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The structure of amylosucrase from *Deinococcus radiodurans* has an unusual open active-site topology

Amylosucrases (ASes) catalyze the formation of an α -1,4-glucosidic linkage by transferring a glucosyl unit from sucrose onto an acceptor α -1,4-glucan. To date, several ligand-bound crystal structures of wild-type and mutant ASes from *Neisseria polysaccharea* and *Deinococcus geothermalis* have been solved. These structures all display a very similar overall conformation with a deep pocket leading to the site for transglucosylation, subsite -1. This has led to speculation on how sucrose enters the active site during glucan elongation. In contrast to previous studies, the AS structure from *D. radiodurans* presented here has a completely empty -1 subsite. This structure is strikingly different from other AS structures, as an active-site-lining loop comprising residues Leu214–Asn225 is found in a previously unobserved conformation. In addition, a large loop harbouring the conserved active-site residues Asp133 and Tyr136 is disordered. The result of the changed loop conformations is that the active-site topology is radically changed, leaving subsite -1 exposed and partially dismantled. This structure provides novel insights into the dynamics of ASes and comprises the first structural support for an elongation mechanism that involves considerable conformational changes to modulate accessibility to the sucrose-binding site and thereby allows successive cycles of glucosyl-moiety transfer to a growing glucan chain.

1. Introduction

The transfer of glucose onto a growing α -1,4-glucan chain most commonly involves a 'high-energy' glucosyl donor in the form of a nucleotide glucosyl derivative. This reaction is found in most organisms (Cid *et al.*, 2002). A small group of bacteria are able to perform glucan synthesis through an additional route that utilizes sucrose as a glucosyl donor. This reaction is catalyzed by amylosucrases (ASes; EC 2.4.1.4), which belong to a family consisting mainly of glycoside hydrolases: glycoside hydrolase family 13 (GH13, <http://www.cazy.org>). Along with other sucrose-specific enzymes, such as the sucrose hydrolases (SHes), ASes cluster into subfamily 4 (Stam *et al.*, 2006). Although hydrolysis does occur as a side reaction, the ASes are efficient in glucan synthesis (Potocki de Montalk *et al.*, 2000), whereas the SHes are very efficient hydrolases and do not synthesize glucan (Kim *et al.*, 2004). Both reactions occur through a covalent glucosyl-enzyme intermediate (Jensen *et al.*, 2004), which reacts with water in the case of hydrolysis or an acceptor sugar moiety in the case of transglucosylation. This implicates that differences in active-site topology determine the outcome of the catalyzed reaction. In overall terms, compared with SHes, ASes are able to bind and present the reducing-end hydroxyl group of the glucan chain to the glucosyl intermediate in a way that protects the covalent intermediate from attack by water.

Both ASes and SHes have been well characterized in terms of crystallization and three-dimensional structure (Skov *et al.*, 2000, 2001, 2002; Mirza *et al.*, 2001; Jensen *et al.*, 2004; Kim *et al.*, 2008; Champion *et al.*, 2009, 2012; Guérin *et al.*, 2012). Overall, they consist of five domains (Fig. 1): N [residues 1–97 in AS from *Neisseria polysaccharea* (NPAS)], B (185–260), B' (395–460) and C (555–628) arranged around a central (β/α)₈-barrel termed domain A, which harbours the two catalytic carboxylates involved in the α -retaining

mechanism. Furthermore, structures in complex with substrates have provided details of substrate-binding determinants. Carbohydrate moiety-binding subsites are labelled according to the point of cleavage (and in the case of ASes also transfer), which by definition occurs between subsites -1 and $+1$ (Davies *et al.*, 1997). A sucrose complex of an inactive variant of NPAS showed that the substrate was bound at the bottom of a deep pocket (Mirza *et al.*, 2001), while a crystal structure of maltoheptaose in complex with an inactive variant of NPAS suggested that the binding of an acceptor glucan chain in subsites $+2$ and $+3$ would effectively block the entrance to subsite -1 (Skov *et al.*, 2002). This observation has led to the important question of how sucrose enters the binding pocket during elongation. In general, two mechanisms are possible: either the transfer cycle occurs by the release of glucan from AS after elongation or through

conformational changes that enable the entry of a new sucrose molecule. All AS structures published to date display the same overall conformation with little significant difference. The apparent lack of structural flexibility is in favour of the first mechanism. However, the enzymes have so far all been crystallized with either a ligand or a ligand-mimicking buffer/crystallization component in subsite -1 .

