

## Recognition of RNase Sa by the Inhibitor Barstar: Structure of the Complex at 1.7 Å Resolution

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### Abstract

The 1.7 Å resolution structure of RNase Sa complexed with the polypeptide inhibitor barstar is reported here. The crystals are in the hexagonal space group  $P6_5$  with unit-cell dimensions  $a = b = 56.9$ ,  $c = 135.8$  Å and the asymmetric unit contains one molecule of the complex. RNase Sa is an extracellular microbial ribonuclease produced by *Streptomyces aureofaciens*. Barstar is the natural inhibitor of barnase, the ribonuclease of *Bacillus amyloliquefaciens*. It inhibits RNase Sa and barnase in a similar manner by steric blocking of the active site. The structure of RNase Sa is very similar to that observed in crystals of the native enzyme and its complexes with nucleotides. Barstar retains the structure found in its complex with barnase. The accessible surface area of protein buried in the complex is about 300 Å<sup>2</sup> smaller and there are fewer hydrogen bonds in the enzyme–inhibitor interface in RNase Sa–barstar than in barnase–barstar, providing an explanation of the reduced binding affinity in the former. Previous studies of barstar complexes have used mutants of the inhibitor and this is the first structure which includes wild-type barstar.

### 1. Introduction

A number of micro-organisms secrete a ribonuclease from the cell. The functional role of these enzymes has not been clearly established. Those studied so far all appear to have the same overall fold (Hill *et al.*, 1983; Ševčík *et al.*, 1990), but there is only low sequence identity between many members of the family, with substantial deletions and insertions in the surface loops. Some of the organisms produce an intracellular polypeptide inhibitor of their ribonuclease to prevent it being active prior to secretion, which would be extremely harmful to the cell. This is in some ways comparable to the role of the trypsin inhibitor in the pancreas.

One of the best characterized microbial ribonucleases is barnase, produced by the bacterium *Bacillus amyloliquefaciens* (Hartley *et al.*, 1972). Barnase has 110 amino acids and no cysteine residues. The sequence is known and the three-dimensional structure of the free enzyme (Mauguen *et al.*, 1982) and its complexes with d(GpC) (Baudet & Janin, 1991), 3'-GMP (Guillet *et al.*, 1993) and d(CGAC) (Buckle & Fersht, 1994) have been

determined. In addition the structure of the free enzyme (Bycroft *et al.*, 1991) and its complex with 3'-GMP (Meiering *et al.*, 1993) have been determined using NMR. The enzyme has been extensively studied to probe its physicochemical properties, activity, folding and stability (Mossakowska *et al.*, 1989; Serrano *et al.*, 1990, 1991, 1992; Meiering *et al.*, 1991, 1992; Clarke & Fersht, 1993; Chen *et al.*, 1993; Fersht, 1993; Buckle *et al.*, 1993).

Of special interest with regard to barnase is that the polypeptide inhibitor, barstar, secreted by the same organism has been well characterized and cloned (Hartley, 1988). Barstar has 89 amino-acid residues with two cysteines at positions 40 and 82, which are almost completely buried in the protein and do not form a disulfide bond. Growth of barstar crystals was reported but these were not of sufficient quality for three-dimensional analysis (Guillet *et al.*, 1993; Raghunathan *et al.*, 1994). However, the three-dimensional structure of wild-type barstar has been solved by NMR (Lubienski *et al.*, 1994). Stabilization of barstar by chemical modification of the cysteines was studied by Ramachandran & Udgaonkar (1996), unfolding by Khurana & Udgaonkar (1994) and dynamics of buried tryptophan by Swaminathan *et al.* (1996).

Barstar binds tightly to barnase, forming a one-to-one non-covalent complex (henceforth known as Baba) (Hartley & Smeaton, 1973; Hartley, 1989). The dissociation coefficient for the complex of wild-type barnase with wild-type barstar is  $6 \times 10^{-14}$  M and that for wild-type barnase with the C40A,C82A barstar double mutant is  $2 \times 10^{-13}$  M (Hartley, 1993). Early attempts to determine the three-dimensional structure of Baba with wild-type inhibitor were unsuccessful as the crystals grew as aggregates (Guillet *et al.*, 1993) perhaps due to the presence of a mixture of both oxidized and reduced forms of barstar (Buckle *et al.*, 1994). Finally diffraction-quality crystals of Baba with both barstar cysteine residues, Cys40 and Cys82, mutated to alanines were used and the structure independently determined at 2.6 Å (Guillet *et al.*, 1993) and 2.0 Å resolution (Buckle *et al.*, 1994). Subsequent studies were oriented towards understanding the role of individual residues in the barnase–barstar interface by protein engineering (Jones *et al.*, 1993; Schreiber & Fersht, 1993, 1995; Schreiber *et al.*, 1997).

*Streptomyces aureofaciens* bacteria secrete extracellular ribonucleases which are members of the extended microbial family (Hartley, 1980; Hill *et al.*, 1983). RNases have been isolated and characterized from three strains of *S. aureofaciens* to date and referred to as RNase Sa, Sa2 and Sa3. The sequences vary considerably between these different strains. RNase Sa, the best characterized of this subfamily, is a single-chain protein with 96 amino acids and one disulfide bridge (Shlyapnikov *et al.*, 1986). The primary structures of RNase Sa2 (Nazarov, unpublished) and Sa3 (Homerova *et al.*, 1992) have been deduced from the nucleotide sequences of their genes. Sa2 and Sa3 have sequence identity with Sa of 56 and 69%, respectively. The limited number of sequence differences make these enzymes a good system for a site-directed mutagenesis study aimed at understanding the differences in their properties. These enzymes are also being used as model systems for the study of protein conformational stability (Pace *et al.*, 1998).

