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Structure of Sulfur-Substituted Rhodanese at 1.36 Å Resolution

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Abstract

1.36 Å resolution X-ray diffraction data have been recorded at 100 K for bovine liver sulfur-substituted rhodanese, using synchrotron radiation. The crystal structure has been refined anisotropically to a final R factor of 0.159 ($R_{\rm free}$ = 0.229) for 53 034 unique reflections. The model contains 2 327 protein atoms and 407 solvent molecules, with a good geometry. The high resolution allows full details for helices, β-sheets, tight turns and of all inter- and intramolecular interactions stabilizing the enzyme molecule to be given. The situation at the active site is described, particularly in regard to the network of hydrogen bonds made by S^{γ} and S^{δ} of the sulfur-substituted catalytic Cys247 and surrounding groups and solvent molecules. The replacement of the precipitant ammonium sulfate with cryoprotectants in the crystal-suspending medium led to the removal of the sulfate ion from the enzyme active site. Only limited changes of the enzyme structure have been found as a result of the drastic change in the crystal medium.

1. Introduction

Rhodanese (thiosulfate:cyanide sulfurtransferase, E.C. 2.8.1.1) is a mitochondrial enzyme that is widely distributed in nature. However, its biological function has not clearly been established as yet. *In vitro* the enzyme catalyzes the transfer of a sulfane S atom from thiosulfate to the nucleophilic acceptor cyanide in a two-step reaction, as follows,

$$\mathrm{E} + \mathrm{S_2O_3^{3-}} \leftrightarrow \mathrm{ES} + \mathrm{SO_3^{2-}}$$
$$\mathrm{ES} + \mathrm{CN^-} \rightarrow \mathrm{E} + \mathrm{SCN^-}.$$

In this reaction rhodanese cycles between two stable catalytic intermediates: sulfur-free (E) and sulfur-substituted persulfide-containing (ES) enzyme. The persulfide group is substantially stabilized by a large number of hydrogen-bonding interactions with neighboring groups at the enzyme active site (Ploegman *et al.*, 1979). The crystal structure of rhodanese, which has been determined at 2.5 Å resolution (Ploegman, Drent, Kalk, Hol *et al.*, 1978; Ploegman, Drent, Kalk & Hol, 1978, 1979), and subsequently refined at 2.1 Å (Hol *et al.*, 1983) and 1.9 Å resolution (Gliubich *et al.*, 1996), reveals that the protein consists of two domains possessing highly similar conformations, despite their quite dissimilar amino-acid sequences. The active site containing the catalytic Cys247, which is involved in the persulfide group formation, is located close to the interface of the two domains. Removal of the transferable S atom from the persulfide group by cyanide (Ploegman *et al.*, 1979; Gliubich *et al.*, 1996) as well as chemical modifications of the catalytic sulfhydryl group (Gliubich *et al.*, 1996) has been found to lead to limited structural changes, which are mainly confined to the enzyme active site. We describe here the crystal structure of sulfur-substituted rhodanese at 1.36 Å resolution.

2. Experimental

2.1. *Data collection and processing*

Single crystals of sulfur-containing rhodanese were obtained by means of the sitting-drop vapor-diffusion method, in the presence of approximately 1.9 *M* ammonium sulfate, as previously described (Gliubich *et al.*, 1996). In order to exchange the ammonium sulfate used as the precipitant, the crystals were first soaked for 20 min in a solution containing 28% PEG 6000, 0.05 *M* sodium phosphate, pH 7.6, and then for 15 min in a cryoprotectant solution containing 22% PEG 6000, 22% (*v*/*v*) glycerol, 0.04 *M* sodium phosphate. The soaking in the cryo-protectant solution did not cause any modification of the cell parameters determined previously (Ploegman, Drent, Kalk & Hol, 1978; Gliubich *et al.*, 1996): the space group is $C2$ and cell dimensions are a = 156.23, b = 49.04, c = 42.25 Å, β = 98.6°.

