

Crystal structure of the ribosomal protein S6 from *Thermus thermophilus*

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The amino acid sequence and crystal structure of the ribosomal protein S6 from the small ribosomal subunit of *Thermus thermophilus* have been determined. S6 is a small protein with 101 amino acid residues. The 3D structure, which was determined to 2.0 Å resolution, consists of a four-stranded anti-parallel β-sheet with two α-helices packed on one side. Similar folding patterns have been observed for other ribosomal proteins and may suggest an original RNA-interacting motif. Related topologies are also found in several other nucleic acid-interacting proteins and based on the assumption that the structure of the ribosome was established early in the molecular evolution, the possibility that an ancestral RNA-interacting motif in ribosomal proteins is the evolutionary origin for the nucleic acid-interacting domain in large classes of ribonucleic acid binding proteins should be considered.

Key words: amino acid sequence/ribosomal proteins/RNA interaction/X-ray crystallography

Introduction

Ribosomes are the ribonucleoprotein organelles on which the mRNA-directed synthesis of proteins takes place. They consist of a large and a small subunit which assemble during the initial stages of protein synthesis. Prokaryotic ribosomes comprise three molecules of RNA and >50 protein molecules (Wittman, 1982). Ribosomal RNA from a large number of sources have been sequenced (Gutell, 1993). As for the proteins, all of the *Escherichia coli* proteins have been isolated and sequenced (Wittman, 1982). The structures of six ribosomal proteins from various sources have been solved, namely a C-terminal fragment of L7/L12 (Leijonmarck *et al.*, 1980), L30 (Wilson *et al.*, 1986), S5 (Ramakrishnan and White, 1992), L6 (Golden *et al.*, 1993), L9 (Hoffman *et al.*, 1994) and S17 (S.W.White, personal communication). The latter is an NMR structure, while the former five were determined by X-ray crystallography. Most ribosomal proteins are relatively small (<150 amino acid

residues) basic molecules which interact with ribosomal RNA.

Almost all biochemical characterization of S6 stems from *E.coli* (ES6) which is situated on the platform of the small ribosomal subunit facing the large subunit (Kahan *et al.*, 1981; Stöffler and Stöffler-Meilicke, 1984; Capel *et al.*, 1987). ES6 undergoes an unusual post-translational modification as two to six glutamic acids are added at the C-terminus (Hitz *et al.*, 1975; Schnier *et al.*, 1986). It thereby acquires a variable length of 131 – 135 amino acid residues and is the most acidic protein in the small ribosomal subunit (pI = 4.8; Kaltschmidt and Wittman, 1970). The

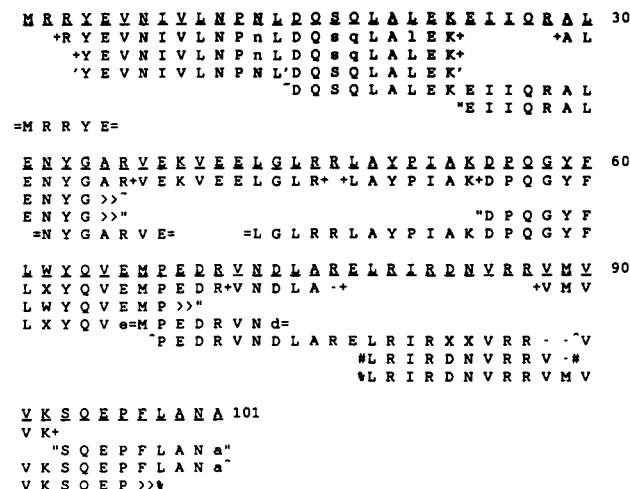


Fig. 1. The primary structure of ribosomal protein S6 from *T.thermophilus*. (++) , tryptic peptides; (= =), endoproteinase Glu-C peptides; (" ") , endoproteinase Lys-C peptides; (' ') , endoproteinase Asp-N peptides from tryptic peptide; (""""), BrCN peptides; (# #), endoproteinase Glu-C peptide from BrCN peptide; (~ ~), endoproteinase Asp-N peptide from BrCN peptide; (% %), endoproteinase Glu-C peptide from endoproteinase Lys-C peptide; >> indicates that the sequencer run was interrupted. Lower case letters indicate an uncertain residue; - indicates that the corresponding residue was not determined in that particular peptide.

