Crystal structure of the transcription factor sc-mtTFB offers insights into mitochondrial transcription

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Abstract

Although it is commonly accepted that binding of mitochondrial transcription factor sc-mtTFB to the mitochondrial RNA polymerase is required for specific transcription initiation in *Saccharomyces cerevisiae*, its precise role has remained undefined. In the present work, the crystal structure of sc-mtTFB has been determined to 2.6 Å resolution. The protein consists of two domains, an N-terminal α/β -domain and a smaller domain made up of four α -helices. Contrary to previous predictions, sc-mtTFB does not resemble *Escherichia coli* σ -factors but rather is structurally homologous to rRNA methyltransferase ErmC'. This suggests that sc-mtTFB functions as an RNA-binding protein, an observation standing in contradiction to the existing model, which proposed a direct interaction of sc-mtTFB with the mitochondrial DNA promoter. Based on the structure, we propose that the promoter specificity region is located on the mitochondrial RNA polymerase and that binding of sc-mtTFB indirectly mediates interaction of the core enzyme with the promoter site.

Keywords: Transcription factor; mitochondrial RNA polymerase; mtf1; mtTFB; mitochondrial transcription

Eucaryotic organisms possess a distinct small mitochondrial chromosome. In *Saccharomyces cerevisiae*, this chromosome is circular and ~80 kb in size and primarily encodes for two ribosomal RNAs, a number of tRNAs, and some of the proteins that function in the cell's respiratory and oxidative phosphorylation pathways. Transcription of the mitochondrial genome is performed by an enzyme that is structurally and functionally distinct from the nuclear RNA polymerase (RNAP). The genes of the mitochondrial RNAPs of humans, *S. cerevisiae*, *Xenopus laevis*, and a number of plants have been identified. The encoded proteins display a large degree of sequence conservation particularly in their C-terminal halves (Masters et al. 1987; Bogenhagen and Insdorf 1988; Tiranti et al. 1997; Weihe et al. 1997). All of these enzymes belong to a family of bacteriophage and

bacteriophage-like RNAPs, the best-characterized member of which is T7 RNAP. The crystal structures of T7 RNAP as well as its initiation and elongation complexes have been reported (Sousa et al. 1993; Cheetham and Steitz 1999; Chen 1999) and provide valuable insight into the workings of the enzyme. Although the mitochondrial and bacteriophage RNAPs are evolutionary related, they function differently. Unlike mitochondrial RNAPs, which recognize their promoter and initiate transcription only in the presence of one or more transcription factors (Schinkel et al. 1987), the bacteriophage RNAPs do not require auxiliary factors for specific transcription.

The mitochondrial RNAP of *S. cerevisiae* is currently the best-characterized mitochondrial RNAP. The functional enzyme consists of two proteins: a 153-kD core enzyme related to bacteriophage RNAPs and a single 39.5-kD transcription factor named sc-mtTFB (Schinkel et al. 1987). Both proteins are nuclear encoded, synthesized in the cytoplasm, and imported into the mitochondrion via N-terminal targeting sequences (Kelly et al. 1986; Lisowsky and Mi-

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chaelis 1988). In vitro transcription assays have shown that the presence of sc-mtTFB is necessary and sufficient for the core enzyme to perform specific transcription (Jang and Jaehning 1991; Mangus et al. 1994; Carrodegua et al. 1996). Disruption of the gene for either of these proteins results in the loss of mitochondrial DNA and a petite phenotype (Greenleaf et al. 1986; Shadel and Clayton 1995), underlining their importance for the organism.