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A medium-resolution (1.8 Å) set of data was first obtained at room-temperature using a HI-STAR area detector, mounted on a Siemens M18XHF-SRA rotating anode. The latter was operated at 4.5 kW (50 kV, 90 mA), with an apparent focus size of 0.3 × 3 mm. The Cu $K\alpha$ radiation was selected by a graphite monochromator. Using a crystal-to-detector distance of 14 cm, 2720 frames of 0.25° rotation around ω were measured, for a total rotation of 680°. Images were evaluated with the program *SAINT* (Siemens Industrial Automation), resulting in 21 852 independent reflections for a maximum resolution of 1.8 Å.

High-resolution data were collected at the diffraction beamline of the ELETTRA synchrotron (Trieste, Italy). The ring was operated at 1.99 GeV, the current during data collections was between 140 and 100 mA. A 1 Å wavelength was selected and the (111) face of a silicon double crystal was used as a monochromator. The specimen crystal was mounted in a loop and immediately frozen at 100 K by flushing with liquid nitrogen, the temperature being controlled by an Oxford Cryosystems Cryostream. Data were recorded using an imaging-plate scanner (MAR Research, diameter 180 mm), positioned at a distance of 90 mm from the crystal. Oscillations of 1.0° at a constant dose for a mean time of 5 min were performed, for a total of 89°. Since many of the strong reflections were saturated, one crystal was used for low-resolution data, with the detector positioned at a distance of 170 mm: 89 oscillations of 1.5° for a total of 133.5° were measured for a shorter exposure time.

Images were processed with *MARXDS*2.1 (Kabsch, 1988). Cell dimensions (a = 154.364, b = 49.312, c = 41.676 Å, β = 99.81°) are slightly, but definitely, different from those found for the crystals at room temperature. Table 1 gives the statistics for data collection and the percentage of reflections measured as a function of resolution.

Table 1. *Summary of data collection.*

(*a*) Parameters for data measurements. Synchrotron data were measured at 100 K, using three crystals and a wavelength of 1.0 Å.

Maximum resolution (Å)	1.36
No. of measurements	131477
Independent reflections	56060
$R(I)_{\rm merge} = (\sum \lvert I - \langle I\rangle\rvert / \sum \langle I\rangle) \times 100$	6.76

(*b*) Independent reflections as a function of the resolution, completeness percentages and mean ratio of the intensities over σ in the shell.

Resolution interval (Å)	Number of independent reflections	%	$\langle I/\sigma(I)\rangle$
∞–2.06	17935	93	45
2.06–1.64	14731	78	17
1.64–1.43	13218	70	7
1.43–1.36	10176	54	4
∞–1.36	56060	74	—

2.2. *Refinement*

The atomic coordinates for sulfur-substituted rhodanese, refined at 1.9 Å resolution (Gliubich *et al.*, 1996), were used as the starting model. The initial R factor for the rotating-anode data was 0.30 and was reduced to 0.165 after several cycles of restrained least-squares refinement with the program *TNT* (Tronrud *et al.*, 1987) and manual rebuilding. Solvent molecules were added as water. When synchrotron data were used, the R factor for all reflections was 0.32 and, after 20 cycles of refinement, was reduced to 0.25. Refinement was then accomplished with *SHELXL*93 (Sheldrick, 1993), using the restraint parameters automatically generated by the program. Each refinement macrocycle in general consisted of ten cycles of automatic minimization using the conjugate-gradient option, followed by map calculation (with $|2F_{\rm obs} - F_{\rm calc}|$ or $|F_{\rm obs} - F_{\rm calc}|$ coefficients) and manual rebuilding of the model. 5% of the total reflections were not considered in minimization; instead, they were used at the end of each step to calculate the $R_{\rm free}$ factor (Brünger, 1992). After the first two macrocycles of refinement, the R factor was reduced to 0.24 ($R_{\rm free}$ = 0.28). The introduction of H atoms in calculated positions, using the appropriate *HFIX* option of the program, reduced it to 0.19 in two additional macrocycles ($R_{\rm free}$ = 0.24). From this stage, restrained anisotropic thermal parameters for all atoms, except H atoms, were introduced. In particular, the components of the anisotropic displacement of 1–2 and 1–3 bonded atoms were restrained to be equal, within a standard deviation of 0.02 Å; moreover, atoms closer than 1.7 Å were