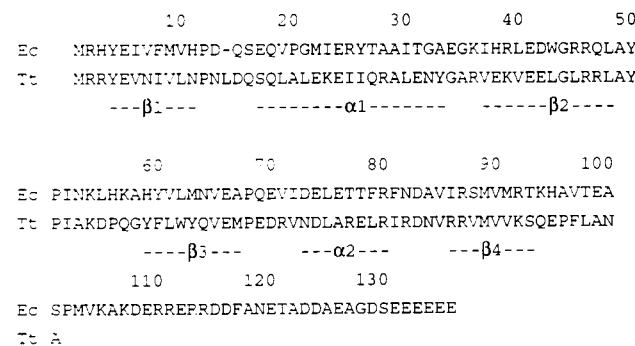


Fig. 2. Comparison of the primary structure of *T.thermophilus* S6 with that of *E.coli* S6. A gap has been introduced to maximize homology. The comparison was based on the table in Gribskov and Burgess (1986). The elements of secondary structure are indicated.

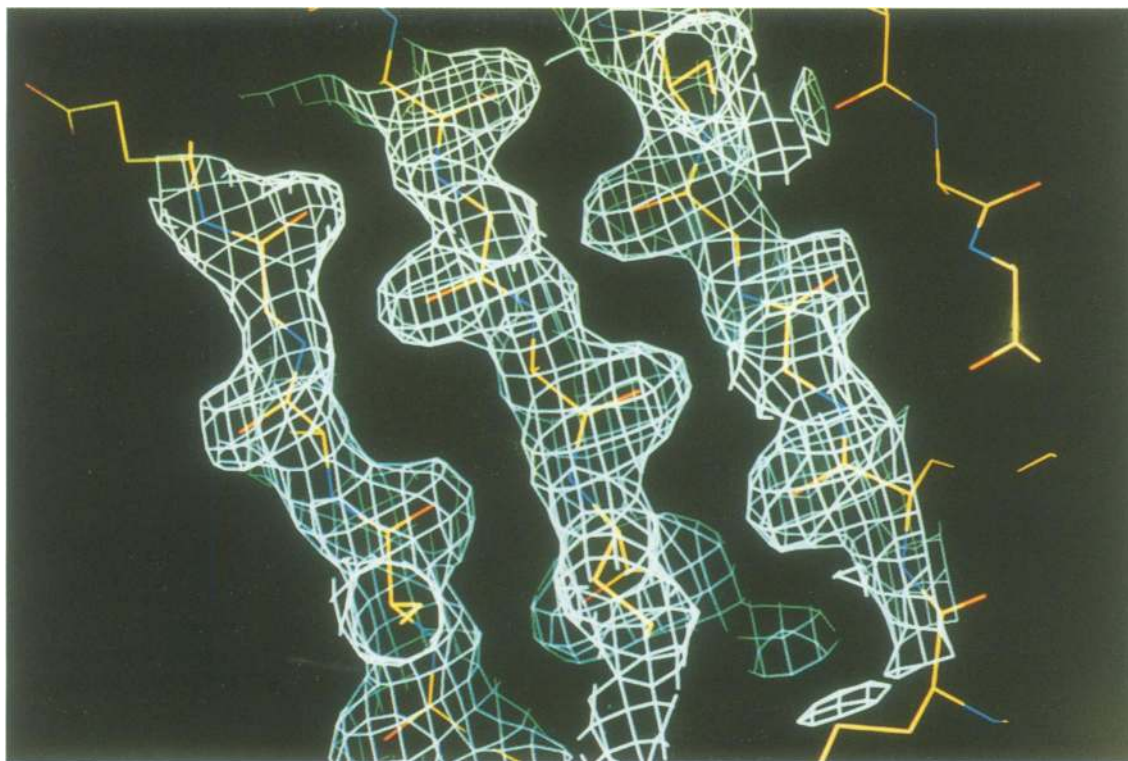


Fig. 3. Strands 2, 3 and 1 of the β -sheet's solvent flattened MIR electron density with polyaniline built into it. The carbonyl oxygens are readily observed.

sequence of S6 from *Thermus thermophilus* presented in this study consists of only 101 amino acid residues and lacks the trailing glutamic acids whereby it becomes almost neutral, $pI = 6.6$.

