Sec. 1, Heping East Road, Taipei 11677, Taiwan, and ^cDepartment of Medical Research and Education, Taipei Veterans General Hospital, No. 201, Sec. 2, Shipai Road, Taipei 11217, Taiwan. *Correspondence e-mail: shliaw@ym.edu.tw

Trehalose synthase (TS) catalyzes the reversible conversion of maltose to trehalose and belongs to glycoside hydrolase family 13 (GH13). Previous mechanistic analysis suggested a rate-limiting protein conformational change, which is probably the opening and closing of the active site. Consistently, crystal structures of *Deinococcus radiodurans* TS (DrTS) in complex with the inhibitor Tris displayed an enclosed active site for catalysis of the intramolecular isomerization. In this study, the apo structure of the DrTS N253F mutant displays a new open conformation with an empty active site. Analysis of these structures suggests that substrate binding induces a domain rotation to close the active site. Such a substrate-induced domain rotation has also been observed in some other GH13 enzymes.

1. Introduction

Trehalose [α -D-glucopyranosyl-(1 $\rightarrow$ 1)- α -D-glucopyranoside] is used by a wide variety of organisms for various key functions such as a carbon/energy source and a bacterial cell-wall component, as well as for signalling and for the protection of proteins and membranes (Barry *et al.*, 1998; Elbein *et al.*, 2003; Avonce *et al.*, 2006). In mycobacteria, trehalose is part of a toxic lipid in the cell wall which has been identified as the main virulence factor of tuberculosis (Hunter *et al.*, 2006), and hence enzymes that are involved in the biosynthesis of this disaccharide may serve as drug targets (Edavana *et al.*, 2004; Kalscheuer *et al.*, 2010). In addition, trehalose stabilizes various biomolecules and hence has been widely used in the food, cosmetic, medical and other biotechnological industries (Ohtake & Wang, 2011). Trehalose synthase (TS) catalyzes the simple conversion of maltose to trehalose, with a side reaction of hydrolysis (Nishimoto *et al.*, 1996). This process has a number of advantages and hence could be suitable for the industrial manufacture of trehalose (Schiraldi *et al.*, 2002).

TS belongs to glycoside hydrolase family 13 (GH13), which includes a diverse range of carbohydrate-metabolizing enzymes (Cantarel *et al.*, 2009). The GH13 enzymes share a catalytic (β/α)₈-barrel and a C-terminal β -sandwich (domain C) as their structurally conserved core. A common subdomain B and a variable number of subdomains that are unique to particular members are inserted within the (β/α)₈-barrel. Mechanistic analysis of TS from *Mycobacterium smegmatis* (MsTS) illustrated that this enzyme catalyzes the disaccharide isomerization intramolecularly using a double-displacement mechanism, and suggested that a protein conformational

Table 1

Macromolecule-production information.

Source organism	<i>D. radiodurans</i> R1
DNA source	pET-23a(+) overexpression vector
Forward primer†	CTCGGGCCACTGGAACGCCTCGGCAAGC
Reverse primer†	GCTTGCCGAGGCGTTCCAGTGGCCCGAG
Cloning vector	pET-23a(+)
Expression vector	pET-23a(+)
Expression host	<i>Escherichia coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MVPTQAHPEWYKSAVFYELSVRTFQDNGD GKGDFPGLTSRLDYLKNLGVDCWLWLPW FSPPLRDDGYDVADYRGIHPDLGLTDDF KVFLREAHARGLWVIGDLVTNHTSSDHP WFQAARRGPTLPDGPNEYHDYVWSDE GKEYADTRIIFTDETVSNWTLDEQAGKY YWHRFASQPDNLNPNKVVVEELHGAAR FWDLDGLDGRVDAVPYLIEREGETSCEN LPETHEILKGFRAMVDREYPGRLLLAEA FQWPEEVVEYFGTEAEPEFHMCFNFVPM PRLYMSLKREDTSSIREIMGRLPKIPSF GQWCIFLRNHDELTEMLVTDDEAFMYA AYAPDARMKINVGIRRLAPLLDNDRRR IELLNTVLLALPGSPVLYGDEIGMGDD LGLPDRNGVRTPMQWNAGTSGGFSTAQP SDCFFPPIQDPVYGFGRVNVQSQLQDPS SLLKWTARQLELRRAPAFAGHDLTFIE TGNPAILAFTRQYDGETLLIVSNFAGNA QAGLLDLAPFVGRAPVTLSGASPLPVVT GNGQYPVVMGKYDYYWLRNLSRVDKLAA ALEHHHHHH

