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A Sialidase Mutant Displaying *trans*-Sialidase Activity

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Trypanosoma cruzi, the agent of Chagas disease, expresses a modified sialidase, the *trans*-sialidase, which transfers sialic acid from host glycoconjugates to β-galactose present in parasite mucins. Another American trypanosome, *Trypanosoma rangeli*, expresses a homologous protein that has sialidase activity but is devoid of transglycosidase activity. Based on the recently determined structures of *T. rangeli* sialidase (TrSA) and *T. cruzi trans*-sialidase (TcTS), we have now constructed mutants of TrSA with the aim of studing the relevant residues in transfer activity. Five mutations, Met96-Val, Ala98-Pro, Ser120-Tyr, Gly249-Tyr and Gln284-Pro, were enough to obtain a sialidase mutant ($TrSA_{5mut}$) with *trans*-sialidase activity; and a sixth mutation increased the activity to about 10% that of wild-type TcTS. The crystal structure of $TrSA_{5mut}$ revealed the formation of a *trans*-sialidase-like binding site for the acceptor galactose, primarily defined by the phenol group of Tyr120 and the indole ring of Trp313, which adopts a new conformation, similar to that in TcTS, induced by the Gln284-Pro mutation. The transition state analogue 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (DANA), which inhibits sialidases but is a poor inhibitor of *trans*-sialidase, was used to probe the active site conformation of mutant enzymes. The results show that the presence of a sugar acceptor binding-site, the fine-tuning of protein–substrate interactions and the flexibility of crucial active site residues are all important to achieve transglycosidase activity from the TrSA sialidase scaffold.

Keywords: *trans*-sialidase; sialidase; *Trypanosoma cruzi*; *Trypanosoma rangeli*; protein engineering

Introduction

Sialic acids are nine-carbon monosaccharides and comprise a family of about 40 members found in the outermost position of oligosaccharide chains of glycoproteins and glycolipids.[1] Advances in fields as different as cell adhesion and morphogenesis[2,3] lymphocyte homing and inflammation,[4] viral, bacterial, and, protozoarian pathogenesis and nutrition[5], and regulation of the immune system[6,7] stress the biological importance of sialic acids[4,8–10] and of the enzymes, sialidases and sialyltransferases, that regulate their presence and cell surface distribution.

Trypanosoma cruzi, the agent of Chagas disease and *Trypanosoma brucei*, the agent of the disease known as sleeping sickness in humans (*T. brucei* spp. *gambiense* and *T. brucei* spp. *rhodesiense*) and ngana in domestic animals (*T. brucei brucei*), are unable to synthesize sialic acid. Instead, these parasites express a glycosylphosphatidylinositol (GPI)-anchored surface *trans*-sialidase (TS) that scavenges sialic acid from host glycoconjugates.[11,12] The TS from *T. cruzi* (TcTS) has been extensively studied. TcTS was suggested to be involved in relevant processes such as parasite survival from the complement-mediated host immune response,[13] host cell invasion[14–16] and *T. cruzi* pathogenesis.[17,18] The involvement of TcTS in these processes is likely due to its capacity of sialylating the mucin molecules that cover the parasite surface with a dense protective layer.[19] TcTS has a globular catalytic domain and a C-terminal extension of tandemly repeated 12 amino

Abbreviations used: TS, *trans*-sialidase; TcTS, *Trypanosoma cruzi trans*-sialidase; TbTS, *Trypanosoma brucei trans*-sialidase; TrSA, *Trypanosoma rangeli* sialidase; GPI, glycosylphosphatidylinositol; MuNANA, 2′-(-4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; DANA, 2, 3-didehydro-2-deoxy-*N*-acetylneuraminic acid; wt, wild-type.

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acid residue motifs termed SAPA (for shed acute phase antigen) that is not essential for the enzymatic activity.[20]

The catalytic domains of TcTS and *T. brucei* TS are modified sialidases (E.C. 3.2.1.18) with unusually efficient transglycosidase properties.[21,22] Recently, we have obtained the 3-D structure of TcTS[23,24] and that of the closely related *Trypanosoma rangeli* sialidase (TrSA),[25,26] a strict hydrolase, which is 70% identical with TcTS at the amino acid level.[27,28] The enzymes fold into two structural domains that interact through a large hydrophobic interface. The N-terminal domain displays the characteristic six-bladed β-propeller fold of viral and bacterial sialidases and includes the active center, whereas the C-terminal domain has a β-sandwich fold with a lectin-like topology. The structural comparison of the TcTS and TrSA suggests that some amino acid changes close to the active center might be important for TS activity. In the complex of TcTS with the acceptor substrate lactose, the disaccharide makes stacking interactions with the aromatic side-chains of Tyr119 (replaced by serine in TrSA) and Trp312 (which is conserved in TrSA but adopts a different conformation, probably because of the substitution TrSA-Gln284→TcTS-Pro283[29]). Another important difference involves the structural environment of the catalytic nucleophile Tyr342.[26,30] While this region displays a rigid topology in sialidases, the tyrosine side-chain presents two distinct orientations in different crystal structures of TcTS,[23] suggesting that nucleophile flexibility might be important for the trans-glycosylation reaction.

Information derived from the sequence and structural comparisons of TrSA and TcTS had been previously used to construct point mutants of TrSA with the aim of obtaining *trans*-sialylation activity using the TrSA scaffold.[25,29,31] However, these point mutants did not display TS activity, probably because a combination of several essential amino acids is actually required for the enzyme to transfer sialic acid. Here, we report the construction and analysis of a number of TrSA mutant proteins, and demonstrate that mutations at six positions allow the obtention of a TrSA enzyme with a significant level of TS activity. Analysis of these mutant proteins defines the residues relevant for trans-glycosidase activity, with potential implications for the design of tools that may be useful in carbohydrate synthesis and in the design of TcTS inhibitors.

Results

Obtaining a TrSA mutant with TS activity

The structural comparison of the TrSA and TcTS active sites showed that most relevant residues for catalysis and substrate-binding are confined to the first 200 amino acid residues of the proteins. Therefore we constructed chimeric proteins exchanging the N-terminal segment of TcTS and TrSA, and assayed their hydrolytic and transfer activities (Figure 1A). We found that one of these chimeras was inactive, while the other (with the N terminus of TcTS and the C terminus of TrSA) had a strict sialidase activity. When the Gln284-Pro mutation was included in this second chimera, both TS and sialidase activities could be detected (Figure 1A). Previous results have highlighted the importance of Pro283 (the structural homologue of Gln284 in TrSA) for TcTS activity.[32] The proline side-chain is in contact with Trp312, a conserved residue that is part of the TcTS lactose-binding site.[23] Therefore, a bulkier residue at the equivalent Pro283 position (such as Gln284 in TrSA) could affect the Trp side-chain orientation and interfere with substrate binding. Nevertheless, a single point mutant of TrSA having a proline residue at position 284 lacked TS activity, implying that this is not the only amino acid required to achieve *trans*-sialylation activity (Figure 1A).