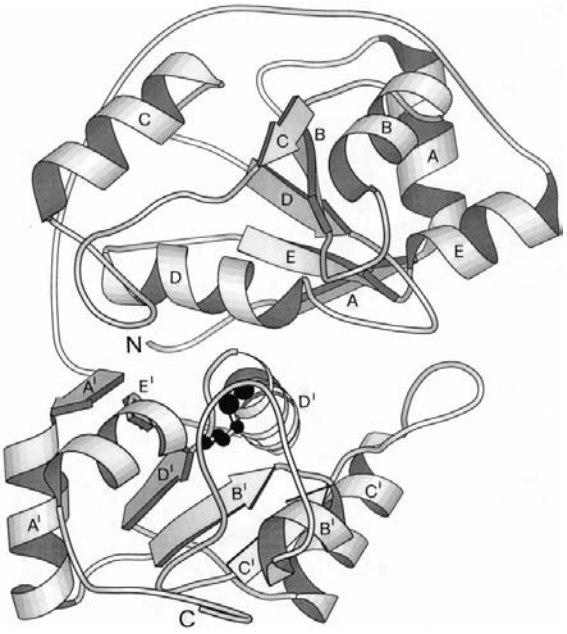

Fig. 1. Cartoon drawing of the rhodanese structure, with the conventional numbering system for α-helices and β-strands. Atoms of the catalytic residue Cys247 are represented as small spheres.

Table 2. *Statistics for the refined models.*

R_T^{free} factor is defined as: $R_T^{free} = (\sum_{hkl \subset T} |F_{obs}| - k|F_{calc}|) / \sum_{hkl \subset T} |F_{obs}|)$. The test set T is a random selection of 5% of the observed reflections and $hkl \subset T$ means that all reflections belong to T. The r.m.s. deviations on the usually reported parameters are obtained by means of the program *SHELXL*93 (Sheldrick, 1993).

Crystal data	$C2$, $Z = 4$	
Cell parameters (Å, °)	$a = 154.364$	
	$b = 49.312$ (5)	
	$c = 41.676$ (8)	
	$\beta = 99.81$ (7)	
	Number of reflections	R factor (%)
Used in refinement	53034	15.9
With $F > 6\sigma(F)$	42221	14.1
For R_{free}	2792	22.9
Goodness of fit	1.14	
Weighting scheme	$1/[\sigma^2(F_{obs}^2) + (0.444\ P)^2]$	
Protein atoms	2327	
Solvent molecules	407	
	Target restraint value	
1–2 distances (Å)	0.03	
1–3 distances (Å)	0.03	
Planarity (Å)	0.2	
Chiral volume (Å^3)	0.2	

restrained to have the same U^{ij} components, with a standard deviation of 0.10 Å, except for the terminal atom in the chain, for which a standard deviation of 0.2 Å was allowed. At each step, the peaks automatically recognized by the *SHELXL* software as water molecules were visually checked on a graphic system and eventually added. About ten macrocycles of minor manual adjustments and solvent revision brought the structure to the final R value of 0.159 ($R_{free} = 0.229$). At each stage of refinement, the weighting scheme suggested by the output of the program itself was used. After the last cycle, the highest residual density in the Fourier-difference map was 0.44 e Å^{-3}. Statistical data for the final model are reported in Table 2.

