

Complex of ribonuclease Sa with a cyclic nucleotide and a proposed model for the reaction intermediate

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The structure of the complex of ribonuclease from *Streptomyces aureofaciens* (RNase Sa) with *exo* guanosine 2',3'-cyclophosphorothioate has been refined against 0.2-nm resolution synchrotron data using, as a starting model, coordinates from the RNase Sa: 2'-GMP complex. The refinement was based on all data over 1.0–0.2 nm and converged to a crystallographic *R* factor of 11.9%. This is the first structure of a microbial ribonuclease complexed with a 2',3'-cyclophosphorothioate, which is a thio analogue of the intermediate of the two-step reaction. However, *exo* guanosine 2',3'-cyclophosphorothioate is bound in a non-functional mode and is not hydrolysed. This structure therefore does not provide direct evidence on the identity of the amino acid residues responsible for catalytic cleavage of the substrate. However, based on present and previous results, a plausible model is proposed for the complex of the cyclic intermediate which acts as substrate for the second step of the catalysis.

RNase Sa is one of the increasingly numerous members of the microbial ribonuclease family originally described by Hartley (1980) and Hill et al., (1983). The enzyme consists of 96 amino acid residues and its relative molecular mass, determined from the primary sequence is 10054 (Shlyapnikov, et al., 1986). RNase Sa is a highly specific endoribonuclease which hydrolyses the phosphodiester bond of RNA at the 3'-side of guanosine nucleotides (Zelinkova et al., 1971). The structures of native RNase Sa and its complexes with 3'-GMP and 2'-GMP have been reported previously (Sevcik et al., 1991; 1993). The structures of the two complexes clearly showed the guanine-specific recognition site, formed by main-chain atoms of residues 38–40 and the carboxylic group of Glu41.

The sequences for a number of microbial RNases have been determined. These are either bacterial or fungal. Three-dimensional structures are known for a number of bacterial enzymes, including RNase Ba from *Bacillus amyloliquefaciens* (Mauguen et al., 1982), Bi from *Bacillus intermedius* (Pavlovsky et al., 1983), St from *Streptomyces erythreus* (Nakamura, et al., 1982) and the present RNase Sa. In addition, several fungal RNase structures are known: RNase T₁ from *Aspergillus oryzae* (Heinemann and Saenger, 1982), C₂ from *Aspergillus clavatus* (Polyakov et al., 1987) and Pb₁ from *Penicillium brevicompactum* (Borisova et al., 1986). The primary structures have been aligned taking the three-dimensional structures into account (Sevcik et al., 1990). The similarity is higher within either the bacterial or fungal groups than between members of the two groups. Only five residues

are totally conserved over all the sequences. In RNase Sa these are Asn39, Glu41, Glu54, Arg69 and His85. The OE atoms of Glu41 H-bonded to the guanosine base. Asn39 appears important in maintaining the conformation around the binding site for the nucleotide. The side chains of Glu54, Arg69 and His85 are proposed as catalytic residues (Hill et al., 1983).

The bacterial RNase Sa has similar enzymic properties to the representative fungal RNase T₁ (Both et al., 1991, Yakovlev et al., 1992) in spite of very low similarity in both sequence and structure, except for the conserved structural motif composed of a three-stranded antiparallel β -pleated sheet and the nucleotide-binding site (Sevcik et al., 1990). There is a difference at the catalytic site of the two enzymes; RNase Sa has no residue equivalent to RNase T₁ His40, which appears to be essential for the catalytic activity in RNase T₁ (Steyaert et al., 1990).