Even though ES6 alone cannot bind to the 16S RNA, S6 and S18 in *E.coli* form a complex protecting a portion of the 16S RNA (Held *et al.*, 1974; Prakash and Aune, 1978). S6 from *T.thermophilus* displays unspecific RNA affinity (S.E.Sedelnikova, unpublished data). S6 might, as a part of the complex with S18, have a specific interaction with the 16S RNA. It has been expected that ribosomal proteins have a common origin, but sequence alignment studies have given poor support for this hypothesis (Jue *et al.*, 1980; Wittman-Liebold *et al.*, 1984). However, the similarity in tertiary structure between L30 and the C-terminus of L7/L12 has been pointed out as support for this proposal (Leijonmark *et al.*, 1988). In the present article, the structure of S6 from *T.thermophilus* is described and found to be similar to several other ribosomal and nucleic acid binding proteins, lending further support for this hypothesis.

Results and discussion

Primary structure

The primary structure determination of protein S6 from *T.thermophilus* is depicted in Figure 1. It has 101 amino acid residues and a calculated molecular weight of 11 973. The N-terminus is not blocked. Since the C-terminal Ala could hardly be seen with Edman sequencing, an independent confirmation was necessary. To that end, the C-terminal BrCN-generated peptide was analyzed also by plasma desorption mass spectrometry. A mass of 1304.1 (MH⁺) was found, corresponding to the predicted mass of

VVKSQEPFLANA (1302.5). The protein lacks Cys, His and Thr, whereas the four amino acids Arg, Glu, Leu and Val together constitute nearly half of the polypeptide chain. A comparison with protein S6 from *E.coli* reveals only a weak, but nevertheless clear, homology with 27% sequence identity (Figure 2). The *E.coli* gene sequence has 30 residues at the C-terminus which are lacking in *T.thermophilus*. Furthermore, TS6 does not contain the stretch of post-translationally added glutamic acid residues in the C-terminal.

Electron density map and quality of the model

The structure was solved by multiple isomorphous replacement (MIR). The initial MIR map was of high quality (Figure 3) and the polypeptide chain was readily traced through the electron density map except for the four C-terminal residues. The refined structure has a crystallographic *R*-factor of 18.3% and shows good stereochemistry (see Materials and methods).

Overall structure

The molecule consists of a single domain with an anti-parallel β -sheet with two α -helices packed on one side, leaving the opposite side of the sheet exposed. The connectivity scheme is $\beta_1-\alpha_1-\beta_2-\beta_3-\alpha_2-\beta_4$. The folding pattern gives the molecule a pseudo 2-fold axis between the central β -strands perpendicular to the β -sheet which superimposes the secondary structure elements. The most conserved region between the *E.coli* and *T.thermophilus* proteins (residues 48–54) occurs in the extended loop region between β -strands 2 and 3 forming a 'hook' which partly folds over the β -sheet, thereby giving it a concave nature (Figure 4).