The sequence comparison of TrSA with the two trypanosomal *trans*-sialidases of known primary structure, TcTS and *T. brucei* TS (TbTS) is shown in Figure 1B for the β-propeller domain. The arrows indicate the residues close to the substrate-binding site (see also Figure 2) that were changed to generate the TrSA mutants in the search of sialyltransferase activity. Among them, Tyr119 was earlier found to be crucial for TcTS activity.[25] However, previous results showed that the corresponding mutation Ser120-Tyr in TrSA, or even a triple mutant bearing essential residues for *trans*-sialylation (TrSA Ser120-Tyr, Gly249-Tyr and Gln284-Pro), did not result in the appearance of TS activity.[29] The crystal structures of TrSA and TcTS in complex with the inhibitor 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) revealed that a conserved aspartic acid residue (Asp96 in TcTS, Asp97 in TrSA) makes different hydrogen bonding interactions with the sialic acid moiety of the substrate in each case.[23,26] These structural differences, which appear to be critical in determining the precise placement of the sialic acid in the reactive center, may arise from amino acid substitutions at the two adjacent positions: Val95 and Pro97 in TcTS are replaced by Met96 and Ala98 in TrSA (Figure 2). Moreover, the residues at these two positions are conserved in TcTS and TbTS (Figure 1B), suggesting that they may be important for TS activity. While the TrSA double mutant (Met96-Val, Ala98-Pro) did not show TS activity (Table 1), the TrSA protein bearing the five mutations at positions 96, 98, 120, 249 and 284 resulted in the first TrSA displaying TS activity. The specific activity of this mutant, $TrSA_{5Mut}$, was about 1% of the activity present in recombinant wild-type TcTS (Table 1).

Crystal structure of $TrSA_{5mut}$

In order to further validate the conformations of mutated amino acids, the crystal structure of

$TrSA_{5mut}$, alone and in complex with DANA, were determined at 2.8 Å and 3.1 Å resolution, respectively (Table 2). As expected, the structure is similar to that of wild-type TrSA (PDB code 1N1S), with rms deviations of 0.42 Å for the 389 C^{α} positions of the catalytic domain and 0.57 Å for all 628 residues. The inhibitor DANA binds to the catalytic center in a similar way as observed for wild-type TrSA (Figure 3). In particular, the C2 atom of DANA (the anomeric carbon in sialic acid) is positioned immediately above the hydroxyl group of Tyr343 (distance 2.9 Å) and the OH-4 hydroxyl of DANA makes strong hydrogen bonding interactions with both the guanidinium group of Arg54 (2.6 Å) and the carboxylate group of Asp97 (2.9 Å), as observed in the TrSA–DANA complex.[26] These interactions are not observed in the TcTS–DANA complex, where the OH-4 group interacts with Arg53 and the proton donor Asp59, while Asp96 (equivalent to Asp97 in TrSA) makes hydrogen bonding interactions with the glycerol moiety of the inhibitor.[23]

The five mutated residues have well-defined electron density. In particular, the two Tyr sidechains, Tyr120 and Tyr249, in the $TrSA_{5mut}$–DANA complex have similar ($\chi1$, $\chi2$) dihedral angles as observed for Tyr119 and Tyr248 in TcTs. In the

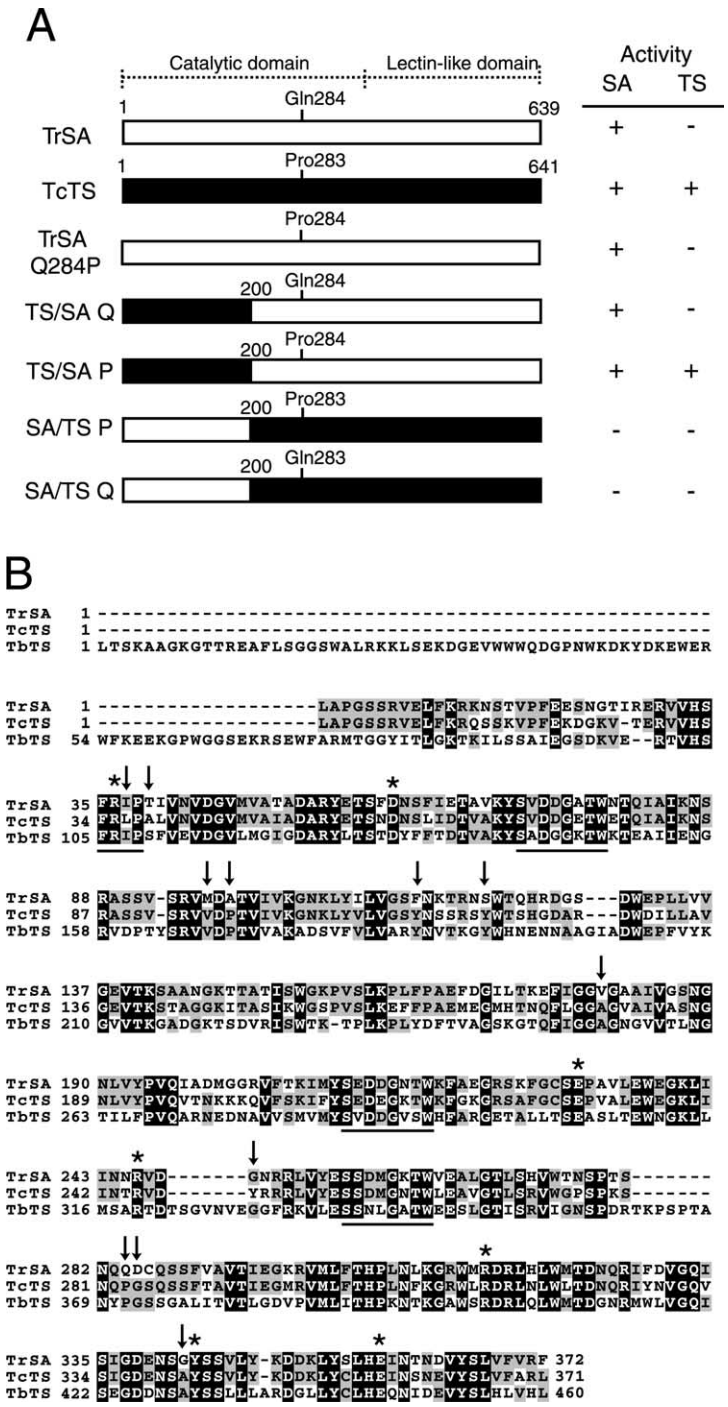

Figure 1. Sialidase and *trans*-sialidase activity of chimera proteins between TcTS and TrSA. A, The top two bars represent the open reading frames of the recombinant *T. cruzi trans*-sialidase and *T. rangeli* sialidase. Chimeric proteins between these two genes are showed below, dark and white bars represent TcTS and TrSA-derived sequences, respectively. The amino acid present at the 283/284 position is indicated. The sialidase and *trans*-sialidase activities were assayed in crude extracts by measuring the fluorescence of 4Mu released from 0.2 mM MuNANA (sialidase activity) and, the amount of sialic acid transfer from 1 mM sialyl-lactose to 40,000 dpm of [^{14}C]lactose (*trans*-sialidase activity). B, Sequence alignment of trypanosomal sialidase/*trans*-sialidase. Amino acid residues conserved among the three proteins are shown in dark and those residues conserved in two proteins in gray. An arrow indicates the residues known to be relevant for *trans*-sialidase activity that were mutated in TrSA. An asterisk indicates the position of strictly conserved residues in the whole sialidase superfamily. TrSA has one extra amino acid at position 27 as compared with TcTS. As a consequence, the numbering of residues between both proteins differs by one. FRIP and Asp motives, which are conserved in sialidases, are underlined.