† The nucleotides for the designed DrTS mutation are underlined.

change is the rate-limiting step; this is likely to be the opening and closing of the active site (Zhang *et al.*, 2011). Consistently, our previous structure of *Deinococcus radiodurans* TS in complex with the inhibitor Tris (DrTS–Tris) revealed an enclosed active-site conformation to facilitate such intramolecular isomerization and minimize disaccharide hydrolysis (Wang *et al.*, 2014), whereas the apo structures of MsTS and *M. tuberculosis* TS (MtTS) showed an occluded substrate-binding cleft (Caner *et al.*, 2013; Roy *et al.*, 2013). However, such an occluded conformation has never been observed in other GH13 enzymes. To date, all attempts to obtain an apo DrTS structure have been unsuccessful. To obtain the open conformation of the apo enzyme, we mutated several residues involved in formation of the closed conformation. Here, we report the crystal structure of the N253F mutant of DrTS, which displays a novel open conformation with an empty active site. Comparison with several other GH13 members suggests that the present new open active-site topology represents the apoenzyme prior to substrate binding.

2. Materials and methods

2.1. Macromolecule production

The DrTS gene in our study was cloned from *D. radiodurans* strain ATCC 13939, which was obtained from the American Type Culture Collection (Wang *et al.*, 2007), while the reported DrTS sequence (Filipkowski *et al.*, 2012) was from *D. radiodurans* strain DSMZ 20539 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen. Three residues differ between these two DrTSs: Trp97, Ile313

Table 2

Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well VDX plate, Hampton Research
Temperature (K)	288
Protein concentration (mg ml ⁻¹)	30.0
Buffer composition of protein solution	20 mM HEPES pH 7.5, 100 mM NaCl, 3.3% glycerol, 1 mM DTT
Composition of reservoir solution	0.3 M Tris–HCl pH 7.0, 7% PEG 4000, 0.2 M sodium acetate trihydrate
Volume and ratio of drop	4 µl; 1:1 ratio
Volume of reservoir (µl)	500

and Val380 are present in our DrTS, while Arg97, Thr313 and Ile380 are present in the latter DrTS. The crystal structures show that Trp97 is exposed on the protein surface, whereas Ile313 and Val380 are buried. The activities of these two DrTSs have been shown to display a distinct temperature-dependence (Wang *et al.*, 2007; Filipkowski *et al.*, 2012).

Isolation of the recombinant protein was carried out as reported previously (Wang *et al.*, 2014). Mutational analysis was carried out using a QuikChange site-directed mutagenesis kit (Stratagene). Luria–Bertani broth cultures were grown at 310 K and induced with 0.4 mM isopropyl β -D-1-thiogalactopyranoside at 289 K for 16 h. The cell pellets were resuspended in lysis buffer consisting of 20 mM sodium phosphate, 500 mM NaCl pH 7.4 and lysed using a French press. The crude extract was applied onto a nickel–nitrilotriacetic acid column (GE Healthcare). After washing with 20–60 mM imidazole, the protein was eluted with 250 mM imidazole and dialyzed at 277 K. An additional Sephacryl S-300 gel-filtration column (HiPrep 16/60 Sephacryl S-300 HR, GE Healthcare) was used to improve protein purity when necessary. Macromolecule-production information is given in Table 1.

2.2. Activity assay

The activity of DrTS was assayed in a reaction mixture consisting of 40 µl purified DrTS at 0.25 mg ml⁻¹ and 160 µl 100 mM maltose/trehalose solution in 20 mM sodium phosphate pH 7.4 at 293 K for 2 h (Wang *et al.*, 2014). The activity assay was carried out in triplicate. The reaction was terminated by heating the mixture in boiling water for 15 min. The amounts of maltose, trehalose and glucose in each reaction mixture were measured using a high-performance liquid-chromatography system (Schambeck SFD 2100) equipped with a refractive-index detector (Schambeck SFD RI 2000) at a flow rate of 1 ml min⁻¹. A carbohydrate-analysis column (6.0 × 150 mm, Shodex SZ5532) equilibrated with 75% acetonitrile, 24% Milli-Q water and 1% formic acid was used.