Electron-density maps were calculated with the *X-PLOR* program (Brünger *et al.*, 1987) and displayed on an IRIS 4D Graphics Workstation (Silicon Graphics) using the program *TOM* (Jones, 1978). Stereochemistry and quality of the final models were examined with the program *PROCHECK* (Laskowski *et al.*, 1993) and the location of hydrogen bonds and interatomic contacts was analyzed with programs *CONTACT* and *ACT* contained in the *CCP*4 package, version 2.14 (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

3.1. *Quality of the model*

The conformation of the polypeptide chain of the enzyme is quite well defined in the electron-density map, with the exception of a few N-terminal (1–3) and C-terminal (291–293) residues. This is confirmed by the mean thermal parameter: mean overall B factors of 16 and 19 Å^2 for main-chain and side-chain atoms, respectively, have been determined. No other area of the molecule appears to be disordered, except for some positively or negatively charged side chains, listed in Table 3. A Ramachandran plot (Ramachandran *et al.*, 1963) shows that 91.5% of the residues fall in the most favored regions, 8.1% in the additionally allowed regions and only one residue (His2) in a 'generously allowed' region. However, it should be noted that the latter residue is located in the disordered N terminus. The other geometric tests reported by the *PROCHECK* program (Laskowski *et al.*, 1993) do not show any outliers, except for Gly291, which is also present in one of the two aforementioned disordered areas.

Table 3. *Side-chain atoms that appear to be disordered.*

These atoms are considered disordered since there is no clear electron density for such atoms in the map with contour levels drawn at 1σ, and it was not possible to establish the existence of multiple conformations. It should be noted that residue 1–3 and 290–293 are completely disordered (see text).

Residue	Atoms
Glu62	C^γ, C^δ, $O^{\varepsilon 1}$, $O^{\varepsilon 2}$
Lys66	C^γ, C^δ, C^ε, N^ζ
Glu148	C^γ, C^δ, $O^{\varepsilon 1}$, $O^{\varepsilon 2}$
Lys153	C^δ, C^ε, N^ζ
Arg158	C^δ, N^ε, C^ζ, NH1, NH2
Arg175	C^γ, C^δ, N^ε, C^ζ, NH1, NH2
Arg205	C^β, C^γ, C^δ, N^ε, C^ζ, NH1, NH2
Glu226	C^γ, C^δ, $O^{\varepsilon 1}$, $O^{\varepsilon 2}$
Arg229	N^ε, C^ζ, NH1, NH2
Lys236	C^γ, C^δ, C^ε, N^ζ
Lys241	C^ε, N^ζ

3.2. *Structure of rhodanese and comparison between its two structurally homologous domains*

The refined structure of sulfur-substituted rhodanese does not substantially differ from the previously determined medium-resolution structures and the relatively small r.m.s. deviations between equivalent Cα atoms may be ascribed to a large number of small local differences, mainly due to the substantially higher resolution of the present data. The overall structure (Fig. 1) appears to be stabilized by 198, 112 and 33 intramolecular hydrogen bonds between main-chain–main-chain, main-chain–side-chain and side-chain–side-chain atoms, respectively. Several additional intramolecular hydrogen bonds appear to be mediated by water molecules. Moreover, seven salt bridges are formed between charged side chains.

The rhodanese molecule consists of two nearly superimposable domains, possessing quite different amino-acid sequences. In our model, the superposition of the two domains results in an r.m.s. deviation between Cα atoms of 2.05 Å^2.

Ten helices are present in the protein molecule: five (helices *A*–*E*) are located in domain I (residues 1–142)

and five (helices A'–E') in domain II (residues 159–293). Helices B (42–50) and A'(163–174) fully correspond to classical α-helices, according to the torsion-angle values and hydrogen-bond pattern. Helices A (11–22), C (76–87), D (107–119) E (129–137), C'(224–235) and E'(274–282) are also classical α-helices, but they shift towards the 3_{10}-helix in the last turn. Although the torsion-angle values of the short helix B'(183–189) are characteristic of an α-helix, the main-chain carbonyl O atom of Gly185 forms a bifurcated hydrogen bond with the main-chain NH groups of Gly189 and Thr190. Two portions for helix D'(251–264) can be distinguished on the basis of torsion-angle values typical of a 3_{10}-helix (residues 252–256) and of a classical α-helix (residues 256–261).