structure of unliganded $TrSA_{5mut}$, the aromatic side-chain of Tyr120 is not well defined in the electron density map, probably because it adopts alternate conformations as observed in some TcTS structures.[24] Comparison of the active site of $TrSA_{5Mut}$ with that of TrSA and TcTS shows that the lactose-binding site, found in TcTS but not in TrSA, is also present in $TrSA_{5Mut}$. This site is created by the Ser120-Tyr substitution and the movement of the side-chain of Trp313 (Figure 3A) from the external orientation observed in wt TrSA to a new position closer to Pro284, as observed in TcTS.[23]

Additional substitutions increase the transglycosylation efficiency

The five amino acid substitutions at TrSA positions 96, 98, 120, 249 and 284, directly affecting substrate binding, produced a protein with significant TS activity. However, this activity is still low compared with wt TcTS, indicating that additional factors are also important for transglycosylation. One of these factors may be the intrinsic flexibility of Tyr342 in TcTS,[23] which has been recently shown to act as the catalytic nucleophile.[24,30] To investigate this issue, we analyzed the effect of introducing additional amino acid substitutions that could contribute to increase the active site flexibility in $TrSA_{5Mut}$. The positions studied include the residue Gly342 in TrSA (Ala341 in TcTS), which is part of the nucleophile-containing loop, and Ile37 (Leu36 in TcTS), whose side-chain displays an alternate conformation in the TcTS structure and is in contact with the aromatic ring of the nucleophile.[23] Two other amino acid differences that may contribute to create a cavity in TcTS near the active center were also tested, namely the substitution of Val180 in TrSA by a smaller residue (Ala179) in TcTS, and that of Asp285 (TrSA) by Gly284 in TcTS[26] (Figure 2B). Three of the above substitutions (Gly342-Ala, Val180-Ala, and Asp285-Gly) correspond to conserved substitutions in both *T. cruzi* and *T. brucei* *trans*-sialidases (Figure 1B). A fifth mutation analyzed was the replacement of Phe144 in TrSA by Tyr113 in TcTS (Figure 2), a residue that is part of the hydrophobic pocket that binds the *N*-acetyl group of sialic acid and might also contribute to modulate substrate binding.

Introduction of these mutations, separately, in the $TrSA_{5Mut}$ framework increased from three- to tenfold the TS activity of $TrSA_{5Mut}$ (Table 1). The most interesting results were obtained with the mutations at positions 37 or 342. These mutations rendered enzymes that showed 10–11% of TS activity as compared with wt TcTS recombinant enzyme (Table 1). On the other hand, the introduction of Val180-Ala, Asp285-Gly or Phe114-Tyr mutations in the $TrSA_{5Mut}$ framework resulted only in a modest increase of the TS activity. Prompted by these results, a genetic construction including simultaneously the Ile37-Leu and Gly342-Ala

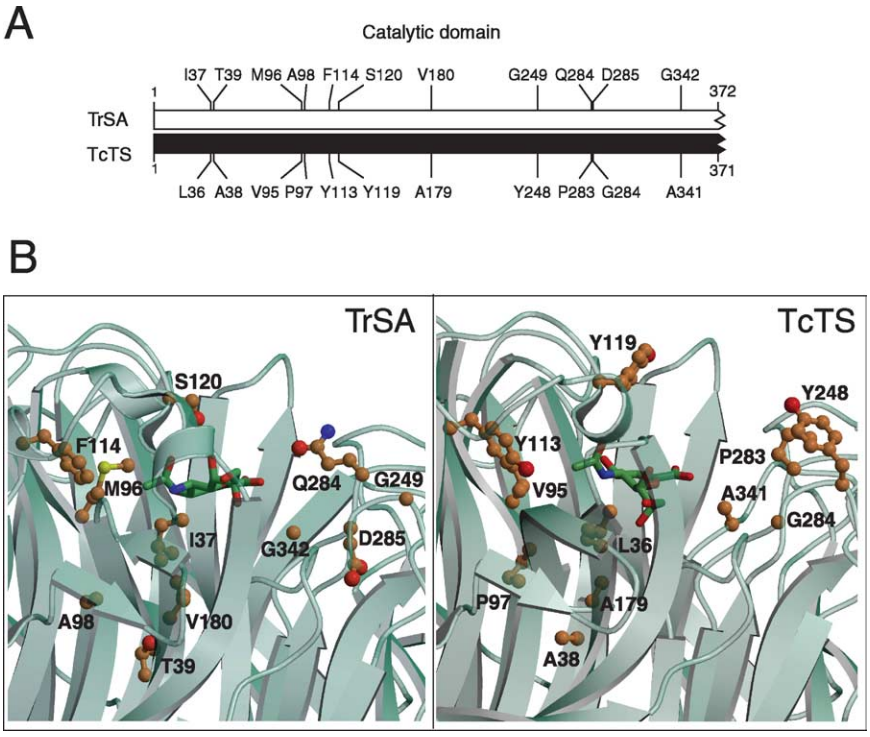

Figure 2. Amino acid differences between sialidase and *trans*-sialidase. A, The bars represent the catalytic domains of TrSA (white bar) and TcTS (black bar). Selected amino acid residues that differ between the two enzymes and may be important for *trans*-sialidase activity are indicated. B, Location of the above amino acids in the corresponding three-dimensional structures (PDB codes 1MS8 and 1N1T). The inhibitor DANA is also shown within the active site cleft.

Table 1. *trans*-Sialidase activity of TrSA mutant proteins

Protein	TS activity (pmol min^{-1} (μg protein)$^{-1}$)[a]	AppK_m (mM)[b]	AppV_{max} (nmol min^{-1} (μg protein)$^{-1}$)[b]
TrSA wt/TrSA Ser120Tyr/TrSA Gln284Pro/ TrSA Met96Val, Ala98Pro/TrSA Gly249Tyr, Gln284Pro/TrSA Ser120Tyr, Gly249Tyr, Gln284Pro	Undetectable	n.d.	n.d.
$TrSA_{5Mut}$	3.84 ± 0.18 (0.9)	n.d.	n.d.
$TrSA_{5Mut}$ Ile37Leu	47.20 ± 5.50 (11.3)	9.44 ± 2.00	14.70 ± 1.11
$TrSA_{5Mut}$ Gly342Ala	45.28 ± 3.30 (10.9)	6.90 ± 2.57	6.39 ± 1.30
$TrSA_{5Mut}$ Val180Ala	10.15 ± 0.21 (2.4)	n.d.	n.d.
$TrSA_{5Mut}$ Phe114Tyr	11.11 ± 0.57 (2.7)	n.d.	n.d.
$TrSA_{5Mut}$ Thr39Ala	26.62 ± 0.72 (6.4)	n.d.	n.d.
$TrSA_{5Mut}$ Asp285Gly	12.13 ± 0.78 (2.9)	n.d.	n.d.
TcTs wt	416.71 ± 47.43 (100)	0.24 ± 0.03	3.83 ± 0.42

trans-Sialidase activity was measured using sialyl-lactose and lactose as donor and acceptor substrates, respectively (see Materials and Methods for details). The values are the mean and standard deviation of three independent measurements.

