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Crystallization and preliminary X-ray crystallographic studies of the alkanesulfonate FMN reductase from *Escherichia coli*

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The alkanesulfonate FMN reductase (SsuE) from *Escherichia coli* catalyzes the reduction of FMN by NADPH to provide reduced flavin for the monooxygenase (SsuD) enzyme. The vapor-diffusion technique yielded single crystals that grow as hexagonal rods and diffract to 2.9 Å resolution using synchrotron X-ray radiation. The protein crystallizes in the primitive hexagonal space group *P*622. The SsuE protein lacks any cysteine or methionine residues owing to the role of the SsuE enzyme in the acquisition of sulfur during sulfate starvation. Therefore, substitution of two leucine residues (Leu114 and Leu165) to methionine was performed to obtain selenomethionine-containing SsuE for MAD phasing. The selenomethionine derivative of SsuE has been expressed and purified and crystals of the protein have been obtained with and without bound FMN. These preliminary studies should lead to the structure solution of SsuE. It is anticipated that this new protein structure will provide detailed structural information on specific active-site regions of the protein and insight into the mechanism of flavin reduction and transfer of reduced flavin.

1. Introduction

For many bacterial organisms, inorganic sulfate is an important metabolite in the biological synthesis of sulfur-containing macromolecules. Inorganic sulfur is poorly represented in aerobic soil; therefore, bacteria in soil environments must have alternative sources for obtaining this element. When *Escherichia coli* is deprived of inorganic sulfur or cysteine, a set of sulfate-starvation-induced proteins are produced at increased levels (Quadroni *et al.*, 1996). These proteins include an FMN reductase (SsuE) and an $FMNH_2$-utilizing monooxygenase (SsuD; Eichhorn *et al.*, 1999, 2002). This two-component system is involved in the acquisition of sulfur through the reduction of alkanesulfonates to sulfite and aldehydes (van der Ploeg *et al.*, 1999). In the overall reaction scheme, SsuE is proposed to catalyze the reduction of FMN directly by NADPH to form $FMNH_2$ (Fig. 1). The reduced flavin is then transferred to SsuD, which converts the alkanesulfonate to aldehyde and sulfite in the presence of molecular oxygen.

The alkanesulfonate monooxygenase system belongs to a family of two-component enzyme systems that utilize flavin as a substrate rather than a bound prosthetic group. The number of bacterial flavin-dependent monooxygenases that utilize flavin as a substrate has increased significantly, with new systems continually being identified. An interesting mechanistic feature of the two-component enzyme systems is the transfer of the reduced flavin from the flavin reductase to the monooxygenase component. Typically, flavin cofactors are tightly bound to proteins through covalent or noncovalent interactions. Despite the growing number of these two-component systems that have been identified, the mechanism of flavin transfer

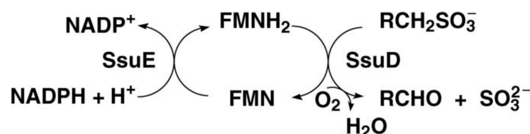
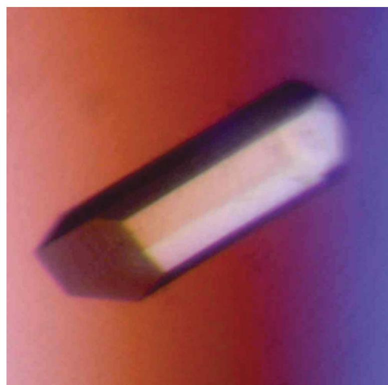


Figure 1
Proposed mechanism of the alkanesulfonate monooxygenase system (Gao & Ellis, 2005). SsuE is the FMN reductase that supplies reduced flavin to the SsuD monooxygenase enzyme.