We have carried out extensive structural studies of these enzymes with the aim of understanding the mechanism of action and structure–function relationship. Several crystal structures of RNase Sa and its complexes are known: native and 3'-GMP complex at 1.8 Å resolution (Ševčík *et al.*, 1991), native and 2'-GMP complex at 1.7 Å (Ševčík, Hill *et al.*, 1993) and the complex with *exo*-guanosine-2',3'-cyclophosphorothioate (2',3'-GCPT), a thio-analogue of the intermediate of the two-step catalytic reaction at 2.0 Å (Ševčík, Zegers *et al.*, 1993). The structure of native RNase Sa and its 2'-GMP complex have been subsequently refined at atomic resolution (Ševčík *et al.*, 1996). The structures of RNase Sa2 and Sa3 have also been determined (Ševčík, Pace, Dauter & Wilson, to be published) and preparation of site-directed mutants is in hand (N. Pace, private communication).

*S. aureofaciens* also produces a specific ribonuclease inhibitor. Unfortunately, isolation of the inhibitor initially proved to be extremely difficult (Krajcikova *et al.*, 1990) and cloning and expression of the inhibitor gene from the Sa2 strain is only now in hand (Krajcikova *et al.*, 1998). Thus, to date structural studies have not been

possible for the complex of RNase Sa with its natural inhibitor, though these are planned as soon as sufficient material is available. The molecular weight of the inhibitor is 14 kDa suggesting substantial differences in the three-dimensional structure compared to barstar. However, all barstar residues which are involved in interactions with barnase and RNase Sa are fully conserved in the Sa inhibitor (Krajcikova *et al.*, 1998).

In the interim we have analysed the complex of RNase Sa with barstar. RNase Sa has relatively low sequence identity with the other members of the family, and the sequence identity between RNase Sa and barnase is only 23%, Fig. 1. However, the active sites of the two enzymes are similar, and barstar, a specific protein inhibitor of barnase, also binds to and inhibits RNase Sa. The components of the RNase Sa–barstar complex (Saba) are not as tightly bound as Baba: its dissociation constant, about  $10^{-10}$  M (Hartley *et al.*, 1996), is four orders higher. Here we describe the three-dimensional structure of Saba at 1.7 Å resolution and relate it to the known structure and properties of Baba. This is the first step in studying polypeptide inhibition of the *S. aureofaciens* subfamily of extracellular RNases. The Baba complex has been accepted as an excellent model for protein stability and folding studies (Buckle *et al.*, 1994) and for study and design of protein–protein non-covalent interactions (Guillet *et al.*, 1993). Additional observations from the Saba complex will complement those studies.

## 2. Experimental

### 2.1. Protein purification

Recombinant RNase Sa was isolated and purified according to the procedure described previously (Hebert *et al.*, 1997). The main steps were extraction of RNase Sa from the periplasmic space by osmotic shock, ion-exchange chromatography using an SP Sephadex C-25 column and gel-filtration chromatography on a Sephadex G-50 column.

The gene for barstar was expressed in *E. coli* strain HB 101 containing plasmid pMT316. Extraction of the

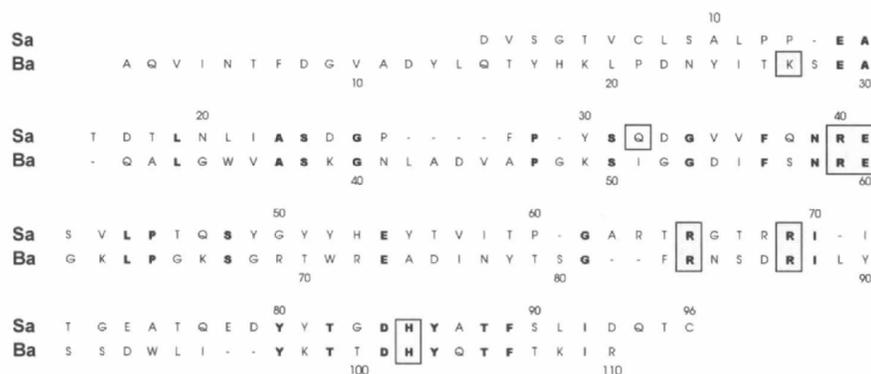


Fig. 1. Alignment of the sequences of RNase Sa and barnase using CLUSTAL V (Higgins *et al.*, 1992). Identical residues are in bold. The key residues (shown below in Fig. 5) which make hydrogen bonds in the interface or are structurally equivalent in the two structures are shaded.

Table 1. *Data-collection parameters*

Beamline	X31, EMBL Hamburg
Wavelength (Å)	0.95
Resolution range (Å)	25.0–1.7
Fully recorded	118438
Partially recorded	22844
Unique reflections	27354
Completeness (%)	100.0
$R(I)_{\text{merge}}$ (%)	4.6
$I/\sigma(I)$	27
Outer resolution range (1.72–1.70 Å)	
$R(I)_{\text{merge}}$ (%)	30.8
$I/\sigma(I)$	3.7

cells by acetone, gel filtration on Sephadex G-75 and ion-exchange chromatography on DEAE-Trisacryl were the main steps involved in the purification of the inhibitor (Hartley & Krajcikova, private communication).