It has been suggested that the mitochondrial RNAP of *S. cerevisiae* functions mechanistically in a manner similar to *Escherichia coli* RNAP (Mangus et al. 1994), which requires σ -factors, most notably σ^{70} , for promoter recognition and transcription initiation. These σ -factors have been shown to interact with the promoter site, to assist in promoter melting, and to give stability to the holoenzyme (Daniels et al. 1990; Juang and Helmann 1994). The *E. coli* transcription factors are released from the core enzyme after synthesis of a short nascent RNA chain, and the transcription reaction is completed in their absence (Mishra and Chatterji 1993). σ -Factor proteins vary widely in size, but sequence comparison among family members revealed four conserved regions crucial for protein function (Dombroski 1997). Interestingly, sc-mtTFB has been reported to display some amino acid sequence similarity with conserved region 2, which is believed to be involved in promoter recognition and melting, and region 3 of the bacterial σ -factors (Jang and Jaehning 1991). Based on these sequence similarities, it has been suggested that sc-mtTFB is a σ -factor-like protein, which, once associated with the core enzyme sc-mtRNAP, guides the mitochondrial RNAP-mtTFB complex to the promoter site, where it also participates in transcription initiation. Gel mobility-shift assay studies of various arrested transcription complexes lent further support to this model (Mangus et al. 1994), as they showed that sc-mtTFB indeed binds to the core enzyme before promoter binding and that sc-mtTFB is released from the sc-mtRNAP-DNA-RNA complex once a short nascent RNA strand has been synthesized. However, site-directed mutagenesis experiments have recently shown that a motif in conserved region 2, which is crucial for promoter recognition in σ^{70} , is nonessential to the function of sc-mtTFB, suggesting that sc-mtTFB is functionally and possibly structurally distinct from σ -factors (Shadel and Clayton 1995).

Before the current work, it was not possible to directly address this question because the structures of sc-mtRNAP and sc-mtTFB were not available. Herein, we report the crystal structure of sc-mtTFB determined at 2.6 Å resolution. This structure now provides insight into the mechanism of mitochondrial transcription initiation and clearly shows that sc-mtTFB is not a σ -factor-like protein. Furthermore, the structural characteristics of sc-mtTFB provide information that allows a redefinition of the role sc-mtTFB plays during the initiation stage of mitochondrial transcription.

Results and Discussion

Crystal structure

The structure of the mitochondrial transcription factor sc-mtTFB has been determined and refined (R value of 0.187) against X-ray diffraction data to 2.6 Å resolution (see Table 1a). The final model consists of residues 4–324 of the mature protein. The N-terminal His-tag, terminal residues 1–3, and amino acids 325–341 are not visible in the electron density map and are presumed to be disordered. The geometry of the final model has 84% of the residues lying in the most favorable regions of the Ramachandran Plot (Ramakrishnan and Ramachandran 1965), with only glycine residues falling into the disallowed regions.

A Ribbon representation of the backbone structure and a protein topology plot for sc-mtTFB are given in Figure 1. Sc-mtTFB consists of two domains, with a large cleft present at the domain interface. The larger N-terminal domain forms an extended α/β -structure and is centered around an eight-stranded, mixed sheet reminiscent of the Rossmann-fold often encountered in mono- and di-nucleotide-binding domains. The smaller C-terminal domain is composed entirely of four helices.

Comparison with *E. coli* σ -factor σ^{70}

Although the reported amino acid sequence similarities between sc-mtTFB and regions 2 and 3 of the bacterial σ -factors (Jang and Jaehning 1991) suggested a relationship between the two proteins, recent mutagenesis and deletion experiments have implied that sc-mtTFB may be functionally distinct from σ -factors (Shadel and Clayton 1995). The sc-mtTFB structure reveals that the proposed homology regions between a σ^{70} (Malhotra et al. 1996) and sc-mtTFB have no structural similarity (Fig. 2). Thus, the suggestion that sc-mtTFB functions as a σ -factor-like protein during initiation of mitochondrial transcription and is directly involved in promoter recognition and binding is not supported by our structural data. In view of the close sequence homology between the core enzyme sc-mtRNAP and T7 RNA polymerase, it appears more likely that, as in the case for T7 RNAP, sc-mtRNAP itself recognizes and binds its promoter, whereas sc-mtTFB merely indirectly facilitates this process. This alternative model is further corroborated by the unexpected structural similarity of sc-mtTFB with a different family of proteins.