Here, the crystal structure of a ligand-free AS from *Deinococcus radiodurans* (DRAS) is presented. Compared with the previously determined AS structures, this structure reveals large changes in the active-site topology. These changes leave subsite -1 completely accessible. The large conformational changes can be attributed to an empty subsite -1 . These important and novel structural findings shed new light on the glucan-elongation mechanism and support the

Figure 1
(a) Side and (b) top views of the overall structure of DRAS. $2mF_o - DF_c$ density contoured at 1σ around the region corresponding to residues 124–151 is shown as a cyan surface. (c) Putative DRAS dimer with the individual protomers shown in green and yellow, respectively. (d) Close-up view of the putative DRAS dimer interface.

hypothesis of an elongation mechanism involving enzyme conformational changes to allow the entry of sucrose to the active site.

2. Materials and methods

2.1. Expression and protein purification

Escherichia coli BL21 cells containing pGST-DRAS were grown in 2×YT medium supplemented with 100 µg ml⁻¹ ampicillin. Gene expression was induced with IPTG at a final concentration of 5 µM when the OD_{600 nm} reached 0.3. The cells were then grown for 15 h at 291 K and harvested by centrifugation (6500 rev min⁻¹, 15 min, 277 K). The pellets were resuspended to a final OD_{600 nm} of 80 in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ pH 7.3) and sonicated. The extract was centrifuged at 12 500 rev min⁻¹ for 20 min at 277 K and DRAS was purified to homogeneity using GST-affinity chromatography followed by dialysis and subsequent tag removal using PreScission protease (Amersham) and reverse affinity chromatography as described previously (Potocki De Montalk *et al.*, 1999; Pizzut-Serin *et al.*, 2005). Notably, DRAS was purified using HEPES buffers, as opposed to NPAS and AS from *D. geothermalis* (DGAS) where Tris was used in the purification and was subsequently observed to bind as a substrate-mimicking ligand in the crystal structure (Skov *et al.*, 2001; Guérin *et al.*, 2012).

2.2. Crystal structure determination

Purified DRAS was concentrated to 3.3 mg ml⁻¹ in PreScission cleavage buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). Crystallization took place using the hanging-drop vapour-diffusion method at 277 K by mixing 2.5 µl protein solution with 2.5 µl reservoir solution (1 M sodium acetate, 0.1 M imidazole, 0.1 M LiCl pH 6.5). A solution consisting of 0.7 M sodium acetate, 0.07 M imidazole, 0.1 M LiCl pH 6.5, 30% glycerol was used as a cryoprotectant before single crystals were mounted in cryo-loops and cooled in liquid nitrogen. Data were collected from a single crystal at 120 K on beamline X11 at DESY, Hamburg, Germany. Data processing to 3.15 Å resolution was performed using *MOSFLM* and *SCALA* (Leslie, 1992; Evans, 1993). The crystals belonged to the tetragonal space group *P*4₃2₁2 with one DRAS molecule in the asymmetric unit. A homology model of DRAS created using *MODELLER* (Šali & Blundell, 1993) and the structure of DGAS (PDB entry 3ucq; sequence identity 75%; Guérin *et al.*, 2012) was used as a search model for molecular replacement using *Phaser* (McCoy *et al.*, 2007). Model building was performed with *Coot* (Emsley & Cowtan, 2004). Refinement was performed using *PHENIX* (Brunger, 2007; Adams *et al.*, 2002). Positional refinement with individual *B* factors in combination with secondary-structure restraints resulted in an *R* value and an *R*_{free} of 18.8 and 25.5%, respectively, with bond-length and bond-angle deviations of 0.014 Å and 2.012°, respectively. At this point TLS refinement was included with ten TLS groups (residues 16–62, 63–164, 165–210, 211–267, 268–329, 330–418, 419–460, 461–556, 557–610 and 611–643) and scale factors for X-ray/stereochemistry and X-ray/ADP weights both set to 0.1. Very poor and incoherent density for residues 1–15, 124–151 and unclear density for residues 229–233 and 529–534 was found and these residues were consequently not modelled. 3σ *mF*_o – *DF*_c density for several conserved (in the DGAS structures with PDB codes 3ucq and 3uer; Guérin *et al.*, 2012) water molecules was observed; however, none were included in the model as they failed to return 2*mF*_o – *DF*_c density upon inclusion. No residues were found in disallowed regions of the Ramachandran plot (using *PROCHECK* at the *ADIT* validation server; <http://deposit.rcsb.org/validate/>). The