With regard to β-strands A–D of domain I and A'–$D'$of domain II, the mean φ values are relatively close to the theoretical ones for parallel β-pleated sheets ($\varphi = -120$, $\psi = 110°$), whilst ψ values are definitely larger and in some cases they are not compatible with those allowed by theoretical calculations for residues branched at C_β (Chothia, 1973). However, if the first amino acid in the strand is not considered, in general mean ψ values decrease significantly.

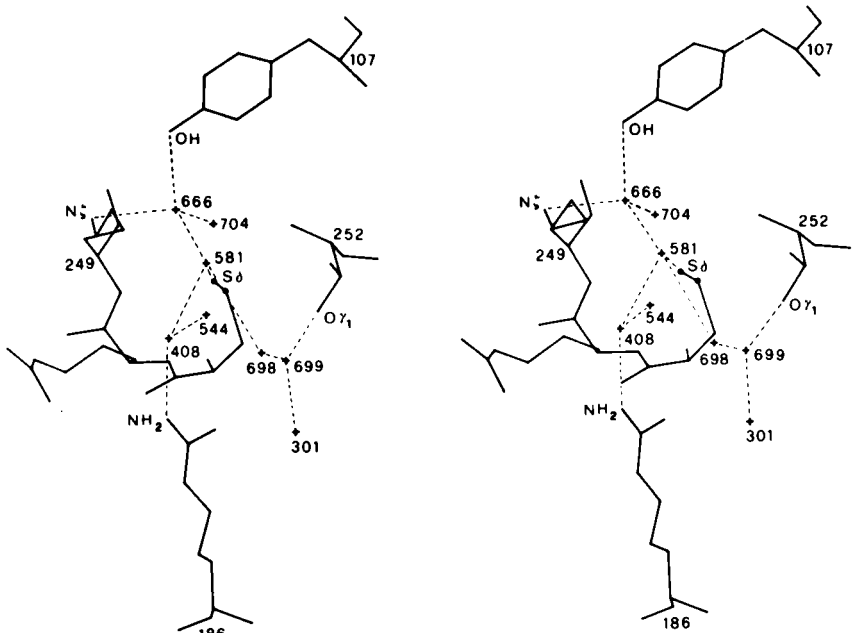

Fig. 2. Stereo drawing of the network of water molecules at the active site of sulfur-substituted rhodanese. Relevant distances are: W704–W666, 2.9 Å; N^{ζ} Lys249–W666, 2.9 Å; W666–W581, 2.7 Å; W666–OH Tyr107, 2.9 Å; S^{δ}–W581, 3.6 Å; W581–W408, 3.1 Å; W581–W698, 3.7 Å; W698–W301, 3.0 Å; W301–W699, 3.1 Å; W699–$O^{\gamma 1}$ Thr252, 2.8 Å; W544–W408, 2.9 Å; W408–NH2 Arg186, 2.9 Å; W698–W699, 2.7 Å.