Sc-mtTFB is related to a family of RNA methyltransferases

Because sc-mtTFB is not related to bacterial σ -factors, it was of interest to identify other possible structural homologs of sc-mtTFB that may help define the functional

Table 1. X-ray crystallography data statistics

Crystal	Native	Xe_1	Xe_2	Xe_2'
a. Data Collection Statistics				
X-ray source	RU-200	RU-200	RU-200	19ID APS
Wavelength (Å)	1.54	1.54	1.54	1.00
Resolution (Å)	2.7	3.2	2.7	2.6
Rotation range (°)	172	300	630	279
Total reflections	112,043	121,545	410,271	202,625
Unique reflections	10,182	6,214	10,390	10,896
Completeness (%)	96.6 [87.8]	95.3 [86]	98 [88.7]	90.8 [63.4]
[last shell]				
I/σ [last shell]	14.0 [4.7]	11.7 [4.5]	17.8 [4.6]	19.5 [4.6]
R _{sym} [last shell]	0.071 [0.20]	0.12 [0.32]	0.11 [0.34]	0.06 [0.17]
b. Phasing Statistics				
Number of Xe sites	—	2	3	
Overall figure of merit	0.58			
(up to 3.2 Å)				
c. Refinement Statistics				
R value (σF cutoff 0.0)		0.187		
Free R value		0.274		
Free R value test set		558 reflections		
RMS deviations from ideality				
Bond lengths (Å)		0.007		
Bond angles		1.4°		
Dihedral angles		22.8°		
Improper angles		0.70°		
Wilson B value (Å ²)		7.7		
Mean B value (Å ²)		43.3		
Coordinate error (Å) ^a		0.28		
Resolution range (Å)		20.0–2.6		
Data cutoff		0.0 σF		
Number of reflections		10896 reflections		
Completeness for range		90.8%		
Number of protein atoms		2677		
Number of solvent atoms		21		

^a Estimated coordinate error from the Luzzati (Luzzati 1952) plot.

role of the protein. A distance matrix alignment (DALI) (Holm and Sander 1995) search of the Protein Data Bank (PDB; Abola et al. 1987) determined that sc-mtTFB is a member of a large group of DNA/RNA methyltransferases. All enzymes of this family share an α/β-domain, with an extended mixed sheet at its center that contains both the catalytic domain and an S-adenosyl-L-methionine (SAM)-binding site. In these enzymes, SAM serves as the donor of the methyl group that is transferred to the target DNA or RNA molecule.

The structure of the α/β-domain is conserved throughout the super family of SAM-dependent methyltransferases; however, the RNA/DNA-binding sites differ markedly. Notably, the C-terminal helix motif observed in sc-mtTFB so far has only been found in ErmC', a 28.9-kD rRNA methyltransferase and member of the Erm family of enzymes. ErmC' from *Bacillus subtilis* confers erythromycin resistance to the organism by catalyzing the di-methylation of N6 of an unpaired adenine base in 23S rRNA (Bechhofer and Zen 1989; Denoya and Dubnau 1987). In ErmC', the

C-terminal domain is thought to constitute a large part of the RNA-binding site (Bussiére et al. 1998). This observation suggests that sc-mtTFB, as ErmC', is an RNA-binding rather than a DNA-binding protein. A superposition (main-chain atoms) of sc-mtTFB and ErmC' structures is shown in Figure 3. Although there is only 15% sequence identity and 25% sequence similarity between the two proteins (Fig. 4), their structural agreement is extensive, with only three regions in the sc-mtTFB structure, for which there is no counterpart in ErmC'. The root mean square deviation for the main-chain atoms between the two structures is 3.1 Å for the 218 structurally similar residues.

Putative SAM-binding site

Identification of sc-mtTFB as a member of a family of SAM-dependent enzymes raises the question of the role sc-mtTFB plays in mitochondrial transcription. The fact that sc-mtTFB is able to mediate transcription in vitro in the absence of added SAM shows that sc-mtTFB does not require SAM to bind the core enzyme or to facilitate mito-

Fig. 1. (a) Ribbon drawing of the sc-mtTFB backbone structure showing the α/β domain and the second domain comprising helices $\alpha 10$ to $\alpha 13$. The image was generated with MOLSCRIPT (Esnouf 1997). (b) Topology plot of sc-mtTFB with the helices displayed as blue cylinders and the strands as pink arrows. A dashed line marks the domain interface. (c) Stereo view of the $2F_o - F_c$ map at 2.6 Å resolution, using phases calculated from the final refined model and contoured at 1σ .

chondrial transcription. However, earlier binding studies of ErmC' have revealed that its RNA-binding affinity is unaffected by the presence or absence of SAM (Su and Dubnau 1990). This could explain the ability of sc-mtTFB to initiate transcription without SAM being present and could still allow for the possibility that sc-mtTFB, in fact, binds SAM and methylates the nascent RNA in vivo. Another possibility is that sc-mtTFB has adapted to its role in mitochondrial transcription and merely serves as a specificity factor without enzymatic activity of its own.