[a] The percentage of *trans*-sialidase activity referred to wt TcTS is indicated in parentheses. n.d., not done.

[b] Apparent kinetic constants for lactose were determined using sialyl-lactose at a fixed concentration of 2 mM and varying the concentration of lactose.

mutations in $TrSA5_{mut}$ was also produced, but the resulting protein could not be purified because it was unstable and precipitated at low salt concentrations (below 200 mM). Nevertheless, this mutant was indeed found to display TS activity, as determined by qualitative measurements done in a non-desalted crude extract (data not shown).

An important point in order to gain TS activity in the TrSA framework is an appropiate reconstruction of the acceptor substrate-binding. The apparent K_m for lactose as an acceptor substrate was determined for $TrSA_{5Mut}$ Ile37-Leu and $TrSA_{5Mut}$ Gly342-Ala (the other mutants with TS activity were not tested, since their high hydrolytic activities make estimation of appK_m not reliable). Both proteins display a higher appK_m for lactose as compared with that observed for TcTS in the same conditions (Table 1). These values imply a low but measurable affinity for lactose, suggesting that the acceptor substrate-binding site is formed in the TrSA mutants, in agreement with the crystallographic results.

The hydrolytic activity of the TrSA mutants

TcTS is a modified sialidase that efficiently transfers sialic acid to β-galactoses, but it also hydrolyzes sialic acid, though with lower efficiency than other sialidases. The hydrolytic activity of wt recombinant TrSA and TcTS as well as that of the TrSA mutants were assayed using MuNANA and, some of them, with sialyl-lactose, as substrates (see Materials and Methods). When sialyl-lactose was used, the kinetic parameters of wt TcTS could not be estimated because the high *trans*-sialidase activity of this protein interferes with the formation of free sialic acid. The same overall conclusions could be drawn with both substrates. Analysis of MuNANA hydrolysis from wild-type recombinant TrSA and TcTS indicated that the catalytic efficiency was

Table 2. Data collection and refinement statistics

	$TrSA_{5mut}$	$TrSA_{5mut}$–DANA
Data collection		
Cell dimensions (Å)	$a=b=94.69$, $c=156.33$	$a=b=95.66$, $c=155.26$
Measured reflections	121,977	100,194
Unique reflections	17,626	13,500
Resolution (Å)	80–2.8 (2.95–2.8)	80–3.1 (3.15–3.10)
Completeness (%)	98.4 (98.2)	99.7 (99.5)
R_{sym}[a] (%)	10.3 (51.4)	11.5 (30.1)
Refinement		
Resolution range (Å)	80–2.8	80–3.1
Reflections used in refinement	16,895	12,736
R-factor[b] (%)	24.3	28.8
Free *R*-factor[c] (%)	33.6	35.3
r.m.s bond lengths (Å)	0.015	0.013
r.m.s bond angles (degrees)	1.43	1.52
Protein residues	628	389
Sugar atoms	0	20

[a] $R_{sym} = \sum_{hkl} \sum_i |I(hkl) - [I(hkl)]| / \sum_{hkl} \sum_i I(hkl)$.

[b] R-factor $= \sum_{hkl} ||F_{obs}(hkl)| - |F_{cal}(hkl)|| / \sum_{hkl} |F_{obs}(hkl)|$.

[c] R_{free} calculated as for *R*-factor for 5% of the data not used in refinement.

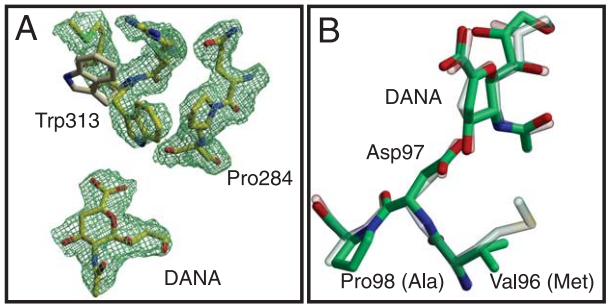

Figure 3. Crystal structure of the $TrSA_{5Mut}$–DANA complex. A, The inhibitor DANA and the new position of the Trp313 side-chain (promoted by the Gln284-Pro substitution) are well defined in the electron density ($2F_o - F_c$) map (contoured at 1 σ). The position of Trp313 in wild-type TrSA is also shown. B, Superposition of DANA as observed in the crystal structures of wild-type TrSA (light green) and $TrSA_{5Mut}$ (green).

about 1000 times lower for TcTS as compared with TrSA. As previously observed,[29] the TrSA point mutant Gln284-Pro is more active than the control, wt TrSA, as indicated by the increase in k_{cat}/K_m, k_{cat} and V_m values with both substrates (Table 3). A decrease in the K_m value of this mutant compared with the wild-type enzyme is consistent with the structural observations. The Gln284-Pro mutation induces a conformational change in the position of Trp313 (Figure 3A), thus likely allowing a more favorable stacking interaction between the Trp side-chain and the aromatic methylumbelliferyl group of MuNANA. The inclusion of two tyrosine residues, Gly249-Tyr and Ser120-Tyr, decreases the catalytic efficiency of the triple TrSA mutant (Ser120-Tyr, Gly249-Tyr and Gln284-Pro).

Substitution of the two amino acid residues, Met96-Val and Ala98-Pro, increased the K_m value for MuNANA (Table 3) but did not affect the k_{cat} value. In contrast, the $TrSA_{5Mut}$ protein showed a marked decrease in the K_m values. Although this protein acquires transferase activity, its catalytic efficiency as a hydrolase remains high, comparable to that of wt TrSA and 1000 times higher than that of wt TcTS. Further increase of TS activity by the inclusion of Ile37-Leu or Gly342-Ala in the $TrSA_{5Mut}$ framework (Table 3) showed a consistent inverse correlation with the hydrolytic efficiency. Other mutations that had little or no effect on the TS activity did not show significant modifications in their hydrolytic activities compared to $TrSA_{5Mut}$. The only exception was $TrSA_{5Mut}$ Val180-Ala, which clearly decreased its hydrolytic efficiency. A possible explanation for this is that the replacement of a buried valine residue by a smaller side-chain creating an internal cavity might destabilize the active conformation of the protein.