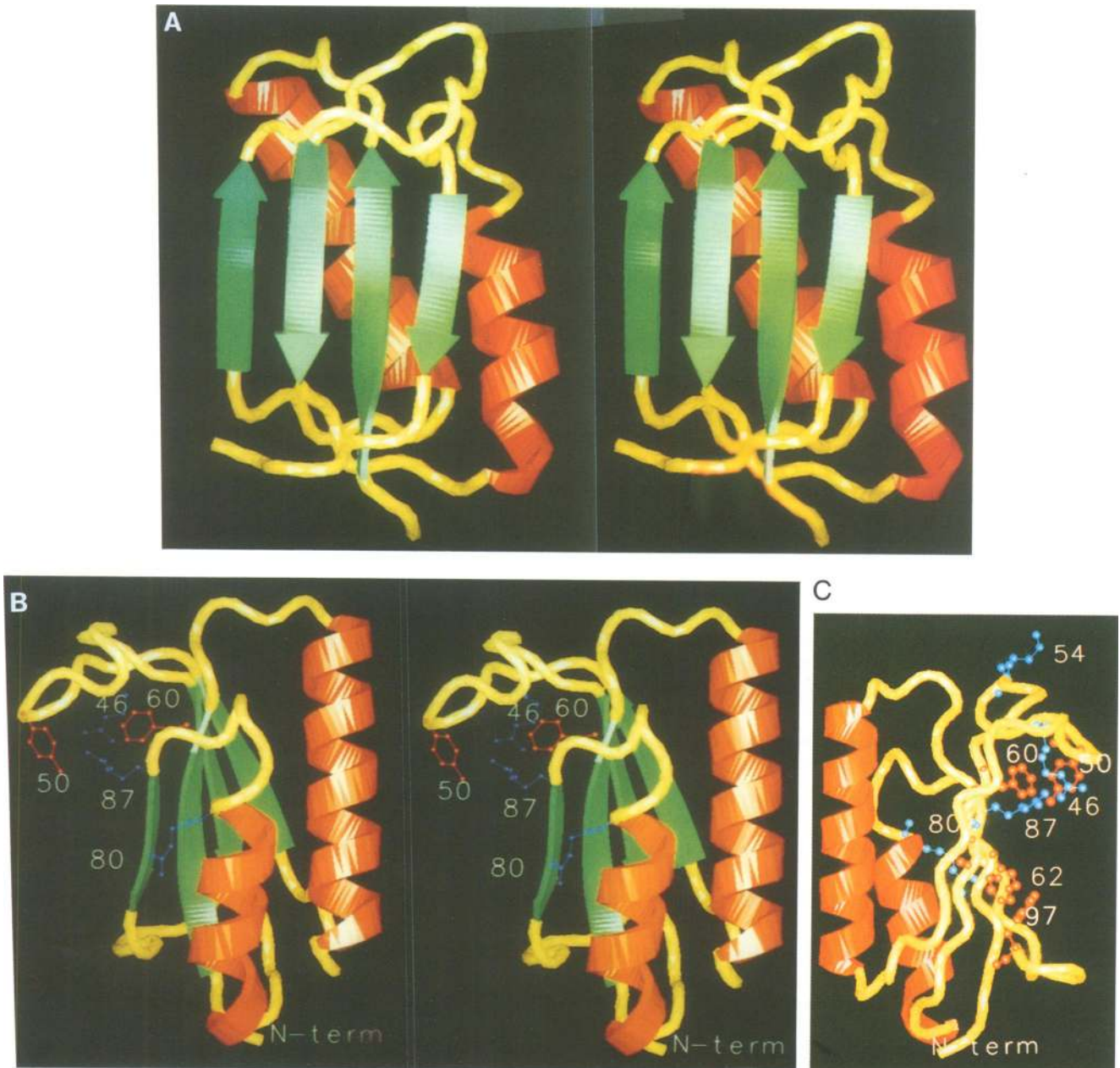


Fig. 4. (A) Stereo picture showing the S6 structure in ribbon style. The N-terminus is to the lower right. (B) Stereo picture showing the concave surface to the left with the residues Arg46, Tyr50, Phe60, Arg80 and Arg87 displayed. These are the conserved residues suggested to participate in RNA interaction. (C) The concave surface with aromatic and basic residues Arg46, Tyr50, Lys54, Phe60, Trp62, Arg80, Arg87 and Phe97.

The C-terminal tail lies over the opposite edge of the β -sheet and further accentuates the concave surface.

The inner β -strands ($\beta 1$ and $\beta 3$) are mainly hydrophobic while the rest of the molecule possesses a large number of charged and polar residues. Interesting features of the S6 structure are the highly exposed, partly conserved aromatic residues Phe60 and Trp62 on $\beta 3$, as well as Tyr50 in the extended loop bridging $\beta 2$ and $\beta 3$. Together with the basic residues Lys54, Arg80 and Arg87 (Figure 4C), they could form a binding site for rRNA explaining the rRNA protection mentioned above.