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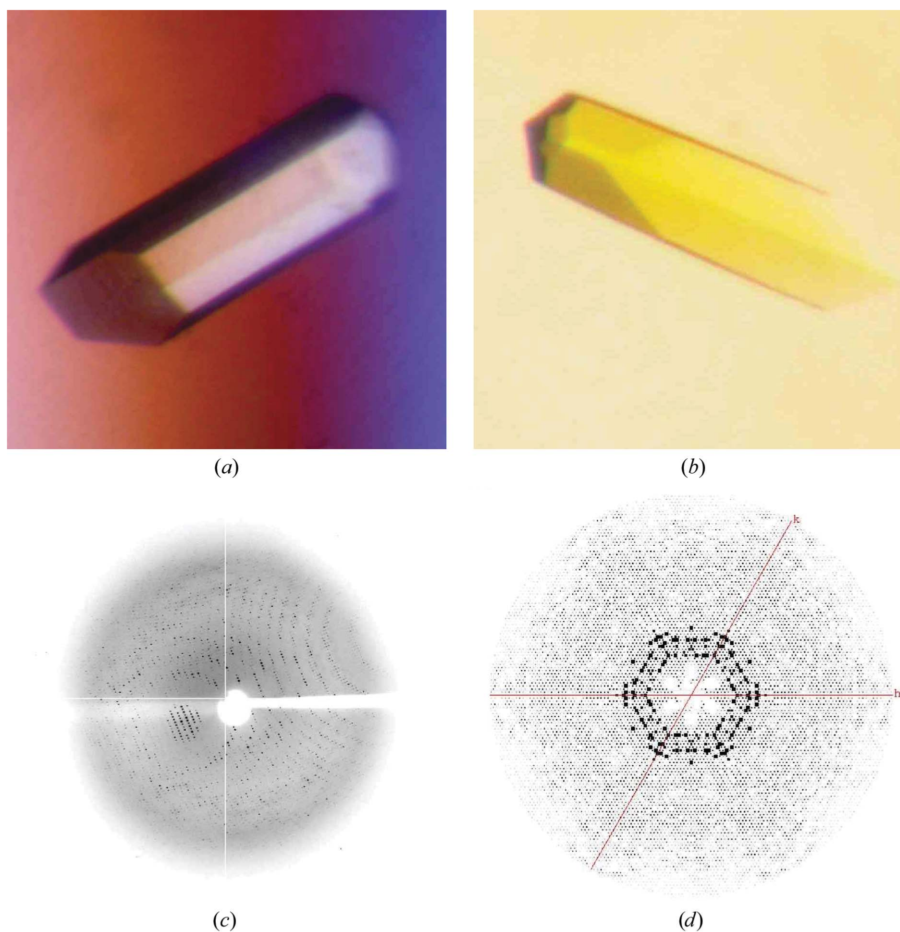
has not been fully elucidated. The mechanism of reduced flavin transfer could either occur by direct transfer involving protein interactions or by a diffusion mechanism. It is expected that protein associations are important during flavin transfer in order to prevent the generation of hydrogen peroxide, superoxide and hydroxyl radicals arising from flavin oxidation. A diffusion mechanism from the reduced flavin to the monooxygenase component would be likely to lead to generation of these reactive oxygen species, eventually leading to cellular damage.

Two classes of flavin reductases are associated with the flavin-dependent monooxygenase family of enzymes (Tu, 2001). Class I enzymes contain a bound flavin as purified and possess a signature flavin spectrum (Covés *et al.*, 1993; Tanner *et al.*, 1996). Alternatively, class II flavin reductases do not contain any flavin prosthetic group and cannot be defined as standard flavoproteins (Fieschi *et al.*, 1995; Fontecave *et al.*, 1989). From previous studies and our own analysis, the SsuE enzyme belongs to the class II flavin reductases. Of the class II reductases, only the three-dimensional structure of the Fre enzyme involved in the reactivation of the iron center in ribonucleotide reductase has been determined (Ingelman *et al.*, 1999). Interestingly, the Fre enzyme is structurally similar to the ferredoxin reductase family, even though there is no significant amino-acid sequence identity between Fre and other enzymes in this family. Although there is a low overall amino-acid sequence identity between SsuE and other class II FMN reductase enzymes, there are highly conserved regions within this family of enzymes that may be involved in substrate binding or protein interactions with the monooxygenase component. This may also explain why flavin reductase proteins unique to a specific two-component system can be substituted with unrelated flavin reductase proteins belonging to other systems (Louie *et al.*, 2003; Gisi & Xun, 2003). Determining the structure of SsuE will provide a basis for further studies aimed at analyzing the catalytic mechanism of flavin reduction and transfer. In addition, the structural role of conserved regions common to all class II reductases may be deduced from the SsuE structure. In this paper, we report the crystallization and preliminary crystallographic analysis of the SsuE enzyme.

2. Methods and results

2.1. Protein expression and purification

Expression and purification of native SsuE followed previously described protocols (Gao & Ellis, 2005). The protein lacks cysteine and methionine residues, consistent with its role in scavenging sulfur from alternate sources during sulfur starvation. The lack of methio-



(*a*) (*b*) (*c*) (*d*)

Figure 2
(*a*) Single crystal of native SsuE measuring approximately 0.15 × 0.15 × 0.5 mm viewed under polarizing light. This crystal was grown by hanging-drop vapor diffusion from a reservoir solution containing 16% PEG 8000, 0.15 *M* lithium citrate. (*b*) Single crystal of L114,165SeMet SsuE co-crystallized with FMN grown by sitting-drop vapor diffusion from a reservoir solution containing 17% PEG 4000, 0.15 *M* lithium citrate. (*c*) X-ray diffraction pattern obtained from a native SsuE crystal collected using synchrotron radiation (λ = 1.00 Å) and an ADSC Quantum 4 CCD detector at NSLS beamline X26C. (*d*) Pseudo-precession image showing the *hk*0 zone of reciprocal space for the unmerged integrated profile-fitted intensities of the SsuE diffraction data; the sixfold symmetry around *l* is clearly visible. The image was generated using *HKLVIEW* (Collaborative Computational Project, Number 4, 1994)