2.3. Crystallization

Crystallization conditions for the N253F mutant are summarized in Table 2. Crystals appeared within 2–3 weeks. Prior to cooling in liquid nitrogen, crystals were transferred into reservoir mother liquor containing 20%(v/v) glycerol.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	NSRRC beamline BL15A1
Wavelength (Å)	1
Temperature (K)	110
Detector	Rayonix MX300HE CCD
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	0.3
Total rotation range (°)	112.2
Exposure time per image (s)	9
Space group	$P2_12_12_1$
a, b, c (Å)	100.40, 132.42, 196.01
α, β, γ (°)	90, 90, 90
Mosaicity (°)	0.3
Resolution range (Å)	30–2.76 (2.86–2.76)
Total No. of reflections	307537 (29225)
No. of unique reflections	67645 (6642)
Completeness (%)	99.9 (99.3)
Multiplicity	4.5 (4.4)
$\langle I/\sigma(I) \rangle$	12.3 (1.8)
$R_{\text{r.i.m.}}^\dagger$	0.134 (0.903)
$CC_{1/2}$	0.993 (0.606)
Overall B factor from Wilson plot (Å ²)	44.6

† Estimated $R_{\text{r.i.m.}} = R_{\text{merge}}[N/(N-1)]^{1/2}$, where N is the data multiplicity.

2.4. Data collection and processing

A diffraction data set was collected on beamline BL15A1 at the NSRRC synchrotron, Taiwan. Data were processed using the *HKL-2000* software package (Otwinowski & Minor, 1997). The crystal belonged to space group $P2_12_12_1$, with four molecules per asymmetric unit. The data-collection and processing statistics are presented in Table 3.

2.5. Structure solution and refinement

The structure of the N253F mutant was determined by molecular replacement with *Phaser* (McCoy *et al.*, 2007) using the coordinates of wild-type DrTS as a template (PDB entry 4tvu; Wang *et al.*, 2014). The model was subjected to manual reconstruction with *Coot* (Emsley *et al.*, 2010) and NCS-restrained refinement using *REFMAC* (Murshudov *et al.*, 2011). The model quality was evaluated using *MolProbity* (Chen *et al.*, 2010) and the structural figures were generated using *PyMOL* (Schrödinger). The coordinates and structure factors have been deposited in the PDB as entry 5gtv. The refinement statistics are summarized in Table 4.

3. Results and discussion

3.1. The structure of the N253F mutant

Gel-filtration results during protein purification indicated that, as for the wild type, the N253F mutant exists as a dimer in solution as well as in the crystal form, with each asymmetric unit containing two dimers. The final model includes residues 6–553, except for a largely disordered region consisting of residues 140–157 in molecule *A*, residues 120–127 and 141–157 in molecule *B*, residues 141–157 in molecule *C* and residues 124–128 and residues 139–157 in molecule *D*. The four N253F mutant protomers in each asymmetric unit do not display

Table 4

Structure solution and refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	30.00–2.76 (2.83–2.76)
Completeness (%)	96.7
σ Cutoff	0.0
No. of reflections, working set	62376 (3030)
No. of reflections, test set	3231 (150)
Final R_{cryst}	0.192 (0.265)
Final R_{free}	0.251 (0.331)
Cruickshank DPI (Å) using R_{free} (25.1%)	0.368
No. of non-H atoms	
Protein	16940
Ion	8
Ligand	0
Water	237
Total	17185
R.m.s. deviations	
Bonds (Å)	0.009
Angles (°)	1.298
Average B factors (Å ²)	
Protein	51.6
Ion	51.2
Ligand	0.0
Water	34.9
Ramachandran plot	
Most favoured (%)	96.1
Allowed (%)	3.9

significant differences, with root-mean-square deviations (r.m.s.d.s) of 0.3–0.5 Å for all C $^\alpha$ atoms. Each N253F mutant protomer consists of the three common GH13 domains [catalytic (β/α)₈-barrel, subdomain B and domain C] and two loop-rich modules, S7 and S8, that are unique to TS (Fig. 1a).

In the wild-type DrTS–Tris complex (Wang *et al.*, 2014), the interaction networks formed between subdomain B, S7 and the active-site residues seal the active-site entrance and hence result in an enclosed active-site conformation (Figs. 1b and 2a). In particular, Arg148 forms salt bridges with Glu223 and Glu324, which form hydrogen bonds to Gln254 and Asn253, respectively, while Asp153 forms two hydrogen bonds to Asn347. However, such an interaction network is absent in the N253F mutant, and hence the mutant displays an open and empty active-site conformation (Figs. 1b and 2b). In addition, some residues in the B β 1–B β 2 loop, residues 140–157 in molecule *A*, residues 141–157 in molecules *B* and *C*, and residues 139–157 in molecule *D*, are disordered (Fig. 1b).