## 2.2. Crystallization, data collection and processing

In structural studies of Baba, a double mutant of barstar was used. For preparation of crystals of the Saba complex, wild-type barstar was used for the first time. The complex was formed by mixing a solution of RNase Sa with a solution of barstar in 1:1 ratio, giving a concentration of each protein in mother liquor drops of 10 mg ml<sup>-1</sup>. In contrast to the preparation of Baba crystals (Buckle *et al.*, 1994) no further purification of complex from residual enzyme or inhibitor was required before crystallization. Crystals were grown by vapour diffusion (McPherson, 1990). Details of the crystallization and data collection have been described previously (Urbanikova & Ševčík, 1998). In brief, the drops contained 20 mg ml<sup>-1</sup> of the complex in 0.1 M cacodylate buffer, pH 7.2 and 15% ammonium sulfate and the wells 30% ammonium sulfate. X-ray data to 1.7 Å resolution using synchrotron radiation were collected at EMBL, Hamburg with a MAR Research imaging-plate scanner. The images were integrated using *DENZO* (Otwinowski & Minor, 1997). The crystals are in space group *P6<sub>5</sub>* with unit-cell parameters  $a = b = 56.95$ ,  $c = 135.80$  Å. This confirmed that there is one Saba complex in the asymmetric unit, with a  $V_M$  of 3.2 Å<sup>3</sup> Da<sup>-1</sup>.

## 2.3. Structure determination and refinement

The structure was solved by molecular replacement using the program *AMoRe* (Navaza & Saludjian, 1997) with RNase Sa (Ševčík *et al.*, 1996; PDB coordinate set 1RGG from the Brookhaven Protein Data Bank, Bernstein *et al.*, 1977) and barstar (Guillet *et al.*, 1993; 1BGS) as search models. Both the rotation and translation function searches resulted in single clear solutions. Rigid-body refinement of the resulting model in the 10–3.5 Å resolution range gave a correlation coefficient of 54% and an  $R$  factor of 42%.

Table 2. *Summary of model properties*

	$\sigma$	Deviation	Number of parameters
Distances (Å)			
Bond lengths (1–2 neighbours)	0.020	0.025	1520
Bond angles (1–3 neighbours)	0.040	0.040	2070
Dihedral angles (1–4 neighbours)	0.050	0.089	528
Planes (Å)			
Peptides	0.040	0.035	185
Aromatic	0.020	0.012	22
Chiral volumes (Å <sup>3</sup> )	0.150	0.257	231
van der Waals contacts (Å)			
Single	0.30	0.19	581
Multiple	0.30	0.26	808
Possible hydrogen bonds	0.30	0.14	50
Torsion angles (°)			
Planar	7.01	12.8	192
Staggered	15.0	17.0	260
Orthogonal	20.0	22.3	22
$B$ factors (Å <sup>2</sup> )			
Main-chain bonds	3.0	2.9	874
Main-chain angles	5.0	4.2	1004
Side-chain bonds	6.0	5.5	646
Side-chain angles	8.0	7.4	1066

For refinement the maximum-likelihood program *REFMAC* (Murshudov *et al.*, 1997) combined with an automated refinement procedure *ARP* (Lamzin & Wilson, 1997) was used. The initial  $R$  factor was 38%. The refinement was carried out against data in the resolution range 25.0–1.7 Å, with 2739 (10%) of the reflections used for  $R_{\text{free}}$  cross-validation (Brünger, 1993). The  $R_{\text{free}}$  reflections were also used to calculate the maximum-likelihood weighting in refinement and map calculation. Introduction of overall anisotropic scaling reduced the  $R$  factor by 0.5%. Refinement converged with  $R = 16.00$  and  $R_{\text{free}} = 18.98\%$ . All data were used in the last refinement cycles which converged to an  $R$  factor of 16.23%. For visualizing and rebuilding of the model, the program *O* was applied (Jones *et al.*, 1991).

The electron density after a few cycles of refinement was already good enough to build all residues for both enzyme and inhibitor. The two residues of barstar, Glu64 and Asn65, which were missing or poorly defined with very high  $B$  factors in the two structures of Baba (Guillet *et al.*, 1993; 1BGS; Buckle *et al.*, 1994; 1BRS) are also disordered in Saba. However, the electron density for the main chain is clear in this region in Saba.

## 3. Results and discussion

### 3.1. Accuracy of the model

The X-ray data collection and refinement statistics are given in Tables 1 and 2. The final  $R$  factor for all data is 16.23%. The r.m.s. coordinate error of the model, as estimated from the  $\sigma_A$  plot (Read, 1986) is 0.061 Å and the coordinate errors based on  $R$  and  $R_{\text{free}}$  given by

REFMAC (Cruickshank, 1996) are 0.078 and 0.088 Å, respectively. The Ramachandran plots (Ramakrishnan & Ramachandran, 1965) for RNase Sa and barstar calculated using PROCHECK (Morris *et al.*, 1992) show that 89% of the residues of barstar and 90% of RNase Sa are in the most favoured region, eight of barstar and eight of RNase Sa residues are in the additionally allowed region. A single barstar residue, Trp44, has dihedral angles in the generously allowed region ( $\varphi = -157^\circ$ ,  $\psi = -42^\circ$ ), almost the same as in Baba. Residues of RNase Sa in Saba which are in the additionally allowed region, have the same dihedral angles in both independent molecules in the structure of RNase Sa at atomic resolution (Sevcik *et al.*, 1996).