TrSA mutants having TS activity are less sensitive to the sialidase inhibitor DANA

Sialidases are known to be inhibited by DANA, an analog of the oxocarbenium transition state of

Table 3. Kinetic constants for the hydrolytic activity of recombinant TrSA mutant proteins

Protein	K_m (mM)[a]	V_m (nmol min^{-1} (μg protein)$^{-1}$)[a]	k_{cat} (s^{-1})[b]	k_{cat}/K_m (M^{-1} s^{-1})[b]
TrSA wt	0.273±0.010	273.11±8.82	151.42	5.55×10^5
	(0.468±0.149)	(126.8±10.4)	(150.46)	(3.22×10^5)
TrSA Ser120Tyr	0.068±0.002	45.48±1.17	27.21	3.98×10^5
TrSA Gln284Pro	0.082±0.002	1175.14±27.60	703.03	86.08×10^5
	(0.086±0.021)	(988.6±77.8)	(1173.16)	(136.41×10^5)
TrSA Met96Val, Ala98-Pro	1.079±0.048	285.98±11.40	171.09	1.59×10^5
TrSA Gly249Tyr, Gln284Pro	0.070±0.001	225.76±4.57	135.06	19.2×10^5
TrSA Ser120Tyr, Gly249Tyr, Gln284Pro	0.122±0.008	159.32±10.33	95.31	7.83×10^5
$TrSA_{5Mut}$	0.039±0.001	71.54±1.32	42.80	10.88×10^5
	(0.108±0.294)	(234.2±32.0)	(277.92)	(25.73×10^5)
$TrSA_{5Mut}$ Ile37Leu	0.200±0.009	106.04±4.43	63.44	3.17×10^5
	(0.248±0.032)	(123.2±12.3)	(145.76)	(5.88×10^5)
$TrSA_{5Mut}$ Gly342Ala	0.107±0.003	38.29±0.87	22.91	2.13×10^5
	(0.230±0.018)	(114.0±7.8)	(125.38)	(5.88×10^5)
$TrSA_{5Mut}$ Val180Ala	0.172±0.029	49.48±8.18	29.60	1.73×10^5
$TrSA_{5Mut}$ Phe114Tyr	0.038±0.001	74.91±1.53	44.82	11.79×10^5
$TrSA_{5Mut}$ Thr39Ala	0.029±0.001	56.03±1.17	33.52	11.65×10^5
$TrSA_{5Mut}$ Asp285Gly	0.025±0.001	52.85±0.92	31.62	12.70×10^5
TcTS wt	0.291±0.022	0.30±0.022	0.18	0.0062×10^5

The molecular mass of recombinant wt TrSA and TcTS are 71.2 kDa and 76.1 kDa, respectively. Sialidase activity using sialyl-lactose as substrate is indicated in parentheses. The others values were obtained with MuNANA as substrate. Activity is expressed as the amount of 4Mu released from MuNANA or the amount of sialic acid released from sialyl-lactose. The values are the mean and standard deviation of three independent measurements.

[a] K_m and V_m values were estimated from a Lineaweaver–Burk plot.

[b] k_{cat} and k_{cat}/V_m parameters were calculated from the K_m and V_m values of the previous columns.

the reaction. Since *trans*-sialidase is not well inhibited by DANA due to a different architecture of the sialic acid binding site,[23,24,26] remodeling of the substrate-binding site in TrSA mutants is expected to affect their sensitivity to DANA. The inhibition properties of the two wild-type enzymes, TcTS and TrSA, and three TrSA mutants are shown in Figure 4. DANA is indeed less effective as inhibitor of TrSA mutants having acquired TS activity as compared with wt TrSA, but without attaining the levels observed with wt TcTS. As evaluated by a Dixon plot, the K_i values for $TrSA_{5Mut}$, $TrSA_{5Mut}$ Ile37-Leu and $TrSA_{5Mut}$ Gly342-Ala were 1.54 mM, 1.01 mM and 0.54 mM, respectively (Figure 4). These values were in-between the K_i of wild-type proteins: 12.3 mM and 1.5 μM for wt TcTS and wt TrSA, respectively. All TrSA mutants devoid of TS activity (Table 1) display K_i values for DANA similar to that of wt TrSA (data not shown). These results indicate a clear, albeit non-linear, correlation between the acquisition of TS activity and the insensitivity to inhibition by DANA.

Discussion

Here, we have shown that it is possible to achieve *trans*-sialylation activity starting from a sialidase scaffold. For this purpose we took advantage of the information obtained from sequence comparison between three proteins (Figure 1B). Two of them are very similar to each other (70% amino acid identities), but one is a strict hydrolase (TrSA) while the other is an efficient sialyl-transferase (TcTS). The third enzyme, TbTS, has also *trans*-sialidase activity but is only 30% identical with the other two.[22] We have shown that only five mutations in the TrSA framework are enough to acquire *trans*-sialylation activity, but at least a sixth one is required to reach an activity equivalent to 10% of that of the recombinant wt TcTS.

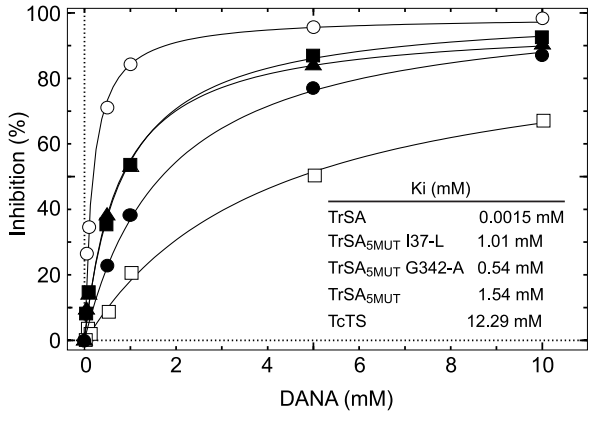

Figure 4. DANA inhibition of TrSA mutant proteins. TrSA wt (open circles), TcTS wt (open squares), $TrSA_{5Mut}$ (filled circles), $TrSA_{5Mut}$ Ile37-Leu (filled squares) and $TrSA_{5Mut}$ Gly342-Ala (filled triangles) purified proteins, were used to measure sialidase activity inhibition by DANA. Proteins were incubated with 0.2 mM MuNANA and different concentrations of DANA and the fluorescence quantified as indicated in Materials and Methods. The inhibition was assumed to be competitive and K_i values were estimated from the intercept of a Dixon plot ($1/v$ *versus* I) with a horizontal line through $1/V_{max}$ (Table 3).

The crystal structures of TrSA and TcTS provide a suitable framework to understand how individual amino acid differences could affect the enzymatic activity. Seven strictly invariant residues (marked with an asterisk in Figure 1B) are directly involved in the catalytic machinery of all microbial sialidases. These include the catalytic residues[30] as well as an arginine triad, which stabilizes the carboxylate group of the substrate. A tryptophan residue (312 in TcTS) was previously shown to be important for TS activity and substrate specificity,[29] and is also conserved among TrSA, TcTS and TbTS. Therefore, the differences in transglycosylation properties must arise from other amino acid substitutions that could modulate substrate binding or affect the conformation of the conserved catalytic residues.