The cleavage of substrate by microbial ribonucleases is a two-step reaction; the first step is a transesterification and results in the formation of the intermediate, guanosine 2',3'-cyclophosphate, which is hydrolysed in the second step to yield 3'-guanylic acid (Takahashi, 1970). Guanosine-2',3'-cyclophosphorothioate (2',3'-GCPT) is an analogue of the reaction intermediate, with a sulphur substituting one of the two free phosphate oxygen atoms. There are two isomers of 2',3'-GCPT, *endo* and *exo* (Fig. 1). The isomers have identical structures except for the phosphate oxygen and sulphur atoms which are interchanged. The *endo* isomer is hydrolyzed by RNase Sa while the *exo* is not (I. Rybajlak, unpublished results). The same has been observed for RNase T₁ (L. Wyns and I. Zegers, unpublished results). Direct structural identification of residues involved in cleaving substrate and their role poses a severe problem: if the contacts between the enzyme and a cyclophosphate were functional, the ligand

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Abbreviation. 2',3'-GCPT, guanosine-2',3'-cyclophosphorothioate.

Enzyme. RNase Sa (EC 3.1.4.8).

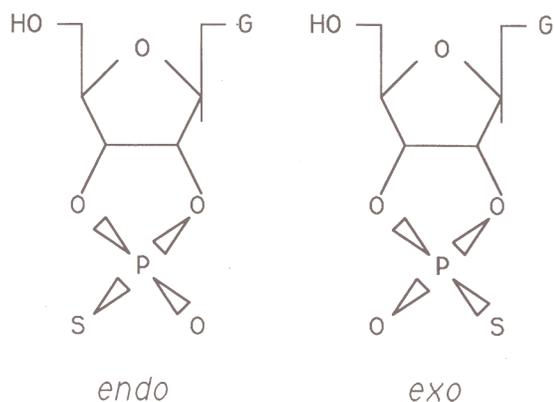


Fig. 1. The chemical structures of the ribophosphate moieties of *endo* and *exo* isomers of guanosine 2',3'-cyclophosphorothioate showing the difference in configuration at the phosphorus atom.

Table 1. Summary of data collection and processing.

Data collected	Value
Beam line	X11
Wavelength (nm)	0.095
Maximum resolution (nm)	0.20
No. measurements	48 382
Fully recorded	41 465
Partially recorded	6 917
Independent reflections	13 932
$R(I)_{\text{merge}} = \sum I - \langle I \rangle / \sum I$ (%)	3.4
Completeness (%)	98.4

would be cleaved and would not be seen in the structure as a cyclophosphate.

Here we present the refined 0.2-nm resolution structure of RNase Sa with *exo* guanosine-2',3'-cyclophosphorothioate. From the structure of this and previously studied complexes we propose a model for the complex with the *endo* isomer and hence for the natural complex with the cyclic reaction intermediate.

MATERIALS AND METHODS

Crystallisation

RNase Sa was isolated from the culture medium of *Streptomyces aureofaciens* essentially as described earlier (Gasperi et al., 1982). Crystals of RNase Sa were grown by hanging drop vapour diffusion from a 3% protein solution in 0.1 M sodium phosphate, pH 7.2, using ammonium sulphate as precipitant (Sevcik et al., 1982). The crystals are in space group $P2_12_12_1$ with unit cell dimensions of $a = 6.47$ nm, $b = 7.87$ nm, $c = 3.91$ nm with two molecules in the asymmetric unit, isomorphous with the native and other complex crystals. *Endo* and *exo* 2',3'-GCPT were synthesised as described previously (Eckstein et al., 1972) and a procedure for their separation developed (A. F. Haikal, unpublished results). The complex was prepared by diffusion of *exo*-2',3'-GCPT into RNase Sa crystals in mother liquor. The concentration of 2',3'-GCPT was about 1.6 mg/ml. Crystals were soaked for three days before mounting in capillaries. Data were collected 6 days later from a single crystal of size approximately $0.5 \times 0.5 \times 0.4$ mm³.