### 3.2. Temperature factors and accessible surface area

The average main-chain and side-chain temperature factors are 16.7 and 20.4 Å<sup>2</sup> for RNase Sa, 23.1 and 30.8 Å<sup>2</sup> for barstar and are shown as a function of residue number in Figs. 2(a) and 2(b), respectively. The temperature factors for water molecules range from 13 to 70 Å<sup>2</sup> with a mean of 40.9 Å<sup>2</sup>. The estimate of the average *B* factor from the Wilson plot (Wilson, 1942) is 20.0 Å<sup>2</sup>. The temperature factors for RNase Sa in the complex are slightly higher in comparison to the structures of native RNase Sa or complexes with mononucleotide inhibitors. In RNase Sa, almost all the chain is well ordered with low *B* values apart from a few side chains on the surface. For barstar, there is one mobile

region around residue 63. The accessible surface area as a function of residue number is shown in Fig. 2(c) for RNase Sa and Fig. 2(d) for barstar, for the individual molecules isolated from the crystal lattice and in Saba showing which residues are buried in the formation of the complex. 673 Å<sup>2</sup> of RNase Sa and 642 Å<sup>2</sup> of barstar contribute to the contacts. The residues from barstar which contribute to the contacts are concentrated in the region between 29 and 45, with a small contribution around 76. In RNase the residues are more widely spread, reflecting the contribution of dispersed parts of the chain to the catalytic and binding sites.

### 3.3. Description of the structure

There is one molecule of the complex in the asymmetric unit which contains 751 RNase Sa and 737 barstar atoms surrounded by 186 water molecules. For both enzyme and inhibitor all residues were modelled. Alternative conformations were built for side chains of Val6 and Gln32 in RNase Sa and Lys1, Glu52 and Glu76 in barstar, all with 0.5 occupancy. For Lys1 of barstar two conformations were also modelled for the main-chain atoms. Gln32 of RNase Sa and Glu76 of barstar are involved in hydrogen bonds in the complex.

This is the first crystal structure which includes wild-type barstar. The CA atoms of Cys40 and Cys82 are 11.5 Å apart, very close to the separation of the CA atoms of Ala40 and Ala82, 11.7 Å, in the double mutant barstar. Cys82 is on the surface of barstar and has no

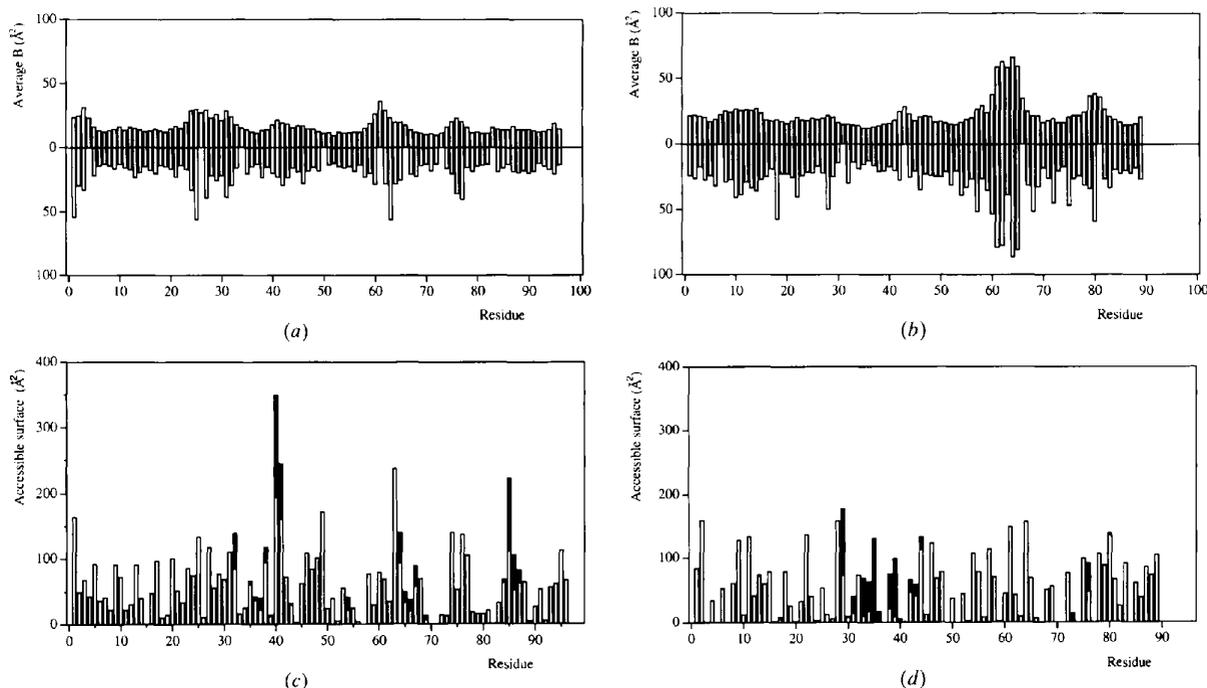


Fig. 2. Average temperature factors for main (top) and side chains (bottom) of (a) RNase Sa and (b) barstar as a function of residue number. The accessible surface areas (calculated with DSSP, Kabsch & Sander, 1983) of (c) RNase Sa and (d) barstar in the individual molecules isolated from the crystal lattice (total bars) and the area buried during complex formation (black).

contacts with RNase Sa. For Cys40 the closest atoms to its SH group are almost all from barstar: Pro27 CD (3.74 Å), Ala36 O (3.43 Å). However, Cys40 SG atom in addition forms van der Waals contact, 3.96 Å, with His85 CE1 of RNase Sa. A similar contact with barnase was postulated for the putative barnase-wild-type barstar complex (Buckle *et al.*, 1994) in partial explanation of the affinity for barnase of the double cysteine to alanine mutant barstar being reduced by about one order of magnitude (Hartley, 1993).

Overlap of barstar from Saba and Baba (1BRS) based on CA atoms gives an r.m.s. displacement 0.52 Å and maximum displacement 1.46 Å. The CA displacements are larger than 1.0 Å for Arg11 (1.05 Å), Asp15 (1.03), Glu57 (1.18), Gln58 (1.20), Gln61 (1.19), Leu62 (1.46) and Thr63 (1.24), none of which are in the interface. The structures are very similar to one another, supporting the idea that the double Cys-Ala mutations in barstar did not introduce substantial structural changes.