The presence of a specific binding site for the acceptor sugar substrate (terminal β-galactose in TcTS) is likely to be a primary requirement for developing TS activity. This binding site is essentially formed in TcTS by two aromatic side-chain residues, Trp312 and Tyr119, that face each other to create a groove capable of binding a lactose molecule.[23] The substitution Ser120-Tyr in TrSA was found, as expected, to be one of the mutations necessary to obtain a TrSA enzyme with *trans*-sialylation activity. Although the single mutation Ser120-Tyr was not enough by itself to obtain a *trans*-sialidase active TrSA, the reverse mutation in TcTS (Tyr119-Ser) decreases 99% the TS activity while significantly conserving its sialidase activity.[25,29] On the other hand, the tryptophan side-chain adopts a different conformation, because the side-chain of Gln284 in TrSA precludes the indole ring of Trp313 from being positioned as in TcTS.[23] Interestingly, the TrSA substitution Gln284-Pro significantly increased the hydrolytic activity[29] (Table 3). The crystal structure of the $TrSA_{5Mut}$ protein showed that the Gln→Pro mutation does allow the side-chain of Trp313 to approach the catalytic center (Figure 3A) as in TcTS. This new orientation may optimize the binding of the aglycone moiety of the sialilated substrate, thus resulting in a lower K_m value and accounting for the improved catalytic efficiency of TrSA Gln284-Pro. However, neither these point mutants nor the triple mutant (Ser120-Tyr, Gly249-Tyr, Gln284-Pro) displayed TS activity, indicating that factors other than the lactose-binding site must be relevant for transglycosylation. When Met96-Val and Ala98-Pro mutations were introduced in TrSA, together with the substitutions discussed above, a first TrSA mutant displaying *trans*-sialylation activity could then be obtained (Table 1). The crystal structure of this protein ($TrSA_{5Mut}$) revealed that all mutated residues adopt a similar conformation as observed in wt TcTS and that the mutations induced no

significant structural changes in the protein, except for the movement of Trp313 discussed above.

The TS activity of the $TrSA_{5Mut}$ protein can be further improved by additional amino acid substitutions. In general, this gain in TS activity is negatively correlated with the hydrolytic efficiency. When the TrSA Gln284-Pro protein, which displays the highest sialidase activity, is taken as the reference hydrolase, kinetic constants (Table 3) show that all TrSA mutants with TS activity have a marked decrease in their hydrolytic activities. The higher TS activity was obtained when the substitutions Gly342-Ala or Ileu37-Leu were introduced into the $TrSA_{5Mut}$ framework. These two residues may indeed affect the flexibility of the tyrosine nucleophile. In particular the side-chains of Leu36 and Tyr342 in TcTS are in contact with each other, and both display an alternate conformation in the crystal structure.[23] A similar situation was found in the hemagglutinin-neuraminidase (HN) enzyme of paramyxovirus,[33] where the movement of the equivalent residues (Tyr526/Ile175) was interpreted as part of a conformational mechanism to switch between the enzymatic active (sialidase) and inactive (hemagglutinin) states of the protein.

In contrast with most sialidases, TcTS has a low affinity for the inhibitor DANA. The structures of the TcTS–DANA and TrSA–DANA complexes provided some clues to understand these observations.[23,26] As a consequence, it was expected that remodeling of the TrSA active site into a *trans*-sialidase-competent conformation might somewhat prevent DANA from inhibiting the enzyme. Consistently, the K_i values of all TrSA mutants lacking TS activity at different concentrations of DANA overlap with that of wt TrSA, whereas the inhibition properties of three mutant TrSA displaying partial TS activity were intermediate between those observed for wt TrSA and wt TcTS (Figure 4). However, the correlation is not simple, since the less sensitive mutant was the one having a lower TS activity ($TrSA_{5Mut}$). A deeper understanding of this behavior must await further mutagenesis and structural studies.

Synthesis of carbohydrates is required in research and industry, and the use of enzymes is an important alternative to chemical synthesis.[34] In this context, glycosyltransferases and nucleotide sugars are one possibility. The second one is to use retaining glycosidases as transglycosidases under special reaction conditions using modified substrates (glycosynthases),[32,35] although generally with low yields. The third possibility could be to use modified glycosidases with a high transglycosidase activity. This is the case of the natural trypanosomal *trans*-sialidases. However, there are very few examples of such effective transglycosidases. We have shown here that it is possible to engineer a sialidase, displaying a strict hydrolytic activity, to obtain a modified enzyme with significant *trans*-sialidase activity. Whether this approach is feasible with other sialidases or, more importantly, with other glycosidases, is difficult to predict. The results from this work indicate that several residues might have to be mutated.

Materials and Methods

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis experiments were carried out on pTrcTrSA4_Nhe,[29] a plasmid expressing the wt TrSA gene. Two complementary oligonucleotide primers containing the desired mutation were extended during temperature cycling by the *Pfu* DNA polymerase in a Perkin Elmer 480 thermal cycler. The reactions were set up in tubes containing 10–50 ng of purified wild-type plasmid, 500 ng of each oligonucleotide primer, 0.4 mM dNTPs and, three units of *Pfu* DNA polymerase in 1X *Pfu* buffer supplied with the enzyme. The tubes were incubated for three minutes at 93 °C, followed by 25 cycles of 30 seconds at 93 °C, one minute at 55 °C, 12 minutes at 68 °C and, a final extension of ten minutes at 72 °C. Reaction products were digested with 20 units of DpnI endonuclease (New Englands Biolabs), which digests methylated and hemi-methylated DNA. The digestion products were used to transform *Escherichia coli* DH5-α F′ Iq cells. Constructs were entirely sequenced to confirm the presence of the desired mutations.

Construction of chimeric proteins

Chimeric proteins were generated by a PCR method using the wild-type TcTS clone, pTrcTS611/2; the wild-type TrSA clone, pTrcTrSA4_Nhe; the TcTS Pro283Gln clone and, the TrSA Gln284Pro clone.[29] The first 605 nt corresponding to the 5′ end of TcTS was amplified using two oligonucleotides, LapNheTS GagatctcGCTAGCC-TGGCACCCGGATCGAGCCGA (lower case is the NheI restriction site) and TS198, TCGTAACCTGCACAGGG-TACACAAG. The 1366 nt corresponding to the 3′ end with TS188, ACGGGAATCTTGTGTACCCTGTGCA and TGA, GTGGAATTCAGGCACTCGTGTCGCTGCTGCT-GTC. The 608 nt corresponding to the 5′ end of TrSA was amplified with LapNheSA, GAagatctGCTAGCTT-GGCGCCCGGCTCAAGCCG (lower case is the NheI restriction site) and SA198, CCGCAATTTGCACAGGG-TACACAAG. The 1356 nt corresponding to the 3′ end with SA188, ATGGGAACCTTGTGTACCCTGTGCA, and SialRAstop, CCATCGATCTAGACTAAGCGGCAGTGC-CAGCGC. Underline indicates the sequence identical between TcTS and TrSA. PCR tubes containing 500 ng of each oligonucleotide, 1 ng of plasmid template, 200 μM each dNTP and two units of Vent DNA polymerase (New England Biolabs) in 1X ThermoPol buffer supplied with the enzyme, were subjected to the following thermal cycles: one cycle of three minutes at 93 °C followed by 35 cycles of 30 seconds at 93 °C, 60 seconds at 55 °C and 60 seconds at 72 °C. The corresponding fragments were separated in an agarose gel and purified using QIAEX II gel extraction kit (Quiagen) following the manufacturer's instructions. To construct chimeric genes, the corresponding 5′ and 3′ fragments were combined in a PCR reaction containing 50 ng of each purified fragment together with the corresponding oligonucleotides: LapNheTS or LapNheSA for the 5′ end of TcTS or TrSA, respectively; and TGA or SialRAStop for the 3′ end of TcTS or TrSA, respectively, together with two units of Vent DNA polymerase in 1X ThermoPol buffer. The cycling conditions were the same as above except that 25 cycles were

used. Fragments containing the N terminus of TrSA and C terminus of TcTS were digested with NheI and KpnI restriction enzymes and ligated to NheI/KpnI-digested pTrcTS611/2. Fragments containing the N terminus of TcTS and the C terminus of TrSA were digested with NheI and SalI, and ligated to NheI/SalI-digested pTrcTrSA4_Nhe. Generated constructs were entirely sequenced prior to protein expression experiments.