SAM-dependent methyltransferases reportedly share nine sequence motifs that are conserved to variant degrees. Residues in motifs I–IV are involved in binding with SAM. Motif I is usually characterized by a G-X-G motif that is preceded by four residues toward the N terminus by a glutamate/aspartate. The aspartate and the two glycines (G56 and G58) are conserved in sc-mtTFB. The second glycine is not present in the other two mtTFBs (Fig. 4). In the ErmC'-SAM complex structure, the carbonyl group of the first glycine hydrogen bonds with the methionine of SAM,

Fig. 2. Comparison of the structures of sc-mtTFB and *Escherichia coli* transcription factor σ^{70} . Shown are supposed homology regions 2.1 through 2.4. As apparent from the image, there is no structural homology between those regions, particularly region 2.4, which has been shown to be involved in promoter recognition in σ^{70} . The drawing was created with MOLSCRIPT.

whereas the second interacts with the ligand hydrophobically. The second motif usually contains a conserved glutamate residue, E59 in ErmC', that forms hydrogen bonds with the ribose moiety of SAM. Usually this glutamate is followed by a hydrophobic amino acid that also interacts with the ligand. Although the glutamate is conserved in the mtTFBs, the hydrophobic residue is not; instead, all three proteins feature charged or polar residues in its place. The critical residue within motif III is D84 in ErmC' (D101 in sc-mtTFB) which is hydrogen-bonded to the amino group of the adenine section of the ligand (Fig. 5). Conserved motif IV is of particular interest because it contains not only residues involved in SAM binding but also suspected catalytic residues with a proposed (G/N/S)IP(Y/F) fingerprint sequence for rRNA Mtases (Malone et al. 1995). N101 forms a hydrogen bond with a carboxy oxygen atom of the methionine moiety in ErmC' and is conserved among the mtTFBs (N137 in sc-mtTFB). Not conserved is the proposed fingerprint motif. Motifs V–VIII are only poorly conserved, but this was already observed for ErmC' compared with DNA methyltransferases and suggested that these motifs may not be present in rRNA methyltransferases. In summary, although there is little overall sequence homology between sc-mtTFB and ErmC', three residues (Glu 59, Asp 84, and Asn 101) that form hydrogen bonds with SAM in the ErmC'-SAM complex are conserved in sc-mtTFB (Glu 77, Asp 101, and Asn 137). Furthermore, there is extensive structural agreement between the sc-mtTFB and ErmC' around the SAM-binding site. Therefore binding of SAM by sc-mtTFB seems possible.

RNA-binding site

Like ErmC', sc-mtTFB has a deep cleft located at the domain interface and along one site of the α/β -domain of the

protein, which possesses an extensive region with positive electrostatic potential (Fig. 6). In ErmC', this area, which is apparently unique to the enzymes constituting the Erm family, has been proposed to constitute the RNA-binding site (Bussire et al. 1998). The lysine and arginine residues that form the proposed RNA-binding pocket are not precisely conserved between ErmC' and sc-mtTFB. Instead, some of these amino acids are displaced within the sequence by a residue in one direction or the other (Fig. 4). However, most of these residues are located in structurally flexible regions. Preliminary binding studies in our laboratory support the predicted RNA-binding properties of sc-mtTFB (F. Schubot, unpubl.). The conservation of the structural and electrostatic features in the RNA-binding site in sc-mtTFB is a further indication for its close evolutionary relationship with ErmC', and the promoter specificity of the sc-mtTFB/sc-mtRNAP system is most likely located on the core enzyme sc-mtRNAP itself as suggested during the discussion of the structural comparison between sc-mtTFB and σ^{70} .

Proposed new mechanism of initiation of mitochondrial transcription

Based on the discovery that sc-mtTFB was functionally distinct from σ^{70} and citing the sequence homology of the mitochondrial core polymerase with T7 RNA polymerase, it was hypothesized that the core enzyme itself, rather than sc-mtTFB, might be responsible for the promoter recognition (Shadel and Clayton 1995). The extensive structural homology of sc-mtTFBs with ErmC' supports this alternative model. An analysis of the sc-mtRNAP/T7 RNAP homology regions in the structure of the T7 RNAP promoter

Fig. 3. Stereo plot of the α -carbon backbone superposition of the sc-mtTFB (blue) and ErmC' (red) structures. Both proteins have a similar domain organization and overall structure (root mean square derivative main-chain of 3.1 Å). There are two main regions in sc-mtTFB that have no counterpart in ErmC'. The possible significance of one of those regions is discussed in the text. The drawing was created with MOLSCRIPT.