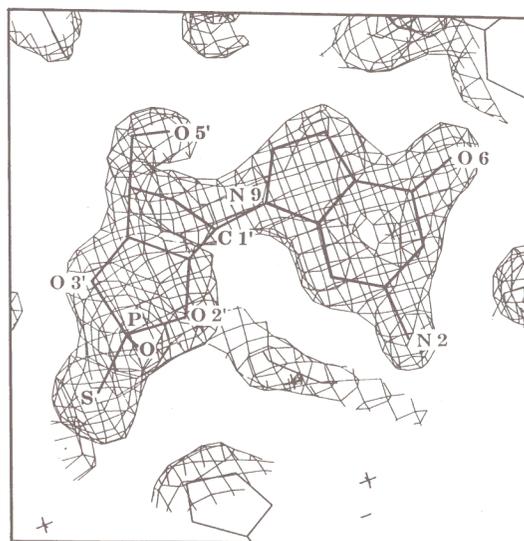


Fig. 2. The $(2F_o - F_o)$ electron density for the *exo* guanosine 2',3'-cyclophosphorothioate in molecule A contoured at the 1.5 σ level.

Table 2. Contact distances less than 0.35 nm between 2',3'-GCTP and the enzyme.

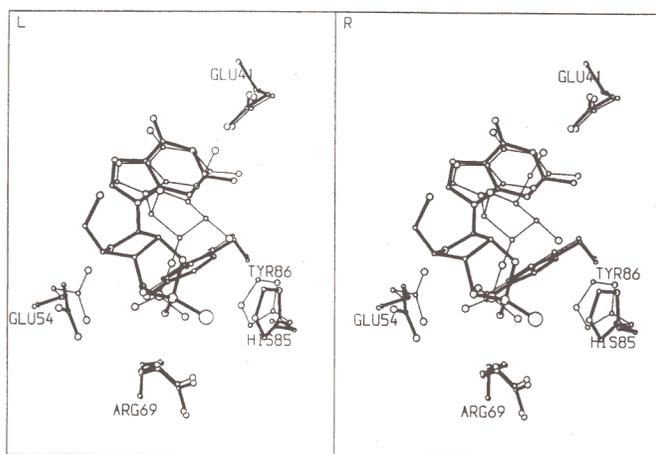
Contact distance determined between			Contact distance for	
			molecule A	molecule B
nm				
O3	NH2	Arg65	0.324	0.294
O1	NH1	Arg69	0.320	—
	NE	Arg69	0.299	0.299
	OH	Tyr86	0.258	0.262
O4'	OE2	Glu54	—	0.202
N7	N	Gln38	0.300	0.292
O6	N	Asn39	0.300	0.267
	N	Arg40	0.274	0.278
N1	OE1	Glu41	0.261	0.272
N2	OE2	Glu41	—	0.290

Data collection and processing

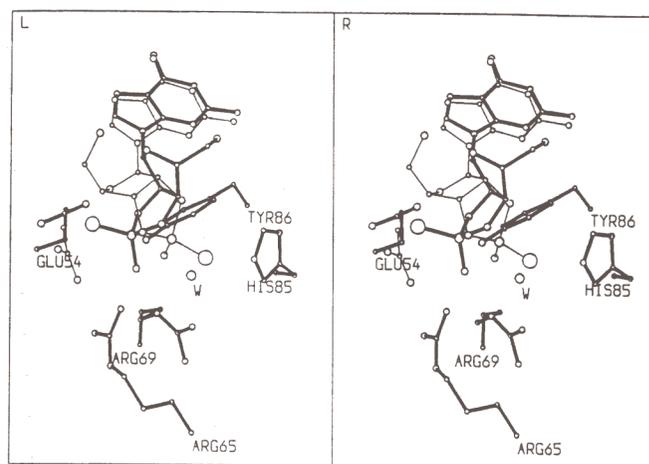
Data to 0.2-nm resolution were collected on the EMBL X11 beam line at the DORIS storage ring, DESY, Hamburg, equipped with an imaging plate scanner. 92° of data were covered by 23 images with 2° rotation followed by 46 images with 1° rotation. The crystal axes were sufficiently offset from the rotation axis to minimize the number of non-measurable reflections in the blind region. A summary of data collection is given in Table 1. The characteristics of the data are very similar to those for RNase Sa complexes with 3'-GMP and 2'-GMP and are not presented in detail. Statistics of the data as a function of resolution are shown in Table S1.