Folding patterns similar to S6 have been observed in other ribosomal proteins [L7/L12 (Leijonmarck *et al.*, 1980), L30 (Wilson *et al.*, 1986), and L6 and L9 (Golden *et al.*, 1993; Hoffman *et al.*, 1994)]. As seen in Figure 5, L30 forms a

basic fold from which all the other proteins can be obtained by insertion or substitution of secondary structure elements. The S6 folding scheme is identical to the topologies of, among other proteins, the RNA binding domain of the U1A snRNP protein (Nagai *et al.*, 1990; Hoffman *et al.*, 1991) and the C-terminal domain of the E2 protein from papilloma virus (Hegde *et al.*, 1992) which binds DNA as well as the enzyme acyl phosphatase (Pastore *et al.*, 1992), ferredoxin (Adman *et al.*, 1973) and mer P, a mercury binding protein in the mercury reductase pathway (P.-O. Eriksson, personal communication). An analysis of α - β sandwiches is presented in Orengo and Thornton (1993). U1A binds to RNA by two regions, RNP1 and RNP2, (Jessen *et al.*, 1991) which are highly conserved in more than 20 RNA binding proteins (Mattaj, 1989). These regions map to the two central

β -strands and the loop between them, and include three exposed aromatic residues similar to that found for S6 (which, however, does not have the RNP conserved sequences). As S6 does not possess a hydrophobic surface suitable for protein-protein interaction and the concave surface of the β -sheet is involved in RNA interaction in other RNA binding proteins, the complex between S6 and S18 is more likely to be formed by elongation of the β -sheet on the β 2 side, as observed for several similar proteins (Gonau *et al.*, 1990; Nagai *et al.*, 1990; Dumas *et al.*, 1992). E2 forms a barrel-like dimer where the β -sheets face the inside of the barrel and the α -helices on the outside bind to the DNA. In S6 the helix residues are poorly conserved and no obvious potential rRNA interaction area is detected among them. Therefore we suggest this binding mode to be a less likely explanation for the possible rRNA interaction of S6.

The observed folding pattern is also similar to the fold of the palm domains (Figure 5) of p66 pol from HIV-1 reverse transcriptase (Kohlstaedt *et al.*, 1992) and the Klenow fragment from Pol I (Ollis *et al.*, 1985). For both these cases, residues important for the catalytic activity map to the loop region connecting the two central β -strands, as discussed above.

Given the wide distribution of the RNP1 and RNP2 signature sequences in RNA-interacting proteins and the structural similarities between DNA and RNA polymerases (Sousa *et al.*, 1993), it is likely that the described folding pattern is common among DNA and RNA binding proteins. As many ribosomal proteins involved in RNA interactions share a related folding motif, the possibility of an ancestral ribosomal protein as a common origin cannot be excluded.

Materials and methods

Preparation of *T.thermophilus* S6

Thermus thermophilus (strain VK-1) cells were grown according to Gogia *et al.* (1986). Ribosome isolation was performed as described previously (Gogia *et al.*, 1986). The ribosomal subunits were separated by hydrophobic interaction chromatography and subsequently purified by centrifugation through a 30% (w/v) sucrose solution containing 1 M NH_4Cl , 50 mM Tris-HCl (pH 7.5 at 20°C), 50 mM MgCl_2 , 2 mM Na_2EDTA and 1 mM dithiothreitol for 10 h at 45 000 rev/min in a 50.2 Ti rotor. The pellets were resuspended and dissolved in buffer A containing 150 mM NH_4Cl , 50 mM Tris-HCl (pH 7.5 at 20°C), 50 mM MgCl_2 , 2 mM Na_2EDTA and 1 mM dithiothreitol. The stirred ribosomes were precipitated by the addition of powdered ammonium sulfate to a final concentration of 3 M. The pellets were stored at 4°C.