The mechanism of inhibition of RNase Sa by barstar is very similar to that found in Baba. One of the barstar helices,  $\alpha 2$ , is positioned in the active-site groove and together with the adjacent loop which connects  $\alpha 2$  to  $\alpha 1$ , completely blocks the active site, Fig. 3. All amino-acid residues of RNase Sa, which are known from the complexes of RNase Sa with 2'-GMP, 3'-GMP and 2',3'-GCPT to be important in binding and cleaving substrate (Glu41, Glu54, Arg65, Arg69, His85) also form hydrogen bonds with barstar except Glu54. The base of the mononucleotide inhibitors in complexes of RNase Sa forms three hydrogen bonds with the main-chain N atoms of Gln38, Asn39 and Arg40 but only one of these, Arg40 N, is involved in binding to barstar through its Asp35 OD1. In addition to the above residues, Gln32,

Table 3. *Hydrogen bonds (Å) between RNase Sa or barnase and barstar*

Barstar	Barnase	1BRS	1BGS	RNase Sa	1AY7
Tyr29 OH	Arg83 O	2.65	2.95	Thr64 OG1	†
Tyr29 OH	Asn84 O	3.13	†	Gly66 N	†
Gly31 N	His102 ND1	3.03	3.03	His85 ND1	2.91
Asn33 ND2	His102 O	3.22	†	His85 O	3.03
Leu34 N	Glu60 OE2	2.84	2.99	Glu41 OE1	2.70
Asp35 OD1	Arg59 N	2.88	2.97	Arg40 N	2.83
Asp39 OD1	Arg83 NH1	2.89	2.97	Arg65 NH2	2.62
Asp39 OD1	Arg83 NH2	2.50	3.00	Gln32 NE2‡	3.00
Asp39 OD2	Arg87 NH2	2.93	3.04	Arg69 NH1	2.86
Asp39 OD2	His102 NE2	2.81	2.88	His85 NE2	2.70
Asp39 O				Gln32 NE2‡	3.15
Thr42 OG1	Lys27 NZ	2.95	2.83	Gln32 NE2‡	2.81
Gly43 O	Arg83 NH1	3.07	3.18		
Glu76 OE1	Arg59 NH1	2.97			
Glu76 OE1	Arg59 NH2	2.98	2.77		
Glu76 OE1§				Arg40 NH1	2.59
Glu76 OE1‡				Gln38 NE2	2.62
Glu76 OE1‡				Arg40 NH2	2.88
Glu76 OE2‡				Arg40 NH1	2.94

† Contact longer than 3.25 Å ‡ In conformation B. § In conformation A.

Gln38 and Arg40 of RNase Sa form hydrogen bonds with barstar. There are 14 hydrogen bonds between RNase Sa and barstar, seven of which are only 'half bonds' as they are formed by residues with two conformations (Gln32 of RNase Sa and Glu76 of barstar), Table 3. This results in an effective total of about 10 hydrogen bonds in the Saba interface. This used a maximum cut-off 3.25 Å: there are a number of potential hydrogen bonds somewhat longer than this. The network of hydrogen bonds is illustrated in Fig. 4(a). Several water molecules are buried in the enzyme-

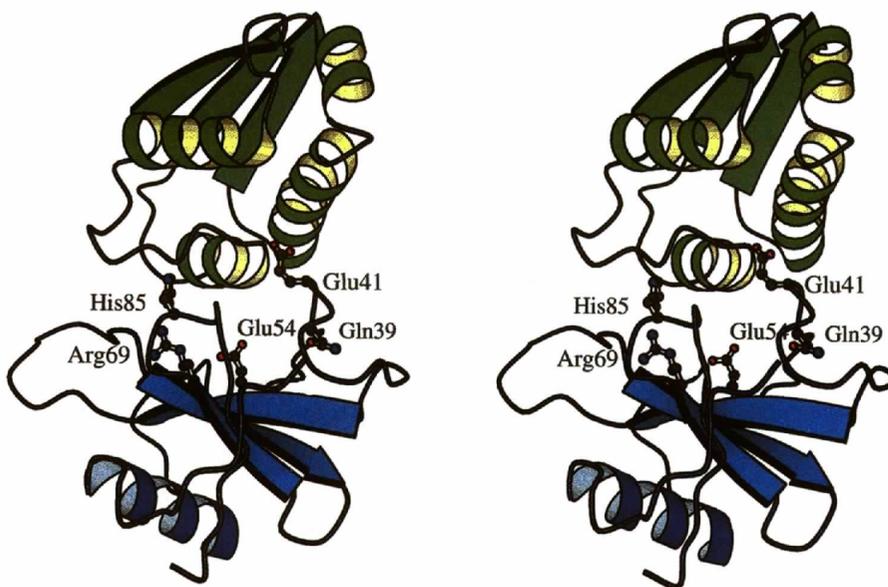


Fig. 3. Stereoview of the Saba complex, with RNase Sa (blue) and barstar (green), prepared using *MOLSCRIPT* (Kraulis, 1991). Active site residues of RNase Sa are shown in ball and stick. The barstar helix  $\alpha 2$  and adjacent loop are clearly positioned in the active site of the enzyme.

inhibitor interface. Only those with a solvent-accessible surface area less than  $10 \text{ \AA}^2$  are considered here, using the accessibility calculated by the program *SURFACE* (Collaborative Computational Project, Number 4, 1994). There are six water molecules involved in RNase Sa–water–barstar contacts and five in RNase Sa–water–water–barstar contacts Table 4. The network of hydrogen bonds mediated through these water molecules, illustrated in Fig. 4(b), is similar to that in Baba, however, the number of water molecules in Saba is lower.