Refinement

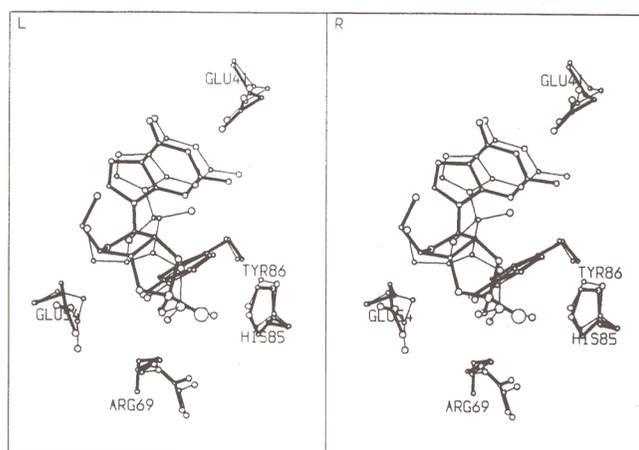
Refinement was carried out by restrained least-squares minimisation (Konnert & Hendrickson, 1980; Baker & Dodson, 1980). No sigma cutoff was applied to the amplitudes. Rebuilding of the model after each refinement step was carried out on an Evans and Sutherland PS300 graphics station



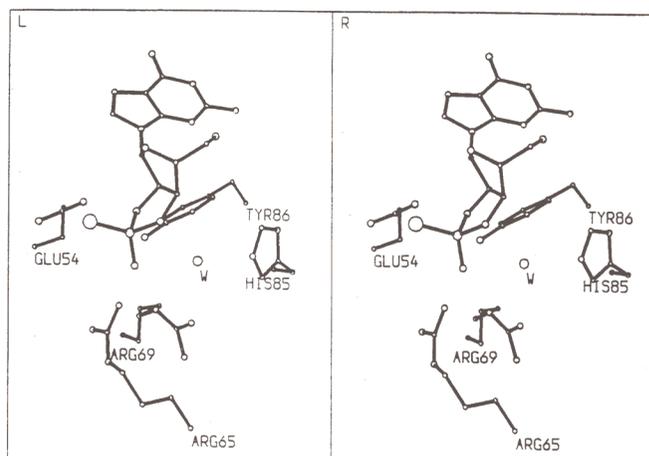
a



a



b



b

Fig. 3. Stereo views of *exo* guanosine 2',3'-cyclophosphorothioate and the neighbouring side chains (thick lines) superimposed on (a) 2'-GMP and (b) 3'-GMP (thin lines) in their corresponding complexes with RNase Sa. The superposition is based on all CA atoms for molecule A. The sulphur atom is represented by a larger circle.

using the program FRODO (Jones, 1978). As a starting model, coordinates of two molecules, referred to as A and B, in the asymmetric unit of the structure of the 2'-GMP complex were used. Only about 75% of the water molecules (290) with low B values were included. No ligand atoms were included in the first stages of refinement. The initial *R* factor was 24%. After a few cycles, the electron density for 2',3'-GCPT at the active site of molecule A became very clear while that at molecule B was weaker. Both 2',3'-GCPT molecules were built into the model with occupancy for all the atoms in molecule B set to 0.5. An occupancy of 0.5 was also given to side-chain atoms of the disordered residues Asp1A, Asp25A, Asp25B, Gln38B, Arg40B and Gln77B, for which the electron density was poor. Two positions were modelled for Ser31A OG and for Cys72A and B SG; the occupancies were estimated from the respective electron densities. Close to Arg63A a sulphate ion was modelled. New water molecules were assigned by an automatic water-search program (V. Lamzin, unpublished results). Their densities were checked after each cycle and those with densities lower