The purification procedure of protein S6 (TS9 according to the original nomenclature; Sedelnikova *et al.*, 1987) has been published elsewhere (Agalarov and Eliseikina, 1989). Briefly, core particles obtained by the treatment of 30S subunits with 3.5 M LiCl were subjected to 6 M LiCl to extract so-called core proteins. The solution containing these proteins was dialyzed against buffer B containing 50 mM Tris-HCl (pH 7.5 at 20°C), 1 mM dithiothreitol and 0.02% (w/v) NaN_3 . The dialyzed solution was loaded onto a 25–30 ml column of CM-Sephacryl CL-6B. As S6 is only slightly acidic it did not bind to this column material, in contrast to most other ribosomal proteins. The protein eluted in the unbound fraction with 85–90% purity. Further purification was obtained by ion exchange chromatography on a DEAE-Toyopearl 650S column (1.5 ml). When buffer B was used the protein did not bind to this column; it eluted with a purity of 95–98%. When buffer C containing 20 mM Tris-HCl (pH 9.0 at 20°C) was used with the same column, the protein was weakly retarded and was eluted with 40 ml of a linear gradient from 0 to 100 mM NaCl in the same buffer. The purity of the S6 thus obtained was at least 98%.

Preparation of peptides for sequence analysis

Digestions of the intact protein with trypsin [treated with 1-*p*-tosylamino-2-phenylethylchloromethyl ketone (Worthington)] or endoproteinase Glu-C (Miles) were carried out in 0.2 M *N*-ethylmorpholinium

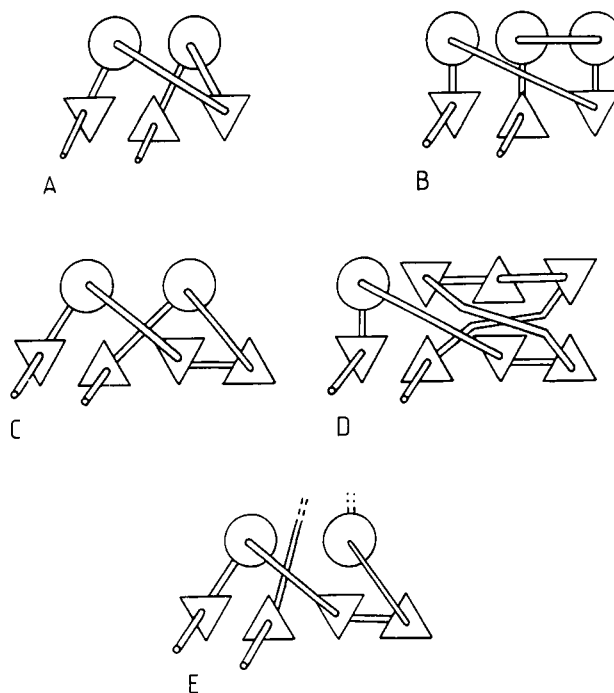


Fig. 5. Folding schemes of some related ribosomal and nucleic acid binding proteins: (A) L30; (B) C-terminal domain of L7/L12; (C) S6, U1A and E2; (D) C-terminal domain of L6; (E) palm domain of the Klenow fragment and p66 of HIV-1 reverse transcriptase. Folds (B–E) can be obtained by the insertion of secondary structure elements between the first helix and the second strand in (A). The ribosomal proteins S17 and S5 do not fit into this scheme.

acetate (pH 8.0) for 24 h at 37°C at a substrate:enzyme ratio of 30:50. Digestions with endoproteinase Lys-C (Boehringer) were performed in 0.2 M Tris-HCl (pH 8.6) for 20 h at 37°C at a substrate:enzyme ratio of 1000:1. Cleavage by BrCN (Villa *et al.*, 1989) was performed using ~100 μg of intact protein in 200 μl 6 M guanidinium chloride and 0.2 M HCl containing 6 mg BrCN. Prior to high-performance liquid chromatography, BrCN was removed by passing a stream of nitrogen over the surface of the solution. Subdigestions on BrCN and endoproteinase Lys-C-generated peptides were carried out with endoproteinase Glu-C in 100 μl 0.2 M Tris-HCl (pH 8.6) and with endoproteinase Asp-N (Boehringer) in 100 μl 0.2 M ammonium carbonate (pH 8.5) for 4–20 h at 37°C using 0.03–0.20 μg enzyme.