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Data statistics	
Space group	<i>P</i> 4 ₃ 2 ₁ 2
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 129.2, <i>c</i> = 122.4
Wavelength (Å)	0.84300
Resolution (Å)	60.00–3.15 (3.32–3.15)
Total reflections	132833 (19357)
Unique reflections	18489 (2640)
Multiplicity	7.2 (7.2)
Completeness (%)	99.9 (100.0)
<i>R</i> _{merge} [†] (%)	8.1 (42.1)
<i>I</i> /σ(<i>I</i>)	7.7 (1.8)
Refinement	
Resolution (Å)	40.60–3.15 (3.32–3.15)
<i>R</i> factor [‡] (%)	20.0 (27.6)
<i>R</i> _{free} [§] (%)	23.6 (31.0)
No. of non-H atoms	
Protein	4668
Water	—
R.m.s. deviations	
Bond lengths (Å)	0.003
Bond angles (°)	0.734
Dihedral angles (°)	13.633
Wilson <i>B</i> factor (Å ²)	81

[†] *R*_{merge} = $\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where *I*_{*i*}(*hkl*) is the *i*th measurement. [‡] *R* factor = $\sum_{hkl} ||F_{obs}| - |F_{calc}|| / \sum_{hkl} |F_{obs}|$. [§] *R*_{free} was calculated using a randomly selected 5% of the reflections that were excluded from refinement.

final structure exhibited an *R* value and an *R*_{free} of 20.0 and 23.6%, respectively, with bond-length and bond-angle deviations of 0.003 Å and 0.743°, respectively. Data-collection and refinement statistics are presented in Table 1. The coordinates and structure factors have been deposited in the Protein Data Bank as entry 4ays.

Multiple sequence alignments were made using *ClustalW2* (Larkin *et al.*, 2007), while graphics and structural alignments of DRAS (PDB entry 4ays), DGAS (PDB entry 3ucq; Guérin *et al.*, 2012) and NPAS (PDB entries 1g5a and 1mw0; Skov *et al.*, 2001, 2002) were made using *PyMOL* (v.1.5.0; Schrödinger LLC).

3. Results and discussion

3.1. Overall structure and putative dimer interface

DRAS consists of the five domains usually observed in the ASes: N (residues 16–79 in DRAS), B (175–251), B' (390–459) and C (563–643) flanking the central domain A (80–173, 252–389 and 460–562) (Figs. 1a and 1b). DRAS displays 75 and 43% sequence identity to DGAS and NPAS, respectively. Structural superposition of these two amylosucrases on DRAS gives overall root-mean-square deviations (r.m.s.d.s) of 1.2 Å (509 C^α atoms aligned) and 1.5 Å (472 C^α atoms aligned), respectively. Notably, the DGAS and NPAS structures align with a lower r.m.s.d. (0.9 Å; 462 C^α atoms aligned), reflecting more similar overall conformations, as discussed below. In contrast to NPAS, DRAS and DGAS have recently been shown to form dimers (Guérin *et al.*, 2012). However, previous reports on DRAS suggested a monomeric state (Pizzut-Serin *et al.*, 2005). The DRAS crystal structure displays a dimeric assembly similar to the DGAS dimer, in both cases with the protomers related by crystallographic symmetry. Despite high sequence conservation between DRAS and DGAS in the seven regions identified as potential dimerization motifs by Guérin *et al.* (2012), the putative DRAS interface is primarily mediated by two regions from domain N, residues Thr22, Leu25, Arg26, Arg29 and Tyr30 and residues Glu73, Leu76 and Leu77, which are involved in hydrophobic interactions with the corresponding residues from the second protomer (Figs. 1c and 1d). A single

hydrogen bond between Glu73 OE1 and Thr22 OG1 is also observed. Finally, residues Thr585 and Pro584 from domain C are in close proximity to Arg333 and His336 from domain A of the other protomer and may play a minor role in dimer stabilization. Analysis of the putative DRAS dimer interface using the PISA server (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html; Krissinel & Henrick, 2007) shows an interface of $2086 \text{ \AA}^2$ and indicates that the assembly is not very strong. In comparison, the DGAS dimer interface is $2950 \text{ \AA}^2$. In addition to the abovementioned hydrophobic patch between the N domains that is fully conserved between the two structures, DGAS residues from domains A and C are extensively involved in the formation of the dimer interface. Furthermore, two salt bridges, between Glu25 and Arg74 and between Asp84 and Arg341, which were proposed to be important for the DGAS dimer (Guérin *et al.*, 2012) are not observed in DRAS. Although all of these four residues are conserved in DRAS, Glu21 (corresponding to Glu25 in DGAS) is involved in an intramolecular salt bridge with Arg24, while the loop containing Arg333 (corresponding to Arg341 in DGAS) adopts a