Fig. 4. Stereo view of the proposed complex of *endo* guanosine 2',3'-cyclophosphorothioate with RNase Sa (thick lines). Only the side chains around the nucleotide are shown. (a) Superimposed on the *exo* isomer observed in the crystal structure. (b) For clarity, without the *exo* isomer. Again sulphur atoms are shown as larger circles. The water molecule proposed to be involved in catalysis is labelled W. Replacement of the sulphur in (b) by oxygen would give the natural reaction intermediate.

than 300 e/nm³ were rejected. Table S2 summarizes the stereochemical restraints which were applied to the parameters of the final model together with the final deviations. Thermal parameters are given in Table S3. The refinement was accomplished in six steps of five cycles each; yielding a final *R* factor of 11.9%. The model contains 745 protein atoms and 23 2',3'-GCPT atoms for each enzyme molecule, as well as 1 sulphate ion and 466 water molecules.

RESULTS AND DISCUSSION

There are no major differences in the protein itself between the present structure and those previously reported; native RNase Sa and its complexes with 3'-GMP and 2'-GMP. The fold of the protein is therefore not further discussed. The (2F_o-F_c) electron density for *exo* 2',3'-GCPT at the active site of molecule A is shown in Fig. 2. 2',3'-GCPT

binds in the same manner but at lower occupancy to molecule B. Therefore, the rest of the discussion is restricted to molecule A. Interactions between 2',3'-GCPT and enzyme are given in Table 2. Stereo views of 2',3'-GCPT and associated amino acid residues superimposed on the 2'-GMP and 3'-GMP complexes are shown in Fig. 3a and b, respectively. Superposition was carried out by least-squares minimisation for all pairs of CA atoms. The base lies in essentially the same position as for 2'-GMP and 3'-GMP and forms an identical network of hydrogen bonds with the enzyme in all three complexes. The ribose and cyclophosphate form an essentially rigid moiety. The conformation of the ribose relative to the base in the *exo* 2',3'-GCPT complex is similar to that for 3'-GMP and quite different from that for 2'-GMP. The free phosphate oxygen forms hydrogen bonds with Arg69 NE and NH1 as in the 3'-GMP complex. The sulphur atom could not form such contacts and thus these bonds determine the orientation of the cyclophosphate, and hence the ribose, in the complex. The sulphur atom points away from the enzyme interior and the closest contact with the enzyme is His85 NE2 at a distance of 0.32 nm. The distances between the 2',3'-GCPT O2' and O3' and the catalytic atoms of Glu54 and His85, respectively, are too long for hydrogen bonds. Indeed, these atoms are placed such that O2' points to His85 and O3' to Glu54 in conflict with the accepted mechanism (Takahashi, 1970). This explains why the ligand is not cleaved. The configuration of the S and O atoms in the phosphate of the *exo* isomer forces it to adopt this non-functional conformation. Thus, the *exo* 2',3'-GCPT complex does not provide direct experimental identification of the residues responsible for catalytic cleavage of the substrate.

However, on the basis of the three known complex structures (*exo* 2',3'-GCPT, 2'-GMP and 3'-GMP), we can develop a plausible model for the productive intermediate, guanosine 2',3'-cyclophosphate or its *endo* thio analogue. *Endo* 2',3'-GCPT is cleaved by RNase Sa and thus the contacts formed in its complex with enzyme must be productive, in contrast to those of the *exo* isomer. The configuration of the S and O atoms is inverted in the *endo* isomer relative to the *exo*.

Building of the model for the *endo* isomer was based on the following.

(a) The position of the base is very similar in the three complexes and results from the specificity of the enzyme. We started by assuming a position for the base as in the 3'-GMP complex, the true reaction product. (b) The two rigid parts of the nucleotides, the base and ribophosphate, can rotate relative to one another around the N9-C1' bond, as is seen in their different mutual orientations in the complexes. (c) It was supposed that two factors determine the orientation of the ribophosphorothioate. These are firstly the bonds between the phosphate oxygen and the residues to which it is bound and, secondly, the relatively large S atom, which is unable to form such bonds, points away from the protein. (d) The conserved residues Glu54, Arg69 and His85 should be involved in catalysis.