### 3.4. Comparison of the Saba complex with the Baba complex

There are 14 residues from the enzyme and 14 from the inhibitor with van der Waals contacts shorter than  $4.0 \text{ \AA}$  in both Baba (1BRS) and Saba. The number of contacts in Baba is approximately 150, in Saba it is lower, about 120. The direct and water-mediated hydrogen bonds found in Baba are listed in Tables 2 and

4 of Buckle *et al.* (1994) using a  $3.25 \text{ \AA}$  cut-off. The direct hydrogen bonds extracted from the Baba coordinates 1BGS and 1BRS are also listed in Table 3 for comparison with Saba. In general, barstar forms hydrogen bonds with the same conserved residues of Saba and Baba. The residues of RNase Sa and barnase involved in hydrogen bonds in the complexes are mainly those which are conserved in the amino-acid sequences of the two enzymes (Arg40–Arg59, Glu41–Glu60, Arg65–Arg83, Arg69–Arg87, His85–His102) or are in structurally equivalent positions (Gln32 and Lys27).

Fig. 5 shows the key enzyme residues involved in hydrogen-bond and salt-bridge interface contacts, based on pairwise superpositions of 35 CA atoms which form the conserved structural core (Sevcik *et al.*, 1990). The residues are identical in the two enzymes, with the exception of Lys27 in barnase whose structural counterpart in RNase Sa is Gln32. This is the only substantial difference in the interface. The structures used are RNase Sa from the present Saba, RNase Sa with free active site (1RGH; Sevcik *et al.*, 1996), native barnase

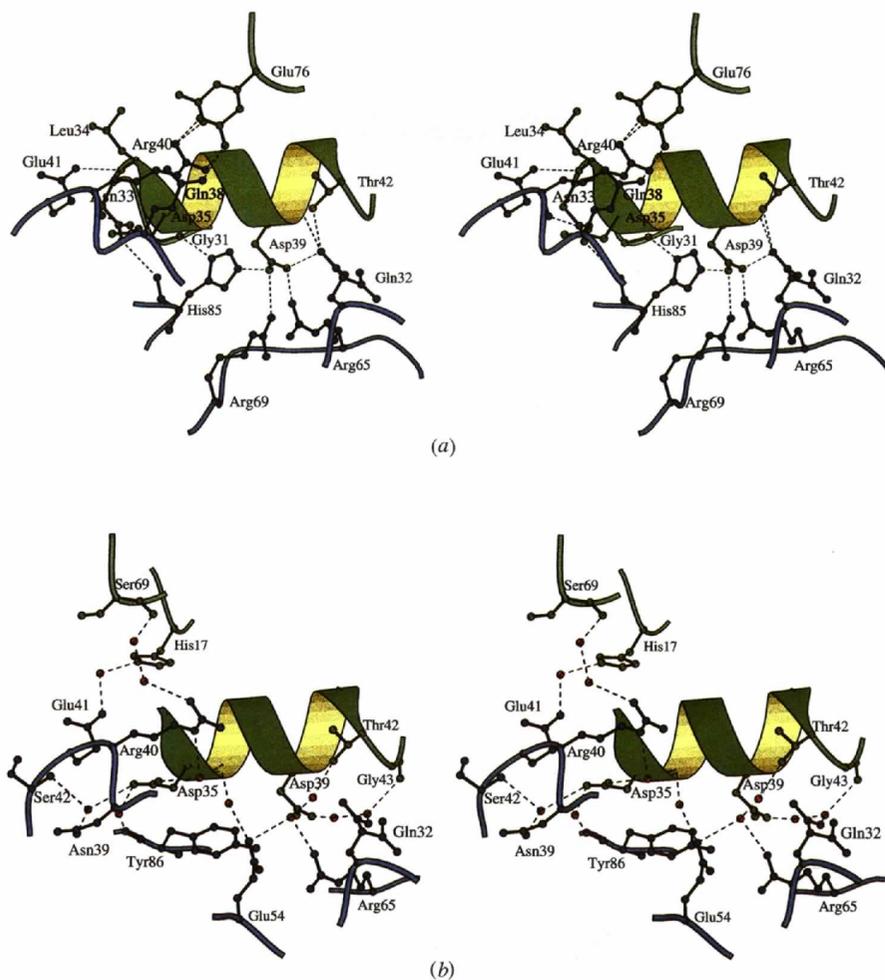


Fig. 4. Stereoview of the network of (a) direct and (b) water-mediated hydrogen bonds in the contact region between RNase Sa and barstar in the Saba complex.

Table 4. Hydrogen bonds (Å) between RNase Sa and barstar mediated through water molecules

RNase Sa	Å	Water	Å	Barstar
Gln32 OE1†	3.21	W176	2.87	Gly43 O
Arg40 NE	3.04	W6	2.62	Asp35 OD1
Glu41 OE1	2.68	W21	2.92	His17 NE2
Asn39 OD1	2.76	W18		
Ser42 N	3.23	W18	3.13	Asp35 OD2
Glu54 OE1	2.93	W4	3.03	Asp35 O
Tyr86 O	2.87	W47	2.45	Asp35 OD2

RNase Sa	Å	Water	Å	Water	Å	Barstar
Gln32 OE1†	3.21	W176	2.71	W128	2.77	Asp39 OD2
Arg40 NH1	3.04	W70	2.55	W71	2.79	Ser69 O
Glu54 OE1	3.06	W20				
Arg65 NH2	3.11	W20	2.72	W101	3.05	Thr42 OG1

† Conformation B.