different conformation to that observed in DGAS. The increased number of polar interactions and the more pronounced shape complementarity observed in the DGAS dimer compared with DRAS are consistent with the higher thermostability of DGAS (Pizzut-Serin *et al.*, 2005; Guérin *et al.*, 2012).

3.2. Extensive changes in active-site topology

The structure of DRAS presented here is devoid of any ligand in subsite -1 (Fig. 2*a*). This is the first such structure, as other AS structures all contain carbohydrate ligands and/or the substrate-mimicking molecules Tris or glycerol (Skov *et al.*, 2001, 2002; Mirza *et al.*, 2001; Jensen *et al.*, 2004; Guérin *et al.*, 2012; Champion *et al.*, 2012). Tris was intentionally avoided in the purification of DRAS in an attempt to obtain a true apo structure. In comparison with previous structures of ASes, which all display a pocket-type topology, the unique ligand-free state of DRAS imposes dramatic differences in the active-site architecture, in particular around subsite -1 (Fig. 3).

Figure 2
The $3.15 \text{ \AA}$ resolution DRAS electron-density map. (a) Stereoview of the final $2mF_o - DF_c$ map (blue) contoured at 1σ and $mF_o - DF_c$ maps contoured at -3σ (red) and 3σ (green). A sucrose molecule from the NPAS-sucrose complex (PDB entry 1jgi; Mirza *et al.*, 2001) is shown to indicate the positions of subsites +1 and -1 and to emphasize that no difference density is present at this position. (b) Stereoview of the final $2mF_o - DF_c$ map contoured at 1σ . Residues Gly224 and Pro215 are labelled to indicate the position of the extended loop in DRAS.

A prototypical GH13 -1 subsite is optimized to facilitate the formation of the covalent glucosyl-enzyme intermediate, and in the case of amylases also to efficiently exclude water in order to facilitate transferase activity. A general feature of subsite -1 is a Tyr residue that provides a stacking platform for the covalent intermediate (Jensen *et al.*, 2004). In the DGAS structure (Guérin *et al.*, 2012) this Tyr residue (Tyr140) is located in a loop region between strand 2 and helix 2 of the A domain (Fig. 3*a*). Another highly important residue in this region is the conserved Asp137, which hydrogen bonds to OH4 of the glucosyl moiety and at the same time engages in a salt bridge with the conserved Arg520. This salt bridge is important for the 'exo'-type activity of ASes, as subsites -2 , -3 *etc.* cannot be formed. In all AS structures elucidated so far, subsite -1

has been situated in a deep pocket. Compared with GH13 hydrolases, for example α -amylase, subsite -1 in ASes is effectively closed by an extended loop of the B domain (Fig. 3*a*). As can be seen from the DGAS structure, two Phe residues from this loop are introduced into the active-site pocket: Phe222 is involved in stabilization of the glucosyl moiety through van der Waals interactions, while Phe225 blocks access of solvent from the nonreducing end. Both Phe residues are conserved in DRAS. In NPAS Phe225 is replaced by a glutamine residue (Gln232) that similarly seals subsite -1 through a hydrogen bond to Asp506.

Most surprisingly, it was found that very weak and incoherent density could be observed for residues 124–151 of DRAS corresponding to the loop region between strand 2 and helix 2. Excluding

Figure 3

Stereo picture showing subsite -1 in (a) ligand-bound DGAS (PDB entry 3ucq) and (b) ligand-free DRAS. The B domain with the extended loop is shown in orange and the B' domain is shown in yellow, while the DGAS region corresponding to the 'missing' region in DRAS (residues 124–151) is coloured cyan. In the DGAS complex (PDB entry 3ucq) the substrate-mimicking ligands Tris and glycerol are accommodated in subsites $+1$ and -1 , respectively.