Rotation of the ribophosphate of the *endo* isomer around the N9-C1' bond by about 120° relative to its position in the *exo* isomer satisfies these requirements. There are H bonds between the phosphate oxygen and the N atoms of either or both of the active-site arginines, Arg69 or Arg65. Which particular H bonds are formed is critically dependent on the exact conformation chosen for the 2',3'-GCPT and of the arginine side chains which are likely to be somewhat flexible. The ribose moiety occupies a position close to that found

in the 2'-GMP complex with the C5' atom pointing away from the active site as expected for the real substrate. The sulphur atom points away from the enzyme and makes no contacts with it. Slight adjustment of the Glu54 side chain allows its OD1 to form a hydrogen bond with the ribose O2'.

In the second step of the catalytic cleavage of substrate, the cyclophosphate is hydrolysed. In this process a water molecule carries out nucleophilic attack on the phosphorus atom. In the model, the distance between His85 NH2 and the P atom, about 0.6 nm, is long enough to be bridged by such a water molecule. In addition, there is a water molecule close to this position in the structure of native RNase Sa. The proposed model for the binding of *endo* 2',3'-GCPT, or indeed the real intermediate, is shown in Fig. 4.

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REFERENCES

- Baker, E. N. & Dodson, E. J. (1980) *Acta Crystallogr. A* **36**, 559–572.
- Borisova, S. N., Vagin, A. A., Nekrasov, Y. V., Pavlovsky, A. G., Strokopytov, B. V. & Vainshtein, B. K. (1986) *Kristallografiya* **31**, 474–477.
- Both, V., Moiseyev, G. P. & Sevcik, J. (1991) *Biochem. Biophys. Res. Commun.* **177**, 630–635.
- Eckstein, F., Schulz, H. H., Rüterjans, H., Haar, W. & Maurer, W. (1972) *Biochem. J.* **11**, 3507–3512.
- Gasperik, J., Prescakova, S. & Zelinka, J. (1982) *Biologia* **36**, 377–381.
- Hartley, R. W. (1980) *J. Mol. Biol.* **15**, 355–358.
- Heinemann, U. & Saenger, W. (1982) *Nature* **299**, 27–31.
- Hill, C. P., Dodson, G. G., Heinemann, U., Saenger, W., Mitsui, Y., Nakamura, K., Borisov, S., Tischenko, G., Polyakov, K. & Pavlovsky, S. (1983) *Trends Biochem. Sci.* **8**, 364–369.
- Jones, T. A. (1978) *J. Appl. Crystallogr.* **11**, 268–272.
- Konnert, J. H. & Hendrickson, W. A. (1980) *Acta Crystallogr. A* **36**, 344–350.
- Mauguen, Y., Hartley, R. W., Dodson, G. G., Dodson, E. J., Bricogne, G., Chothia, C. & Jack, A. (1982) *Nature* **297**, 162–164.
- Nakamura, K. T., Iwahashi, K., Yamamoto, Y., Iitaka, Y., Yoshida, N. & Mitsui, Y. (1982) *Nature* **299**, 564–566.
- Pavlovsky, A. G., Vagin, A. A., Vainshtein, B. K., Chepurnova, N. K. & Karpeisky, M. Y. (1983) *FEBS Lett.* **162**, 167–170.
- Polyakov, K. M., Strokopytov, B. V., Vagin, A. A., Tischenko, G. N., Bezborodova, S. I. & Vainshtein, B. K. (1987) *Kristallografiya* **32**, 918–926.
- Sevcik, J., Dodson, E. J. & Dodson, G. G. (1991) *Acta Crystallogr. B* **47**, 240–253.
- Sevcik, J., Gasperik, J. & Zelinka, J. (1982) *Gen. Physiol. Biophys.* **1**, 255–259.
- Sevcik, J., Hill, C. P., Dauter, Z. & Wilson, K. S. (1993) *Acta Crystallogr. D* **49**, 257–271.
- Sevcik, J., Sanishvili, R. G., Pavlovsky, A. G. & Polyakov, K. M. (1990) *Trends Biochem. Sci.* **15**, 158–162.
- Shlyapnikov, S. U., Both, V., Kulikov, V. A., Dementiev, A., Sevcik, J. & Zelinka, J. (1986) *FEBS Lett.* **209**, 335–339.
- Steyaert, J., Hallenga, K., Wyns, L. & Stanssens, P. (1990) *Biochemistry* **29**, 9064–9072.
- Takahashi, K. (1970) *J. Biochem.* **67**, 833–839.
- Yakovlev, G. I., Moiseyev, G. P., Bezborodova, S. I., Both, V. & Sevcik, J. (1992) *Eur. J. Biochem.* **204**, 187–190.
- Zelinkova, E., Bacova, M. & Zelinka, J. (1971) *Biochim. Biophys. Acta* **235**, 343–344.