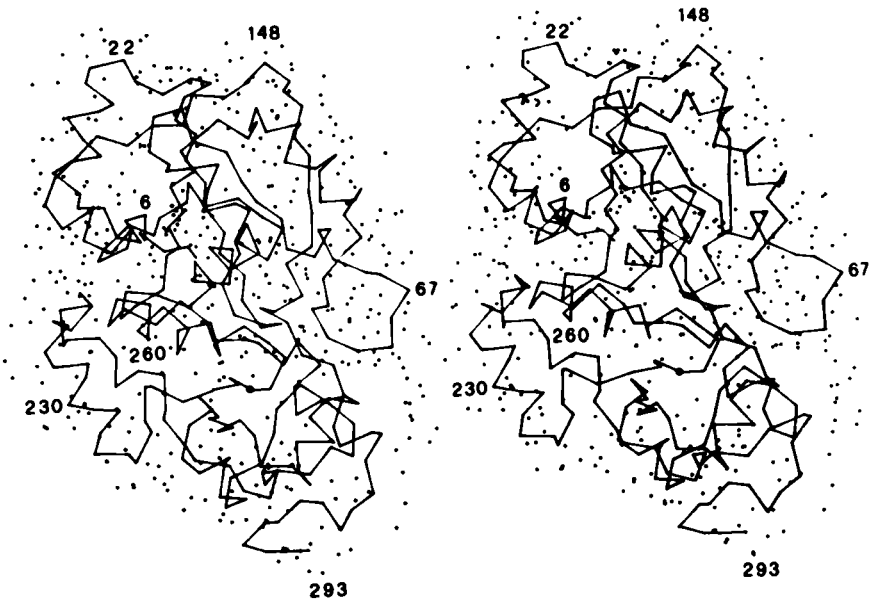

Fig. 3. Stereo drawing of the Cα trace for sulfur-substituted rhodanese surrounded by ordered solvent molecules. A black dot denotes the position of the enzyme active site.

Table 4. *Intermolecular contacts that are not mediated by water molecules.*

Symmetry operations are: (*a*) x, y, z; (*b*) $-x, y, -z$; (*c*) $x+\frac{1}{2}, y+\frac{1}{2}, z$; (*d*) $-x+\frac{1}{2}, y+\frac{1}{2}, -z$; translations of a cell period are indicated by a capital letter; for example, $a + C$ in the first line of the table indicates the identity operator plus a translation of one cell parameter along the *c* axis. If the distance between a hydrogen donor atom and an acceptor atom is less than 3.2 Å, the interaction is considered a hydrogen bond; in case of a distance between 3.2 and 3.6 Å, it is considered a polar interaction; in the case of a distance between a polar and a non-polar atom less than 3.6 Å, it is considered a van der Waals interaction.

Symmetry operation	Hydrogen bonds	Polar interactions	van der Waals interactions
$a + C$	—	—	3
$a + B$	—	—	2
$b + A$	3	—	11
$c - B$	3	4	6
c	11	3	7

Concerning tight turns, only β-turn 52–55 of domain I superimposes well enough on β-turn 204–207 of domain II. The other tight turns are rather different and for some of them there is no correspondence between the two domains.

3.3. *The active site*

A network of hydrogen bonds is formed between both S^{γ} and S^{δ} of Cys247 at the active site of sulfur-substituted rhodanese and main-chain NH groups of surrounding residues. In particular, S^{δ} interacts with four such groups (N 248, N 249, N 251, N 252); S^{δ} and the four peptide N atoms lie roughly in the same plane. Moreover, $O^{\gamma 1}$ 252 and water 581 are at 3.6 Å from S^{δ}, indicating a possible interaction between these atoms. S^{γ} lies above the aforementioned plane and exhibits hydrogen-bonding interactions with N 248, N 250, N 251, N 252 and N 253. Eight water molecules are close to the active site, within a sphere of 8 Å radius from S^{δ} of Cys247: a network of hydrogen bonds forms the connections among them and with side-chain atoms, as shown in Fig. 2. The catalytic Cys247 is at the bottom of a cavity, where a sulfur acceptor substrate, like cyanide, must enter in order to interact with S^{δ} of the persulfide group. The solvent molecules we observe in the crystal structure occupy part of that cavity and some of them are probably displaced by the approaching substrate to allow the reaction to occur. It must be noticed that a sulfate ion was found positioned at the entrance of the active site, near Arg186 and Lys249, for the crystals of both sulfur-substituted and sulfur-free rhodanese (Lijk *et al.*, 1984). We do not observe such a bound anion, possibly because it was removed during the soaking of the crystals in a medium devoid of ammonium sulfate (see §2). The sulfate ion is known to be a strong inhibitor of the sulfur transfer reaction catalyzed by rhodanese. It has been suggested that its interaction with the positively charged side chains of Arg186 and Lys249 would block access and correct orientation at the active site of the two negatively charged enzyme substrates thiosulfate and cyanide (Hol *et al.*, 1983; Lijk *et al.*, 1983). Consistent with this hypothesis, the reaction catalyzed by rhodanese in the crystalline state is greatly accelerated upon replacement of ammonium sulfate with PEG as precipitant in the crystal-suspending medium (R. Berni, unpublished observations).