Fig. 4. ESPrift (Gouet et al. 1999) structure-based sequence alignment of sc-mtTFB with ErmC' and Erm-am (Yu et al. 1997). Also shown are the sequences of two other mitochondrial transcription factors identified from *Saccharomyces kluyveri* and *Kluyveromyces lactis*. Helices are denoted by coils, and strands are denoted by arrows. Residues highlighted in red denote identical residues, and red letters represent conserved residues in four of the five sequences. Labelled below the alignment are the eight conserved sequence motifs that are present in ErmC' (Bussiere et al. 1998) out of the 10 motifs that characterize methyltransferases. Residues constituting motifs I-IV are usually involved in S-adenosyl-L-methionine binding.

complex (Cheetham et al. 1999) provides additional evidence that sc-mtRNAP may directly interact with the promoter. Particularly striking are the positions of conserved

regions VII and VIII that define the N and C termini, respectively, of the specificity loop, which is crucial for promoter recognition (Rong et al. 1998). Apparently, regions

Fig. 5. Stereo view of the *S*-adenosyl-L-methionine (SAM)-binding site in ErmC' (red) together with the superimposed backbone of sc-mtTFB (blue). Also shown are the three residues E59, D84, and D101 in ErmC' that form hydrogen bonds with SAM and are part of conserved motifs II, III, and IV, respectively, and the corresponding residues in sc-mtTFB.

VII and VIII form the scaffolding to position this loop at the core of the catalytic site (Fig. 7). The exposed residues of the loop region are not conserved, but that is not unexpected because the promoter sequences of the two systems are not identical (Masters et al. 1987).

If sc-mtTFB does not provide promoter specificity to the mitochondrial RNA polymerase as previously proposed, what is its function? Possibly, the association of sc-mtTFB

with sc-mtRNAP before promoter binding induces a conformational change that allows the core enzyme to recognize and bind the promoter. In addition, the RNA-binding properties of sc-mtTFB make it tempting to propose an interaction with the nascent RNA chain. This interaction could stabilize the early transcription complex and, as the RNA chain grows, could play a role in the dissociation process of sc-mtTFB from the transcription complex. The question remains as to whether sc-mtTFB is a functional RNA methyltransferase capable of performing RNA methylation or merely serves as a transcription factor.

Fig. 6. Electrostatic surface potential plot of sc-mtTFB generated with GRASP (Nicholls 1992). Regions with positive potential are colored blue, and regions of negative potential are red. This surface potential drawing mirrors that of ErmC'. Also displayed are the residues that form an extensive region of positive potential located mostly at the domain boundary. This area is proposed to be the RNA-binding site. The position of the potential *S*-adenosyl-L-methionine (SAM)-binding motif is also shown.

Binding of Sc-mtTFB to the core RNA polymerase, Sc-mtRNAP

From the current data, it is proposed that the main function of sc-mtTFB during transcription initiation is to interact with the core polymerase, thus permitting promoter recognition and binding by sc-mtRNAP. Although structural data were unavailable, some attempts have been made at characterizing the sc-mtTFB/sc-mtRNAP interface. Studies using a two-hybrid fusion protein approach characterized a number of truncation and point mutations that led to a disruption of the subunit interactions (Cliften et al. 1997). Each of the investigated sc-mtTFB truncation mutants had lost the ability to interact with sc-mtRNAP, implying the presence of an extensive protein/protein interface. Using the structure of sc-mtTFB, the different mutations can now be evaluated based on their specific positions within the protein. The disruptive point mutations, which were grouped

into three clusters (A, B, C), are distributed on different faces of the sc-mtTFB structure. Several are located at the interior of the protein, suggesting that these mutations had an impact on the structural integrity of sc-mtTFB rather than directly affecting core enzyme binding. However, cluster C mutations S218R, I221K, and D225G are all located on the segment constituting helices $\alpha 8$ and $\alpha 9$ (Fig. 1a). This loop-like region, which extends away from the remainder of the protein, is not part of the adjacent α/β -domain. Therefore mutations in its residues are not likely to affect the overall folding of sc-mtTFB. Interestingly, this loop containing the cluster C residues is completely missing in ErmC', although there is otherwise excellent overlap between the two proteins in this region (Fig. 8). Thus, comparison to the structure of ErmC' supports the hypothesis that helices $\alpha 8$ and $\alpha 9$ of sc-mtTFB constitute a crucial part of the sc-mtRNAP/sc-mtTFB interface.