Peptides were usually separated on a Beckman Ultrasphere ODS-column (4 \times 250 mm) with 1-h gradients of 0–80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid at a rate of 1.0 ml/min. Rechromatography of impure peptides was performed under the same conditions, but with 0.1% (w/v) ammonium acetate (pH 6.5) instead of trifluoroacetic acid. In some cases, peptide separations were also done on a Merck Lichrosphere 100 RP18e column (4 \times 100 mm). When large peptides were expected (e.g. with BrCN digestion), a Vydac protein C4 column (4 \times 250 mm) was used.

All sequence determinations were performed on an Applied Biosystems Model 475A pulse liquid protein sequencer, on-line equipped with a Model 120A pth amino acid analyzer. The molecular mass of the C-terminal BrCN-generated peptide was determined with a BioIon plasma desorption plasma mass spectrometer.

Crystallization

The procedure used for crystallization was as described by Sedelnikova *et al.* (1991). The hanging drop technique was used with 10 μl drops of the protein solution equilibrated against 500 μl of a precipitant solution. Drops containing 8–15 mg protein/ml, 0.05 M Tris-HCl (pH 7.5) and 0.1–0.2 M KF were equilibrated against 25% (v/v) methanepentandiol in 0.4 M NaCl. Crystals shaped like rhombic prisms, with dimensions up to 0.4 \times 0.4 \times 0.2 mm, were obtained within 40 h at room temperature.

Data collection and solution structure

X-ray diffraction data were collected on a Siemens area detector equipped with a three-circle goniostat mounted on a Rigaku RU200BEH rotating