3.4. *Solvent structure*

407 solvent molecules, numbered from 301 to 707, have been positioned in our final model (Fig. 3). 266 of them are in the first coordination shell, *i.e.* are close to at least one polar atom of the protein, whilst 141 interact with other solvent molecules only. All solvent molecules were tentatively assumed to be water. If we consider the possibility of hydrogen-bond formation when distances between hydrogen donor and acceptor atoms are shorter than 3.2 Å, most water molecules form three and two hydrogen bonds (118 and 120, respectively), 66 form four hydrogen bonds and 85 only one. 18 solvent molecules do not form any hydrogen bond, according to the previous definition; however, since they are less than 3.5 Å apart from some polar atom and are rather well defined in the electron-density map, they have been included in the refined structure.

3.5. *Crystal packing interactions*

Close intermolecular contacts have been found in the crystal between a reference rhodanese molecule and another five symmetry-related molecules. Molecules are considered in contact with each other when the distance between at least two of their atoms is shorter than 3.6 Å. Table 4 summarizes the number of contacts and indicates that there is a large contact area between two molecules, the reference one and that related by the translation of $\frac{1}{2}$ along the *x* and *y* axes: 11 intermolecular hydrogen bonds are formed between these two molecule as compared to six intermolecular hydrogen bonds between the reference molecule and other two symmetry-related molecules. The contacts with the remaining two symmetry-related molecules involve only a very limited number of van der Waals interactions. Clearly, most protein–protein interactions are mediated by solvent molecules.

The coordinates and structure factors of the protein model have been deposited in the Protein Data Bank for immediate distribution.†

† Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 1RHS, R1RHSSF). Free copies may be obtained through The Managing Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England (Reference: SE0218).

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References

Brünger, A. T. (1992). *Nature* (*London*), **335**, 472–475.
Brünger, A. T., Kuriyan, K. & Karplus, M. (1987). *Science*, **235**, 458–460.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Chothia, C. (1973). *J. Mol. Biol.* **75,** 295–302.
Gliubich, F., Gazerro, M., Zanotti, G., Delbono, S., Bombieri, G. & Berni, R. (1996). *J. Biol. Chem.* **271**, 21054–21061.
Hol, W. G. J., Lijk, L. J. & Kalk, K. H. (1983). *Fund. Appl. Toxicol.* **3**, 370–376.
Jones, T. A. (1978). *J. Appl. Cryst.* **11**, 268–272.
Kabsch, W. (1988). *J. Appl. Cryst.* **21**, 916–924
Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). *J. Appl. Cryst.* **26**, 283–291.
Lijk, L. J., Kalk, K. H., Brandenburg, N. P. & Hol, W. G. J. (1983). *Biochemistry*, **22**, 2952–2957.
Lijk, L. J., Torfs, C. A., Kalk, K. H., De Maeyer, M. C. H. & Hol, W. G. J. (1984) *Eur. J. Biochem.* **142**, 399–408
Ploegman, J. H., Drent, G., Kalk, K. H. & Hol, W. G. J. (1978). *J. Mol. Biol.* **123**, 557–594.
Ploegman, J. H., Drent, G., Kalk, K. H. & Hol, W. G. J. (1979). *J. Mol. Biol.* **127**, 149–162.
Ploegman, J. H., Drent, G., Kalk, K. H., Hol, W. G. J., Heinrikson, R. L., Keim, P., Weng, L. & Russel, J. (1978). *Nature* (*London*), **273**, 124–129.
Ramachandran, G. N., Ramakrishnan, C. & Sasisekharan, V. (1963). *J. Mol. Biol.* **7**, 95–99.
Sheldrick, G. M. (1993). *SHELXL*93, *Program for Crystal Structure Refinement*. University of Göttingen, Germany.
Tronrud, D. E., Ten Eyck, L. F. & Matthews, B. W. (1987). *Acta Cryst.* A**43,** 489–501.