In summary, the crystal structure of the mitochondrial transcription factor sc-mtTFB identified the protein as being similar to the RNA-methyltransferases of the Erm family of enzymes and not, as thought previously, similar to bacterial σ -factors. This finding, together with the striking sequence homology of the core enzyme sc-mtRNAP with bacteriophage RNA polymerases, suggests that the promoter-binding region is part of the latter structure rather than sc-mtTFB. Furthermore, residues involved in the interactions between transcription factor and RNA polymerase were

Fig. 7. MIDASPLUS drawing (Ferrin et al. 1988) of the T7 RNA polymerase-DNA promoter complex (Protein Data Base entry 1CEZ). Shown are homology regions VII (red) and VIII (green), with the sc-mtRNAP and the specificity loop (white) bound to the DNA promoter (cyan/yellow).

Fig. 8. MOLSCRIPT drawing of the extraneous region in sc-mtTFB consisting of helices $\alpha 8$ and $\alpha 9$ (blue; see Fig. 1a,b) with the backbone of ErmC' in the same region depicted in red. The three residues previously shown to be important for subunit interactions with the core polymerase are presented in form of balls and sticks.

identified through structural comparison of sc-mtTFB with ErmC'.

Materials and methods

Details of the cloning, expression, purification, and crystallization of sc-mtTFB are published elsewhere (Schubot et al. 2000). Initial X-ray diffraction data collection on the native and xenon (Xe)-derivative crystals was performed on a Rigaku R-Axis IV area detector at -170°C . The data were indexed, integrated, and scaled using the HKL program package (Otwinowski 1988). Of all the crystals surveyed, the Xe₂ crystal gave the best I/σ -values, and data were recollected on this crystal at 19ID (SBC-CAT) Advanced Photon Source, Argonne National Laboratory using 1.0 Å X-rays. This data set (Xe₂'), which showed good diffraction to 2.6 Å resolution, was used in the final refinement stages for the sc-mtTFB structure. The data collection parameters and processing results are summarized in Table 1a.

For phasing, sc-mtTFB crystals were derivatized under high-pressure Xe using the Molecular Structure Corporation CryoXe-Siter and were subsequently flash-cooled. The first crystal (Xe₁) was placed under 100 pounds per square inch Xe for 10 min, flash-cooled in liquid freon, and mounted in a nitrogen gas cold stream. The Xe₁ crystal diffracted to 3.2 Å, and two Xe sites were identified with SOLVE (Terwilliger and Berendzen 1999). A second derivative crystal (Xe₂) was exposed to 200 pounds per square inch Xe gas for 15 min, flash-cooled in liquid freon, and mounted in a nitrogen gas cold stream. The Xe₂ crystal diffracted to 2.7 Å resolution, and an additional Xe site (15% occupancy) was identified (SOLVE), giving a total of three sites for this derivative.

The SOLVE calculated phases (SIRAS) were then modified using the solvent flattening protocols of SOLOMON (Abrahams and Leslie 1996) and ISIR/SAS (Wang 1985). The resulting electron density maps were of comparable quality, both showing clear protein/solvent boundaries and recognizable features of secondary structure. The solvent flattened map allowed the fitting (Jones et al. 1991) of a polyalanine chain for 250 of the 353 residues. After several rounds of phase combination using SIGMAA (Collaborative Computational Project, Number 4 1994) and model building, the remaining residues and side-chains could be fitted into the

electron density map. The model was refined using simulated annealing (SA) protocol of X-PLOR (Brunger et al. 1989) followed by manual adjustment of the model using SA omit maps. The final refinement statistics are given in Table 1c. Coordinates have been submitted to the PDB (Abola et al. 1987) and stored under the PDB code 1I4W.

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