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Crystallization and preliminary structure determination of *Escherichia coli* Mfd, the transcription-repair coupling factor

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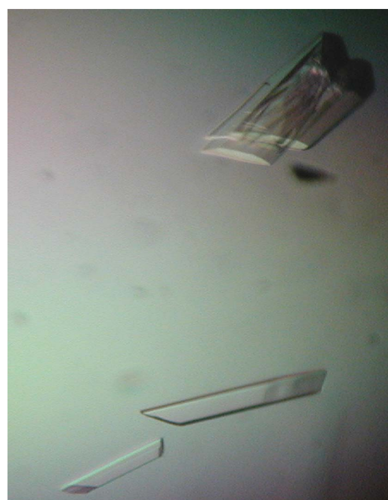
Transcription-repair coupling factors (TRCFs) are SF2 ATPases that couple transcription to DNA-damage repair by recognizing and removing RNA polymerase-elongation complexes stalled at DNA lesions and recruiting the nucleotide excision-repair machinery to the damaged sites. As a first step towards understanding the TRCF mechanism, the 130 kDa *Escherichia coli* TRCF (the product of the *mfd* gene) has been overexpressed, purified and crystallized using an unusual precipitant, pentaerythritol ethoxylate. Initial phases were obtained using single-wavelength anomalous dispersion with a highly redundant 4 Å resolution data set collected from selenomethionyl-substituted crystals and dramatically improved by density modification and phase extension to 3.2 Å resolution. Model building and refinement, which are in progress, will provide insight into transcription-coupled DNA-repair pathways, as this represents the first TRCF to be crystallized to date.



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1. Introduction

Transcription-repair coupling factor (TRCF, the product of the *mfd* gene) is a widely conserved bacterial protein that couples DNA repair with transcription (Selby & Sancar, 1993). TRCF recognizes RNA polymerase (RNAP) stalled at DNA lesions, disrupts the transcription complex to release the transcript and enzyme and recruits the nucleotide excision-repair machinery to the site. The mechanism of RNA release has been illuminated by the discovery that TRCF causes forward translocation of RNAP through an ATP-dependent motor that is homologous to that of the Holliday branch-migration protein RecG (Park *et al.*, 2002).

TRCF is a large (130 kDa) multi-functional protein with a complex structure–function relationship that is currently understood only from sequence analysis and genetic manipulation (Selby & Sancar, 1993, 1995*a*,*b*). A high-resolution crystal structure of TRCF would provide a firm structural basis to design more incisive experiments exploring the interactions between TRCF, the RNAP ternary elongation complex and the nucleotide excision-repair machinery. As a first step towards understanding the structural basis for transcription-coupled repair, *Escherichia coli* TRCF has been overexpressed, purified and crystallized. Initial crystallographic analysis has provided an excellent electron-density map at 3.2 Å resolution.

2. Materials and methods

2.1. Protein overexpression and purification

The *E. coli mfd* gene was amplified from plasmid pMFD19 and subcloned between the *Nhe*I/*Eco*RI sites into a pET28a-based vector as a hexahistidine-fusion protein to generate pAD6. Transformed Rosetta(DE3)pLysS cells were grown to an OD_{600} of ~0.6 in the presence of 50 µg ml^{-1} kanamycin at 310 K and expression was induced with 1 m*M* isopropyl β-D-thiogalactopyranoside. After induction for 4 h at 303 K, cells were harvested by centrifugation, resuspended in buffer IMAC-15 (50 m*M* Tris pH 8.0, 500 m*M* NaCl, 15 m*M* imidazole, 10% glycerol, 2 m*M* β-mercaptoethanol) supplemented with 1 m*M* PMSF and EDTA-free protease-inhibitor cocktail (Sigma) and lysed using a French press. For purification, the clarified lysate was loaded onto an Ni^{2+}-chelating HiTrap column (Amersham

Biosciences) and eluted using a 15–200 m*M* imidazole linear gradient. After overnight digestion of the His-tag with Prescission protease (Amersham Biosciences) and dialysis against buffer IMAC-15, a second subtractive Ni^{2+}-chelating chromatographic step was used to remove uncleaved protein and a tandem GST-affinity column (Amersham Biosciences) was used to remove the protease. The flowthrough was dialysed against low-salt buffer (20 m*M* Tris pH 8.0, 75 m*M* NaCl, 10% glycerol, 10 m*M* β-mercaptoethanol) supplemented with 5 m*M* EDTA to ensure removal of any residual bound nucleotide. The sample was further purified on a Heparin HiTrap column (Amersham Biosciences) using a linear gradient from 100 m*M* to 2 *M* NaCl and finally by size-exclusion chromatography on an SD200 column (Amersham Biosciences) in a buffer consisting of 20 m*M* Tris pH 8.0, 0.5 *M* NaCl and 10 m*M* DTT. For overproduction of selenomethionyl-substituted protein, the *E. coli* Met-auxotroph strain JB(DE3) (a gift from W. Hendrickson) was transformed with pAD6. Protein was overproduced and purified using the same protocol as for native TRCF. The final protein-storage buffer consisted of 20 m*M* Tris pH 8.0, 0.5 *M* NaCl and 20 m*M* DTT.