(Mauguen *et al.*, 1982) and the Baba complex (1BRS; Buckle *et al.*, 1994). Fig. 5(a) shows the two native enzymes with free active sites, for which the 35 CA atoms overlap with an r.m.s. difference of 0.38 Å and maximum 0.88 Å. Arg65 and Arg69 and their counterparts in barnase are in very similar conformations in the two native enzymes. Arg59 and Glu60 are not well defined in native barnase with zero or reduced occupancies assigned at the end of the side chain, and do not qualify for comparison. His85 (His102) has a similar but not identical orientation.

Fig. 5(b) shows the same residues in Saba and Baba, with an r.m.s. 0.40 Å and maximum displacement of 0.99 Å for the same 35 CA atoms. Here the conformations and positions of the key amino-acid residues forming hydrogen-bond and salt-bridge interface contacts are essentially identical in the enzymes, except for barnase Lys27. Indeed in Saba, Gln32 does make a comparable contact, but only in one of its two alternative conformations.

The change of conformation of the residues on complex formation is illustrated in Fig. 5(c) for RNase Sa in its free native form and the Saba complex. The 35 CA atoms overlap with r.m.s. of 0.30 Å and maximum 0.73 Å. His85, Glu41 and Arg40 undergo significant induced fit to accommodate barstar in the active site, and furthermore Figs. 5(a) and 5(b) show that the changes are very similar in both enzymes. This reflects substantial flexibility in both RNase Sa and barnase to achieve tight binding of the inhibitor.

Taking into account that there are only 29 identical residues in the sequences of RNase Sa and barnase leading to substantial differences in tertiary structures, and considering the large effect on binding of barnase to barstar by only one mutation in the interface (Hartley, 1993; Schreiber & Fersht, 1993, 1995), the inhibition of RNase Sa by barstar was unexpected. Indeed, barstar fully inhibits RNase Sa, Sa2 and Sa3 but none of RNase St (*Streptomyces erythreus*), RNase T1 (*Aspergillus*

*oryzae*) or pancreatic ribonuclease A (Hartley *et al.*, 1996). This inhibition led to attempts to express the gene of RNase Sa simultaneously with that of barstar in *E. coli* in a similar way to the co-expression of the barnase and barstar genes (Hartley, 1988) and this indeed reduced the very high toxicity of the enzyme for the host cells allowing relatively high production of the Sa enzyme (Hartley *et al.*, 1996). Bearing in mind that all previous experiments with expression of Sa genes alone failed, it suggests that the presence of the inhibitor is vital to protect the host cells from the lethal effect of RNase Sa if it becomes active within the cell.

RNase St has sequence identity with Sa of 49% and the backbones of the two structures are nearly identical. However, in RNase St Glu39 is the structural counterpart of Gln32 in RNase Sa (Lys27 of barnase). Lys27 and Gln32 make hydrogen bonds to the inhibitor *via* their side-chain N atoms, Table 3. Glu39 in RNase St is presumed to be unable to form an equivalent interaction (Hartley *et al.*, 1996). Mutation experiments at this position in RNase Sa will show whether the presence of Glu instead of Gln at position 32 is responsible for barstar failing to inhibit RNase St.

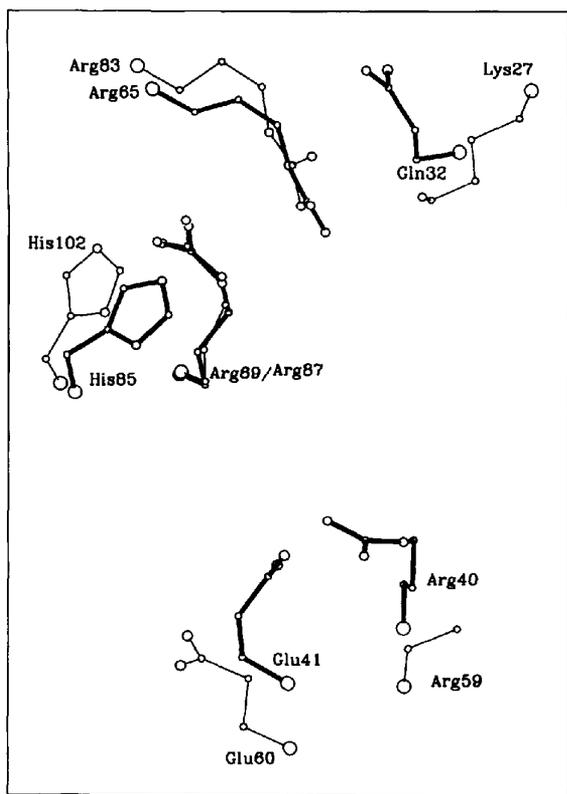
Overlap of RNase T1 on barnase in comparison to that on RNase Sa on barnase reveals a much greater overall difference in the structures and thus it is not surprising that barstar does not inhibit T1. Structural analysis of the RNase A inhibitor shows that its fold and topology are completely different from that of barstar providing a quite different mechanism of inhibition (Kobe & Deisenhofer, 1996).