Recombinant protein expression and purification

The mutagenized clones were used to transform *E. coli* BL21 (DE3) pLysS. Cells were grown in terrific broth modified medium (Sigma) containing 100 μg/ml of ampicillin (Sigma), at 37 °C for 12–16 hours with constant agitation (250 rpm). The culture was diluted 1/50 and resumed under the same conditions up to A_{600nm} 1.0–1.2. Overexpression induction was triggered by adding 0.5 mM isopropyl-thio-β-D-galactopyranoside (Sigma) followed by incubation at 18 °C with constant agitation (250 rpm) for 12–16 hours. Cells were harvested by centrifugation and conserved at −80 °C until needed. After thawing, lysis was achieved in the presence of 20 mM sodium phosphate (pH 7.4), 1 M sodium acetate, 0.5% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 100 μg/ml of DNAse I, and the lysozyme produced by the cells. After ten minutes, the viscosity of the extract was reduced by sonication applying six pulses of 30 seconds on a Brandson 450 sonicator. Supernatants were ultracentrifugated at 100,000*g* for 45 minutes and subjected to iminodiacetic acid Ni^{2+} affinity chromatography (HiTrap Chelating, Amersham Phamacia Biotech AB). The column was extensively washed with 20 mM sodium phosphate (pH 7.4), 1 M sodium acetate and 30 mM imidazole in the above buffer. The protein was eluted with 100 mM imidazole in the same buffer. After dialyzing against 50 mM sodium acetate (pH 5.5), the sample was further subjected to cationic-exchange FPLC (Mono S, Amersham Phamacia Biotech AB) applying a linear NaCl elution gradient. The activity peak was pooled and immediately desalted with a Fast Desalting column (Amersham Pharmacia Biotech AB). Purified proteins were analyzed by SDS-PAGE under reducing conditions, stained with Coomassie brilliant blue R250 (Sigma), and quantified with Kodak 1D 3.0 software using purified BSA as standard.

Enzyme activity assays

Enzyme activity assays were performed with purified protein. When required, the enzymes were diluted in the reaction buffer prior to use. Sialidase activity was determined measuring the fluorescence of 4-methylumbelliferone (4Mu) released by the hydrolysis of 0.2 mM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MuNANA, Sigma). The assay was performed in 50 μl of 50 mM sodium acetate (pH 5.5), 0.2% (w/v) BSA. After incubation, the reaction was stopped with 0.2 ml of 0.5 M Tris (pH 10.5) and fluorescence measured with a DYNAQuant™ 200 fluorometer (Hoefer Pharmacia Inc). The excitation and emission wavelengths were 365 nm and 460 nm, respectively. Suitable modifications were made to the standard reaction to obtain the kinetic constants.

trans-Sialidase activity was measured as the transfer of sialic acid from 1 mM sialyl-α-(2-3)-lactose to 12 μM [D-glucose-1-^{14}C]lactose (55 mCi/mmol) (Amersham), in 30 μl containing 20 mM Hepes-Na (pH 7.5), 0.2% BSA and 30 mM NaCl. After ten minutes at 25 °C, the reaction was stopped by dilution with 1 ml of water and heated for three minutes at 80 °C. QAE-Sephadex (Amersham Pharmacia Biotech) was added and the resin was washed twice with water. Negatively charged compounds were eluted with 0.8 ml of 1 M NaCl and quantified in a WinSpectral 1414 liquid scintillation counter (Wallac).

trans-Sialidase and sialidase activities were determined in the same reaction tube using 5 mM MuNANA as sialic acid donor and 12 μM [D-glucose-1-^{14}C]lactose as acceptor in 30 μl containing 20 mM Hepes-Na (pH 7.5), 30 mM NaCl and 0.2% BSA. After ten minutes at 25 °C, 4 μl was withdrawn, added to 250 μl of 0.5 M Tris (pH 10.5), and 4Mu quantified for sialidase activity. The remaining volume was immediately mixed with 1 ml of water, heated at 80 °C for three minutes, and *trans*-sialidase activity determined by measuring [^{14}C]sialyl-lactose. Inhibition of sialidase activity with 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (DANA, Sigma) was carried out in the assay conditions as described above by adding the inhibitor to the enzyme mix.

Sialic acid measurements

The sialic acid released from the hydrolysis of sialyllactose was quantified by the thiobarbiturate method. Wild-type or mutant proteins were incubated with different concentrations of sialyl-α(2,3)lactose in 50 mM sodium acetate (pH 5.5), 30 mM NaCl, 1% BSA, in a final volume of 30 μl, for ten minutes at room temperature. The enzymatic reactions were stopped by adding 15 μl of a 25 mM $NaIO_4$ solution prepared in 125 mM sulphuric acid. The mixtures were vortexed and allowed to react in a water-bath at 37 °C for 30 minutes (sialic acid oxidation period). Samples were then neutralized by gentle addition of 13 μl of 2% (w/v) sodium arsenite in HCl (0.5 M). Tubes were gently vortexed to complete the reduction reaction. After the total disappearance of yellow color (∼five minutes) 125 μl of thiobarbituric acid (42 mM, pH 9.0) were added and then incubated in a boiling water bath for 15 minutes (chromophore formation). Samples were then cooled in an ice-water bath for five minutes, followed by color stabilization at room-temperature. The samples were centrifuged, and 100 μl were separated by HPLC through a C18 reverse phase column (Waters) using 2 : 3 : 5 water:methanol:buffer (buffer: 0.2% phosphoric acid; 0.23 M sodium perchlorate). Absorbance was measured at A_{549nm}. A sialic acid calibration curve was set and absorbance values were read in the linear range. Under the above conditions the sensitivity of the assay was 100 pmol sialic acid.

Protein crystallization and structure determination

Purified $TrSA_{5Mut}$ was concentrated to 9 mg/ml in 50 mM sodium phosphate, 80 mM sodium acetate (pH 7.0), and crystallized using the hanging-drop, vapor-diffusion method. Suitable crystals for diffraction analysis appeared within four days in drops composed of 5 μl of protein solution plus 5 μl of a reservoir solution containing 16% (w/w) PEG 8000, 50 mM sodium cacodylate and 100 mM ammonium sulfate (pH 6.5). $TrSA_{5Mut}$–DANA complexes were obtained by overnight soaking of native crystals with solutions containing the inhibitor at 10 mM concentration.