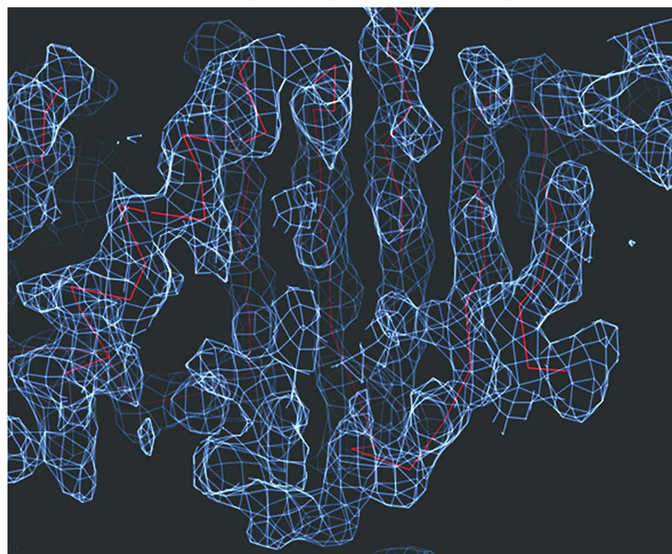
Table 1
Diffraction, data-collection and reduction statistics for TRCF selenomethionyl-substituted crystals.

Values in parentheses are for the highest resolution shell.

	SeMet1	SeMet2
Space group	*C*2	*C*2
Molecules per ASU	2	2
Unit-cell parameters (Å, °)	*a* = 151.7, *b* = 162.2, *c* = 162.1, β = 104.9	*a* = 151.9, *b* = 162.0, *c* = 161.7, β = 105.1
Wavelength (Å)	0.98166	0.97899
Resolution (Å)	40–4.0 (4.14–4.0)	40–3.2 (3.31–3.20)
Reflections measured	228334	300181
Unique reflections	62430	122388
Completeness (%)	99.6 (100)	99.9 (99.9)
Redundancy	3.7 (3.7)	2.5 (2.0)
$\langle I/\sigma(I)\rangle$	10.9 (2.9)	12.4 (2.5)
R_{sym}† (%)	12.9 (50.2)	0.08 (37.7)
SAD figure of merit	0.17	

† $R_{sym} = |I - \langle I\rangle|/\langle I\rangle$, where *I* is the observed intensity and $\langle I\rangle$ is the average intensity of multiple observations of symmetry-related reflections.

(*a*)



(*b*)

Figure 1
(*a*) Crystals of selenomethionyl-substituted TRCF. (*b*) Section of the 3.2 Å resolution electron-density map contoured at 1σ after solvent modification and phase extension using *SOLOMON* (Abrahams & Leslie, 1996). Preliminary model building is shown as a C^{α} trace in red.

2.2. Crystallization

Preliminary crystallization trials were conducted with Crystal Screens I and II, Natrix, PEG/Ion and Index Screens (Hampton Research), which resulted in the identification of a candidate condition in cocktail No. 57 of the Index Screen. After optimization, TRCF crystals suitable for structure determination were grown at 277 K using hanging-drop vapor diffusion against 1 ml 100 m*M* HEPES pH 7.5, 75 m*M* ammonium sulfate, 28% pentaerythritol ethoxylate with a protein concentration of 10 mg ml^{-1} and a 1:1 protein:crystallant ratio. Pentaerythritol ethoxylate, a branched polymer built on the pentaerythritol backbone, is a precipitating agent similar to the low-molecular-weight polyethylene glycols and is a relatively new addition to the battery of protein-crystallization reagents (Gulick *et al.*, 2002). With this precipitant, long clustered plates appeared after 4 d, grew to dimensions of 20 × 40 × 400 µm (Fig. 1*a*) and typically diffracted to ∼5 Å Bragg spacing on an in-house rotating-anode X-ray generator/area detector at cryogenic conditions. These crystals belonged to the monoclinic space group *C*2, had 68% solvent content and contained two molecules (2302 residues) per asymmetric unit. Standard derivatization methods with tantalum and tungsten heavy-metal clusters or with mercury compounds ($HgCl_2$, methylmercury, ethylmercury) destroyed crystal diffraction. Therefore, selenomethionyl-substituted protein was prepared and this yielded isomorphous crystals which were typically thicker and diffracted to higher resolution. Cryoprotection was achieved by transferring the crystals for a few seconds into 100 m*M* HEPES pH 7.5, 75 m*M* ammonium sulfate, 32% pentaerythritol ethoxylate supplemented with 7.5% glycerol and subsequent flash-cooling in liquid ethane.

3. Results

The TRCF structure was solved using single-wavelength anomalous dispersion (SAD), as radiation damage affected the quality of data collected at subsequent wavelengths. An initial 4 Å resolution data set was collected at Advanced Photon Source beamline NE-CAT-8BM (Argonne National Laboratory) at the white line of the selenium *K* absorption edge (Table 1, SeMet1).

Data were processed using *HKL*2000/*SCALEPACK* (Otwinowski & Minor, 1997) and 25 out of 60 possible Se sites were found using *SnB* (Weeks & Miller, 1999). Difference Fourier syntheses were used to locate an additional 23 sites, giving a total of 48 sites. Initial phases calculated from data cut off at 5 Å resolution with *MLPHARE* (Otwinowski, 1991) produced an uninterpretable electron-density map. Density modification as implemented in *DM* also failed to improve the map. However, density modification with solvent flipping and phase extension to 4 Å with *SOLOMON* (Abrahams & Leslie, 1996) yielded a map with excellent connectivity. Subsequently, a 3.2 Å resolution data set was collected at Advanced Photon Source beamline SBC-19ID (Table 1, SeMet2) and these amplitudes were used for density modification and phase extension using *SOLOMON* (Abrahams & Leslie, 1996). The resulting 3.2 Å resolution electron-density map (Fig. 1*b*) was of high quality and contained significant side-chain information. Manual building and refinement of the molecular model are now in progress.

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