3.2. A new open conformation for the N253F mutant

The structure of the N253F mutant has become drastically altered compared with that of the wild-type DrTS–Tris complex (Wang *et al.*, 2014), as illustrated by the r.m.s.d. of 1.1–1.4 Å for 519–527 C $^\alpha$ atoms (Fig. 1b). Analysis of the differences between the open and closed conformations in these two DrTS structures using *DynDom* (Hayward & Berendsen, 1998) showed a well defined domain rotation (Fig. 2). The moving domain as defined by the program mainly consists of residues 57–73 and 99–251, which cover from the β 2– α 2 loop to the β 5 strand. Tight packing of subdomain B (residues 105–184) against the N-terminal side of the (β/α)₈ barrel, particularly against the β 2– α 2 and β 4– α 4 loops, results

in concerted movement of these regions. The moving domain in the N253F mutant structure rotates away from the active site by approximately 9.2° compared with the wild-type DrTS–Tris complex (Figs. 2*a* and 2*b*).

Like the N253F mutant of DrTS, a similar domain rotation was also observed in the apo structures of MsTS and MtTS (Figs. 1*b* and 2*c*), which share $\sim 50\%$ sequence identity with DrTS. However, the tertiary structures of subdomain S7 in MsTS and MtTS are markedly distinct from those in DrTS (Fig. 1*b*), even though there is $>80\%$ sequence identity between them. A segment of subdomain S7, in particular Leu344 in MsTS and Leu352 in MtTS, projects into the active site and hence results in an occluded active-site conformation (Caner *et al.*, 2013; Roy *et al.*, 2013; Fig. 2*c*). As a consequence, the active-site residues adopt different spatial positions; in

particular, the transition-state stabilizer aspartate, Asp342 in MsTS, is shifted away from the active site by ~ 4 Å. Our present N253F mutant structure thus demonstrates a new open conformation.

3.3. Substrate-induced rotation of subdomain B

The apo and substrate-bound structures of the closest structural matches of TS, including sucrose hydrolase (SH), *Halomonas* sp. H11 α -glucosidase (HaG) and amylosucrase (AS), demonstrate that substrate binding causes a TS-like active domain rotation (~ 6.1 – 11.8°) to form the active conformation. The substrate-bound structures of *Xanthomonas axonopodis* SH (XaSH; Kim *et al.*, 2008), HaG (Shen *et al.*, 2015), *Neisseria polysaccharea* AS (NpAS; Skov *et al.*, 2002), yeast isomaltase

Figure 1
(*a*) A ribbon representation of the DrTS N253F mutant monomer. The protein contains a catalytic $(\beta/\alpha)_8$ -barrel (red), with domain C (cyan), subdomain B (blue) and S7 (green) and S8 (yellow) inserted between β_3 and α_3 , between β_7 and α_7 , and between β_8 and α_8 , respectively, within the $(\beta/\alpha)_8$ -barrel. Residues 142–157 in the B β_1 –B β_2 loop are disordered. (*b*) Structural comparison of DrTS and MsTS. Wild-type DrTS (PDB entry 4tvu; Wang *et al.*, 2014), the DrTS N253F mutant and MsTS (PDB entry 3zoa; Caner *et al.*, 2013) are shown in green, red and blue, respectively. The inset illustrates some interaction networks unique to TS that are present in the enclosed active-site conformation in wild-type DrTS–Tris.

Figure 2
Molecular surfaces of (*a*) wild-type DrTS–Tris, (*b*) the DrTS N253F mutant and (*c*) MsTS. The DynDom-defined fixed and moving domains are shaded in yellow and light grey, respectively. The B β_1 –B β_2 loop and a segment in S7 are coloured green and red, respectively. These demonstrate enclosed, open and occluded active-site conformations for TS.

(Yamamoto *et al.*, 2011) and *Rhizobium* sp. MX-45 sucrose isomerase (RhSI; Ravaud *et al.*, 2008), as well as the DrTS–Tris complex (Wang *et al.*, 2014), share a similar relative orientation of subdomain B, particularly the similar spatial position of the β 1– β 2 loop (Fig. 3*a*). In addition, the apo structures of SH from *X. campestris* (XcSH; Champion *et al.*, 2009), HaG (Roy *et al.*, 2013), AS from *D. radiodurans* (DrAS; Skov *et al.*, 2013), the RhSI R284C variant (Lipski *et al.*, 2013) and the DrTS N253F mutant display a similar orientation of subdomain B (Fig. 3*b*). Therefore, despite the low sequence identity (20–38%), and the distinct substrate and reaction specificity of these enzymes, regulation of the opening and closing of the active site by a similar substrate-induced rotation of subdomain B has been maintained during evolution.