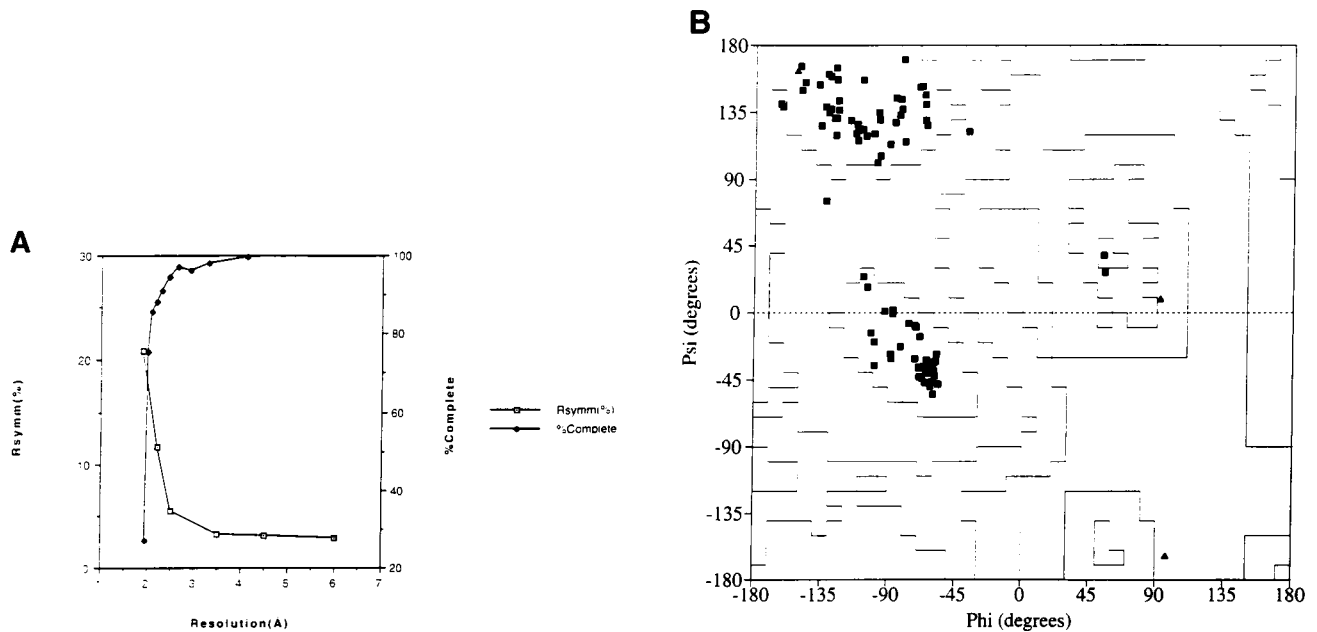


Fig. 6. (A) Completeness and R_s in resolution shells as a function of resolution for the native data set. $R_s = \frac{\sum \Sigma |I - \langle I \rangle|}{\langle I \rangle}$. One crystal was used and 7529 independent reflections (92% at 2.0 Å) were obtained. (B) Ramachandran plot of the S6 structure. The allowed regions are shown in the figure. Glycines are marked as triangles. The plot is produced by the program PROCHECK (Laskowski *et al.*, 1993).

anode. Reflexions were collected at $2\theta = 20^\circ$ and a frame width of 0.25° . The space group is C222 with $a = 106.81$, $b = 52.72$ and $c = 41.12$ Å, the resolution limit being 1.95 Å. Data processing was performed by XDS (Kabsch, 1988a,b). R_s and completeness as a function of resolution are summarized in Figure 6A. Phasing was performed by the MIR method using three similar mercury derivatives (Table I). The MIR phasing was useful to 2.1 Å. All calculations were performed by CCP4 (1979), the program MLPHARE (Otwinowski, 1991) being used for heavy atom refinement and phase calculation. Phase improvement by solvent flattening, histogram matching and adaptation of Sayre's equation to phase invariants was adapted by the program SQUASH (Zhang, 1993). MIR statistics are summarized in Table I. The enantiomorph ambiguity was resolved by incorporating anomalous differences from derivative (a) (see Table I). The enantiomorphs could also be clearly distinguished by the pitch of the α -helices.

The structure was built with the program O (Jones *et al.*, 1991). The entire sequence, except the C-terminal residues 97–101, could be built into the electron density map. Refinement was performed by XPLOR (Brünger, 1992). After initial simulated annealing and subsequent steps of conventional refinement and manual intervention using an Evans and Sutherland graphical workstation, a final model with a crystallographic R -factor of 18.3% [$(F > 1 \sigma, 8.00-1.95 \text{ \AA})$ and r.m.s. bond length deviations of 0.017 Å, bond angle r.m.s. of 3.2 Å, dihedral angle r.m.s. of 24.8° and improper dihedral angle r.m.s. of 1.85°] was obtained. Forty molecules of water have been inserted by inspection of difference electron density maps. The Ramachandran plot is shown in Figure 5B.

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Table I. Figure of merit after joint refinement by MLPHARE (Otwinowski, 1991): 0.71 (centric reflections), 0.50 (acentric reflections)

Measurement	Derivatives		
	EtHgCl (a)	EtHgCl (b)	PCMB (c)
d/Å	2.0	3.3	3.3
No. of observations	31 290	10 699	9837
No. of unique			
Reflections	7659	1783	1838
Completeness (%)	94	90	93
R_s (%) ^a	9.2	5.1	5.1
No. of sites	3	3	2
Phasing power ^b	1.7	2.7	1.2
R_{cullis} ^c	0.67	0.45	0.76

Anomalous differences were incorporated in the refinement for derivative (a). Derivatives (a) and (b) were prepared by soaking the crystals in saturated solutions (< 10 mM) of EtHgCl and precipitant for 72 (a) and 40 (b) h. Derivative (c) was prepared by soaking crystals in 1.0 mM PCMB and precipitant for 40 h.

^a $R_s = \frac{\sum \Sigma |I - \langle I \rangle|}{\langle I \rangle}$.

^bPhasing power = r.m.s. $\langle F_H \rangle / E$, where $\langle F_H \rangle$ = heavy atom structure factor amplitude and E = residual lack of closure.

^c $R_{\text{cullis}} = \frac{\sum ||F_{\text{PH}} \pm F_{\text{P}} - F_{\text{H(cal)}}||}{\sum |F_{\text{PH}} - F_{\text{P}}|}$ for centric reflections.

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