#### 4. Conclusions

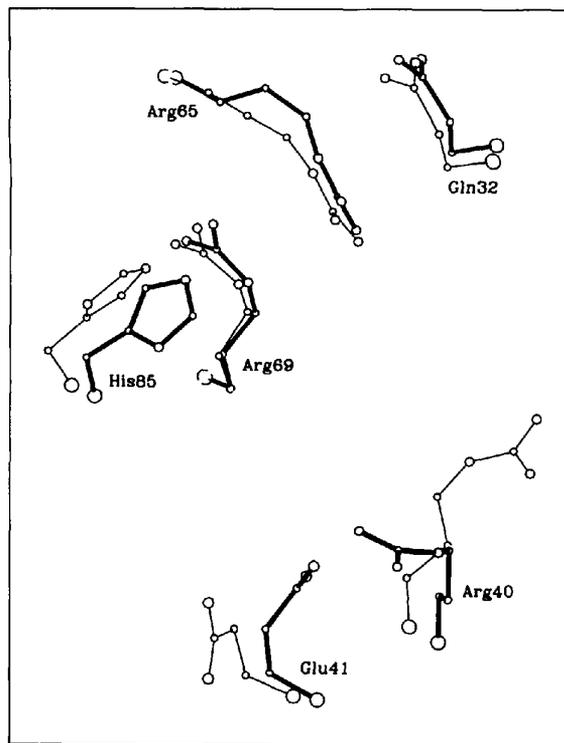
Refinement of the structure of the RNase Sa-barstar complex at 1.7 Å resolution has confirmed its essential similarity to that of the well characterized barnase-barstar. The key residues in the recognition of the inhibitor through hydrogen bonds and salt bridges are almost totally conserved in sequence and conformation, with the exception of Gln32 in RNase Sa (Lys27 in barnase). The latter nevertheless makes an analogous hydrogen-bond contact to barstar through a side-chain N atom. Barnase and its natural inhibitor, barstar, are produced by the same organism. In the RNase Sa-barstar complex the two interacting proteins are produced by different organisms, shedding additional light on the protein-protein interactions in such systems.

Comparison of the interfaces of the Baba and Saba complexes shows the following.

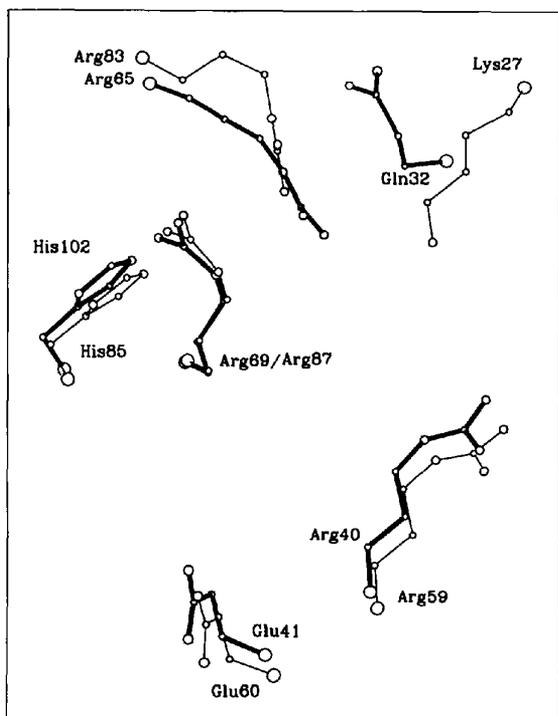
(i) There are about 14 direct enzyme-inhibitor hydrogen bonds and salt bridges in Baba and about 10 in Saba. These bonds are not simple to classify as the error in bond-length estimation is about 0.2 Å at these resolutions and a cut-off of 3.25 Å can somewhat arbitrarily exclude/include some hydrogen bonds. In addition the



(a)



(b)



(c)

Fig. 5. Various pairwise superpositions of the residues in the RNase Sa and barnase active sites, based on the 35 CA atoms belonging to the structurally conserved core (see text). (a) RNase Sa native (thick) and barnase native (thin) with free active sites. Arg59 is truncated as the end of the chain is not defined in the crystal structure. (b) RNase Sa from Saba (thick) and barnase (thin) from the Baba. (c) RNase Sa native (thick) and from Saba (thin).

energies involved are highly dependent on hydrogen-bond angle as well as length.

(ii) There are enzyme-water-inhibitor and enzyme-water-water-inhibitor hydrogen bonds in the interface mediated through 16 water molecules in Baba and through only 11 in Saba. This again is subject to the exact cut-offs of the lengths of contacts and accessible surface areas, and the errors in coordinates. The reduced number of bonds mediated through water molecules probably reflects the smaller area of the interface in Saba.

(iii) In Baba and Saba there are 14 enzyme residues in van der Waals contact with 14 barstar residues. However, the number of contacts in Saba is about 20% lower in comparison to Baba. This reflects the reduction in the surface area buried in the interface from about 1600 Å<sup>2</sup> in Baba to 1300 Å<sup>2</sup> in Saba.

These differences rationalize in general terms that the dissociation constant of Saba is higher than that of Baba by four orders of magnitude, *i.e.* the non-natural Saba complex is less stable, largely because of the smaller accessible surface area buried in the Saba interface rather than a substantial change in the number of hydrogen bonds and salt bridges. In the present analysis we have not attempted to quantify energetically the differences in the interfaces between the two complexes. Much work has been performed on the contribution of the buried surface area, hydrogen bonding and entropic effects on the stability of protein-protein quaternary interactions. Seminal contributions in this area were made by Chothia (1974) and Chothia & Janin (1975) and the field has been followed up by the work of many others *inter alia* Janin (1995, 1996). Several of these studies include the barnase-barstar system, which has been extensively investigated by Fersht and colleagues (*e.g.* Buckle *et al.*, 1994). A theoretical review has recently appeared (Brady & Sharp, 1997). The knowledge of the three-dimensional structure of the Saba complex is an essential step in extending such an analysis to the *S. aureofaciens* RNases and their inhibitors, where the possibilities are enhanced by the existence of enzymes with different sequences and properties in different strains.

The coordinates and structure factors have been deposited in the Brookhaven Protein Data Bank.†

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† Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1AY7).

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