3.4. A snapshot of apo TS

The substrate-bound structures mentioned above share a virtually identical –1 subsite, in which the conserved active-site residues form similar extensive interaction networks to attain the active conformation (Wang *et al.*, 2014). Such interactions are absent in the apo enzymes, and some disordered regions are observed, for example the β 4– α 4 loop in XcSH (Kim *et al.*, 2008) and the β 2– α 2 and β 2– β 3 loops in DrAS (Skov *et al.*, 2013). This may indicate that a lack of extensive interactions causes the surrounding loops to become more flexible (Fig. 3*b*). Similarly, the β 1– β 2 loop is disordered in the DrTS N253F mutant (Figs. 1 and 2*b*). Therefore, like the apo structures of the closest neighbours of TS, with a

less compact and empty active-site architecture and with a lack of a tertiary structural change in subdomain, the DrTS N253F mutant structure, rather than the MsTS and MtTS structures, may represent the open conformation of the apo TS prior to substrate binding.

3.5. Mechanistic implications from the N253F mutant

In GH13 enzymes, the nucleophile Asp attacks the glucose at the –1 subsite to yield the covalent glycosyl-enzyme intermediate. SI and TS are the only two isomerases in the GH13 family and share distinct modules that are unique to these family members inserted within the $(\beta/\alpha)_8$ -barrel (Wang *et al.*, 2014; Ravaud *et al.*, 2008). Both utilize an enclosed active site not only to protect the covalent intermediate from water, but also to allow the cleaved sugar at the +1 subsite to reorient and re-attack the intermediate to complete the isomerization reaction intramolecularly. The randomly mutated RhSI R284C variant displays a decreased isomerase activity and a markedly increased hydrolase activity, and its crystal structure reveals an open conformation, allowing the cleaved sugar at the +1 subsite to be released and more water molecules to enter the active site (Lipski *et al.*, 2013). As a consequence, such an opening of the active site changes the catalysis by the variant from isomerization to hydrolysis.

In contrast, the DrTS N253F mutant shows neither detectable isomerase nor hydrolase activity (Fig. 4). Interestingly, even in the presence of a large amount of the strong inhibitor Tris (300 mM Tris–HCl in the crystallization

Figure 3

A similar substrate-induced rotation of subdomain B. Superposition of (*a*) the substrate-bound and (*b*) the apo structures of SH, AS and DrTS. The β 2– α 2 loop, subdomain B, the β 4– α 4 loop and S7 in the complex of the XaSH E322Q mutant with sucrose (PDB entry 3czk; Kim *et al.*, 2008), the complex of the NpAS E328Q mutant with sucrose (PDB code 4fls; Champion *et al.*, 2012) and DrTS–Tris (PDB entry 4tvu; Wang *et al.*, 2014) are coloured green, blue and red, respectively, in (*a*), while those in XcSH (PDB code 2wpg; Champion *et al.*, 2009), DrAS (PDB entry 4ays; Skov *et al.*, 2013) and the DrTS N253F mutant are coloured green, blue and red, respectively, in (*b*).

Figure 4

Analysis of the products of the TS-catalyzed reaction by HPLC. (a) 75 mM of each of the standards glucose (G), maltose (M) and trehalose (T); (b)–(e) reaction products of wild-type DrTS and the N253F mutant using maltose (b, d) or trehalose (c, e) as the substrate, respectively. Under the assay conditions described in §2, the wild-type DrTS catalyzes the conversion of ~23% of the maltose into ~21% trehalose and ~2% glucose, while ~15% of the trehalose is converted to ~10.8% maltose and ~4.2% glucose. However, no significant product peaks were detected for the N253F mutant.

solution) no inhibitor was bound at the active site in N253F. This may indicate that neither the substrate maltose nor trehalose can bind to this mutant, perhaps owing to an inability to form the closed conformation. The enclosed active-site architecture unique to TS may thus be essential not only for the isomerization reaction but also for substrate binding. Therefore, the residues involved in the formation of the closed conformation may play an important role in controlling the catalytic efficiency of the enzyme.

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