The crystals belong to space group $P4_32_12$. Several tested crystals diffracted X-rays to moderate resolution (∼3 Å) and show small but significant variations in their unit cell dimensions, suggesting the presence of molecular disorder in this particular crystal form. A complete

diffraction data set was collected using synchrotron radiation at the ESRF for unliganded $TrSA_{5mut}$ at 2.8 Å resolution (beamline ID29, $\lambda = 0.9756$ Å) and for the $TrSA_{5mut}$–DANA complex at 3.1 Å resolution (beamline ID14.3, $\lambda = 0.9340$ Å). Data reduction was carried out with the HKL program package[36] (Table 2), and the 3D structure was determined by molecular replacement methods using the program AMoRe[37] and the atomic coordinates of wt TrSA (PDB code 1N1S) as the search model. All crystallographic refinement was carried out with the program REFMAC.[38] From the initial refinement cycles of unliganded $TrSA_{5mut}$, it appeared that the whole C-terminal (lectin-like) domain displayed quite large temperature factors (>100 Å^2), suggesting the presence of disorder. Subsequent TLS refinement of unliganded $TrSA_{5mut}$ was carried out assuming three separate rigid bodies: the catalytic domain (residues 1–376), the lectin-like domain (residues 423–634) and the interconnecting region including an exposed flexible loop (residues 377–422). Using this model, refinement converged to a final *R*-factor of 0.243 ($R_{free} = 0.336$), see Table 2. Analysis of the atomic temperature factors indicated that TLS parameters can account for much of the disorder (or flexibility) of the lectin-like domain, since the total *B*-values were significantly higher for the C-terminal domain compared to the catalytic domain, whereas the residual *B*-values (i.e. without the TLS contribution) displayed a much more homogeneous distribution (Figure 5). When the C-terminal domain was completely excluded from the model, the TLS refinement of the catalytic domain (residues 1–392) as a single rigid body converged to an *R*-factor value of 0.288 ($R_{free} = 0.363$), thus validating the inclusion of all residues in refinement despite the large temperature factor values associated with the C-terminal domain.

Refinement of the $TrSA_{5mut}$–DANA complex was carried out along the same lines described above, but in this case the inclusion of the lectin-like domain resulted in an increase of R_{free} (0.366 for the complete model against 0.353 for the catalytic domain alone) and the electron

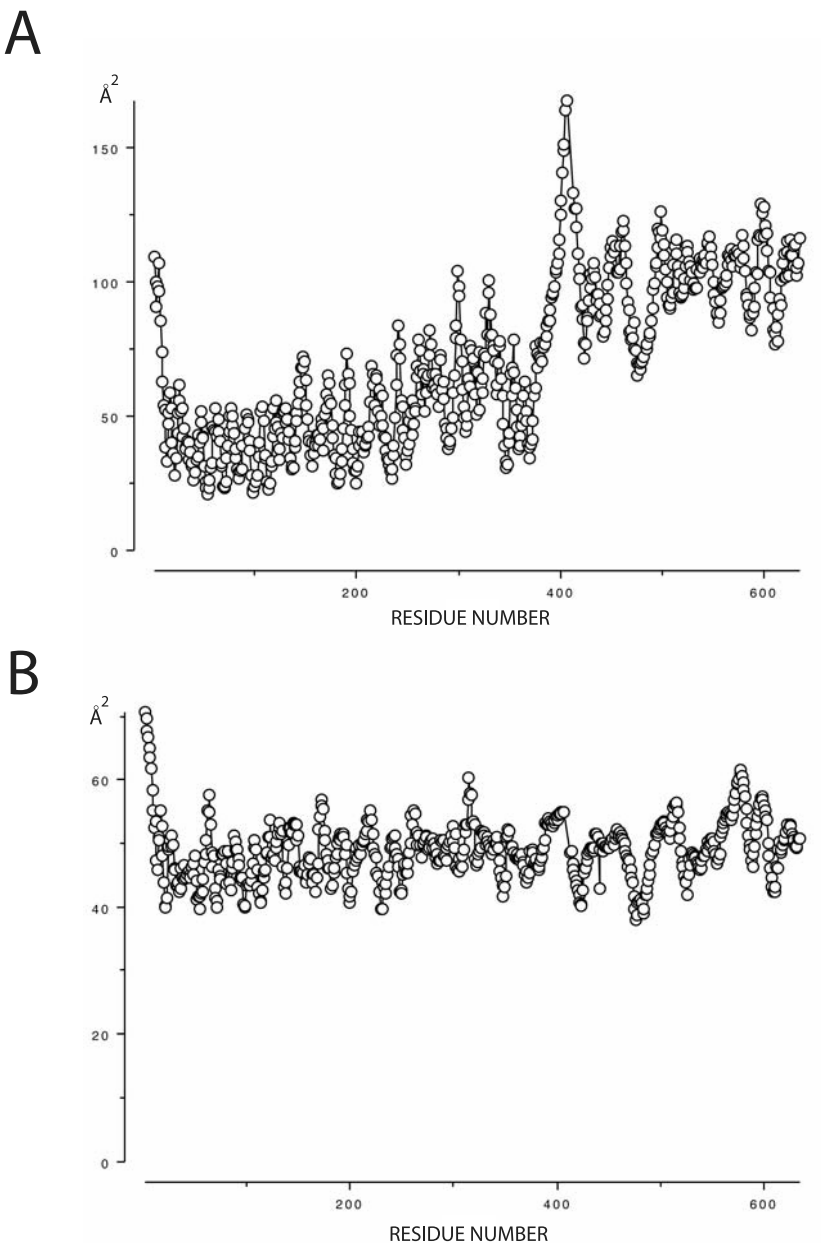

Figure 5. Average *B*-values for $TrSA_{5mut}$. A, Total average *B*-values; B, residual *B*-values, after excluding the TLS contribution arising from rigid-body mobility.

density map was poorly defined for the entire C-terminal domain. Therefore, only the catalytic domain (residues 1–389) was retained in the final model for the complex. The parameters of refinement after the final cycle are given in Table 2.

In both crystal structures, the electron density was well defined for the entire catalytic domain and the bound inhibitor was clearly visible in the electron density map of the complex (Figure 3A). The rms deviation between the free and inhibitor-bound catalytic domains is 0.44 Å for 389 matched C^{α} positions, indicating that binding of the inhibitor does not promote any significant structural change. The two models have a good stereochemistry: only one residue, Val180, in both structures displays main-chain dihedral angles that fall in disallowed regions of the Ramachandran plot, but this is a genuine feature of the structure, since the same values were also observed for the structure of wild-type TrSA at 1.6 Å resolution.[24] Given the limited resolution of this study, no attempt was made to model solvent molecules and all amino acid side-chains far from the active site (in particular those from the lectin-like domain) that displayed little or no density were nevertheless kept in the model, with conformations similar to those observed in the previously determined structure of TrSA at high resolution.

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