Supplementary material to:

Complex of ribonuclease Sa with a cyclic nucleotide and a proposed model for the reaction intermediate

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Table S1. Statistics of the data as a function of resolution.

Resolution	N_{obs}	Completeness	$R(I)$	$>3\sigma$
nm		%		%
2.000–0.820	200	85.8	0.029	96.2
0.820–0.606	327	97.9	0.031	96.8
0.606–0.502	394	97.8	0.029	97.8
0.502–0.439	461	98.3	0.030	97.7
0.439–0.394	520	99.2	0.028	98.9
0.394–0.361	557	99.5	0.026	97.6
0.361–0.335	624	99.4	0.032	98.3
0.335–0.314	640	99.7	0.032	98.1
0.314–0.296	694	99.6	0.037	98.0
0.296–0.281	709	99.7	0.032	97.0
0.281–0.269	759	99.1	0.035	96.5
0.269–0.257	791	99.6	0.039	97.8
0.257–0.247	805	99.3	0.040	96.8
0.247–0.239	856	99.5	0.041	97.1
0.239–0.231	861	99.4	0.045	95.5
0.231–0.223	916	99.7	0.045	96.8
0.223–0.217	921	99.5	0.049	96.2
0.217–0.211	954	99.6	0.048	95.5
0.211–0.205	981	98.9	0.052	95.1
0.205–0.200	967	95.6	0.053	91.7
Overall	13 932	98.4	0.034	96.8

Table S2. Refinement parameters, restraint weighting scheme and standard deviations after the last cycle of refinement. σ , target discrepancy; δ , observed r.m.s. discrepancy. The weight of restraints is $1/\sigma^2$.

Parameter	σ	δ
Resolution limit (nm)		0.20
No. of reflections		13 879
Protein atoms in A/B		745/745
Protein sites in A/B		747/746
No. water molecules		466
No. sulphate ions		1
R factor (%)		11.9
σ_A error estimate (nm)		0.13
Stereochemistry		
Bond length (1–2) (nm)	0.0020	0.0021
Bond angles (1–3) (nm)	0.0040	0.0049
Dihedral angles (1–4) (nm)	0.0050	0.0085
Planar groups (nm)	0.0020	0.0018
Chiral volumes (nm ³)	0.00015	0.00017
Peptide angles ω (degrees)	3.0	3.09

Table S3. Average isotropic atomic temperature factors

	Atomic temperature factor for	
	molecule A	molecule B
	nm ²	
Main chain	0.086	0.105
Side chain	0.124	0.156
Protein	0.104	0.130
2',3'-GCPT	0.171	0.191
Sulphate ion	0.127	
Water molecules	0.437	
Overall	0.191	
B value from Wilson plot	0.105	