Figure 4

Stereo picture showing superimposed structures of ligand-free DRAS (red), ligand-bound DGAS (PDB entry 3ucq; cyan) and NPAS (PDB entry 1mw0; black). For clarity, only the maltoheptaose ligand from the NPAS complex is shown (yellow sticks). The subsite numbering is indicated, with subsites -1 and $+1$ corresponding to the sucrose-binding site.

bulk-solvent correction and anisotropic scaling during map calculation returned more $2mF_o - DF_c$ density for a number of the missing residues (Figs. 1a and 1b); however, it remained impossible to model this region. These residues were excluded from the final model. While proteolytic trimming of the protein cannot be excluded, the presence of electron density in this region, although weak, strongly suggests rather that the protein is intact but highly flexible. To our knowledge, all other GH13 structures determined to date are structurally well defined in this region. Since Asp133 and Tyr136 (corresponding to Asp137 and Tyr140 in DGAS) are part of this disordered region, subsite -1 is only partially formed in the DRAS structure and there is no salt bridge to terminate the binding site after subsite -1 (Fig. 3b). The remaining residues of subsite -1 are well defined and exhibit good $2mF_o - DF_c$ density (Fig. 2a). Moreover, the B' domain of DRAS is shifted approximately 4 Å away from the active site and the extended loop of the B domain (residues Leu214–Asn225) adopts a previously unseen conformation (Figs. 2b and 3b), leaving subsite -1 completely solvent-exposed. In this conformation, the loop interacts with residues Pro435 and Gln436 from the same protomer and with several residues from the N domain of a symmetry-related molecule. Together, the unique structural features observed in the ligand-free DRAS result in a slightly wider active site displaying a groove-like topology and a fully accessible sucrose-binding site (Fig. 4).

SH from *Xanthomonas campestris* is the only other structure of a GH13 subfamily 4 enzyme which has been determined with an empty -1 subsite (Champion *et al.*, 2009). In this structure, the extended loop from the B domain was completely disordered and part of the region corresponding to DRAS residues 124–154 had moved compared with the complexed structures of NPAS (Jensen *et al.*, 2004; Skov *et al.*, 2001, 2002; Mirza *et al.*, 2001), allowing access to subsite -1 from the nonreducing end. Based on this structure, it was speculated that the extent of movement observed was to be expected, since this enzyme is primarily a hydrolase. DRAS, on the other hand, has clearly been shown to be an AS (Pizzut-Serin *et al.*, 2005). The structure presented here therefore suggests that ASes also have highly flexible regions in the absence of bound substrate. This finding is further supported by recent results from MD simulations of DGAS and NPAS that indicated significant mobility of several loops surrounding the active site, in particular the extended loop of domain B, as well as disruption of the salt bridge in subsite -1 in unliganded NPAS (Guérin *et al.*, 2012; Champion *et al.*, 2012).

3.3. Implications for the mechanism of AS glucan elongation

The DRAS structure reveals a high degree of flexibility in the substrate-binding region. This is probably also the case for other ASes, considering the relatively conserved nature of the regions involved (Guérin *et al.*, 2012). While the sucrose-binding subsites -1 and +1 are only partially formed in the ligand-free structure, the acceptor glucan subsites +2, +3 and +4 appear to be largely unchanged; *i.e.* a bound glucan at these subsites would be able to make most of the same interactions with the enzyme in both the open (DRAS) and the closed (NPAS and DGAS) forms. Therefore, it is likely that the glucan is able to remain attached at these sites in both conformations. Additionally, AS is expected to be in close proximity to glucan *in vivo*, as two additional glucan-binding sites distant from the active site have previously been observed on the surface of NPAS (Skov *et al.*, 2002). Moreover, the presence of glycogen has an activating effect on NPAS (Potocki De Montalk *et al.*, 2000). However, based on previous AS structures, which all display the closed

conformation, the presence of a glucan at the reducing-end subsites appeared to efficiently block entry to and exit from the sucrose-binding site (Skov *et al.*, 2002), implying the necessity of glucan release between successive glucosyl-transfer reactions. The major structural changes observed in the ligand-free DRAS structure, however, suggest a much more dynamic nature of the ASes, with considerable conformational changes in the loop regions regulating access to the sucrose-binding site. Such a mechanism would allow multiple cycles of glucosyl transfer onto a growing polymer chain without the need for glucan dissociation from the AS during elongation. Furthermore, it provides a plausible pathway for the escape of the fructose moiety that is released during the transglucosylation reaction, another issue that has so far remained unexplained.

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