nine residues in this protein ruled out standard expression protocols to obtain selenomethionyl SsuE protein for MAD phasing. An alternative approach for derivatization is to substitute leucine residues with methionine for MAD phasing analysis (Gassner & Matthews, 1999). Studies with T4 lysozyme demonstrated that substitution of Met for Leu is most conservative and effective for selenomethionine incorporation and subsequent MAD phasing (Gassner *et al.*, 2003). Two leucine residues at positions 114 and 165 of the SsuE protein were substituted with methionine residues using the QuikChange system (Stratagene, La Jolla, CA, USA). The leucine at position 114 is a methionine residue in the homologous SsuE proteins from *Pseudomonas aeruginosa* and *P. putida*, making this residue ideal for conservative substitution. The SsuE/peT21A plasmid was used as the template with L114M (5′-ACC GTG GCC CAT ATG CTG GCG GTC GAT-3′) and L165M (5′-CGT CTT GAT ACC GCG ATG GAA ACT TTC TGG CAG-3′) forward primers and L114M (5′-ATC GAC CGC CAG CAT ATG GGC CAC GGT-3′) and L165 M (5′-CTG CCA GAA AGT TTC CAT CGC GGT ATC AAG ACG-3′) reverse primers. The L114,165M *ssu*E mutant construct was confirmed by DNA-sequence analysis (Davis Sequencing).

Selenomethionine-containing L114,165M SsuE (L114,165SeMet SsuE) protein was expressed under conditions of methionine-pathway inhibition. A single colony of BL21(DE3) containing the L114,165M SsuE vector was used to inoculate 5 ml LB media containing 100 µg ml^{-1} ampicillin. Following a 7 h incubation at 310 K, the cells were harvested and resuspended in 4 ml M9 media containing 22.2 m*M* glucose as the carbon source. The resuspended culture (1 ml) was used to inoculate (four) 1 l flasks containing M9 media with 100 µg ml^{-1} ampicillin and was incubated at 310 K until the absorbance at 600 nm reached 0.7. Specific amino acids (lysine, phenylalanine and threonine at 100 mg l^{-1}; isoleucine, leucine and valine at 50 mg l^{-1}) were added to block methionine biosynthesis and selenomethionine (60 mg l^{-1}) was added to replace methionine during cell growth. Induction with 0.4 m*M* IPTG was performed 15 min after the addition of the amino acids and the cultures were incubated for an additional 5 h at 291 K before harvesting. The purification protocol for the expressed protein followed the same procedure as previously described for wild-type SsuE. The purified L114,165SeMet SsuE protein possessed similar activity to the wild-type protein. The circular-dichroism spectra of the L114,165SeMet and wild-type SsuE protein were identical, suggesting that these amino-acid substitutions caused no major perturbations in secondary structure.

2.2. Crystallization

Purified wild-type SsuE protein at 8 mg ml^{-1} in 10 m*M* HEPES pH 7.0 was screened for crystallization by vapor diffusion at 293 K using sitting drops (containing 2 µl protein sample and 2 µl reservoir solution) and the sparse-matrix strategy (Jancarik & Kim, 1991) as implemented in Crystal Screens I and II and the PEG/Ion Screen (Hampton Research, Inc.). Large single SsuE crystals grew as hexagonal rods from 15–20% PEG 3350, PEG 4000 and PEG 8000 with 0.1–0.3 *M* lithium citrate in hanging-drop vapor-diffusion experiments (8 µl total drop size over a 1.0 ml reservoir solution). Crystals appeared within a day and grew to full size (∼0.15 × 0.15 × 0.5 mm) in two weeks (Fig. 2*a*). SsuE protein crystals containing oxidized flavin were obtained by both crystal soaking and cocrystallization with FMN (Fig. 2*b*).

Crystals were transferred to a cryoprotectant solution containing 20% glycerol, 20% PEG 8000 and 0.2 *M* lithium citrate prior to flash-cooling at 100 K in the cold stream. Initial X-ray diffraction data from the SsuE crystals were collected in the laboratory using a Rigaku/MSC Saturn92 CCD detector with Cu $K\alpha$ radiation from a Micro007 rotating-anode X-ray generator. The crystals diffracted weakly (∼3.5 Å) using the laboratory X-ray diffraction facility, but several complete data sets were collected. Diffraction data were indexed, integrated and scaled using *d*TREK* (Pflugrath, 1999). Crystals belong to the primitive hexagonal crystal system. A complete data set to 2.9 Å resolution was collected using beamline X26C of the National Synchrotron Light Source, Brookhaven National Laboratory (Fig. 2*c*). The crystal belongs to space group *P*622, with unit-cell parameters $a = b = 183.8$, $c = 181.5$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ (Table 1; Fig. 2*d*). The calculated V_M of the crystal is 2.60 Å^3 Da^{-1}, assuming the presence of eight 21.3 kDa monomers in the asymmetric unit.

Table 1
Summary of X-ray diffraction data collection and processing for SsuE.

Values in parentheses are for the highest resolution shell.

Space group	*P*622
Unit-cell parameters (Å)	$a = b = 183.8$, $c = 181.5$
Temperature (K)	100
Oscillation range per image (°)	0.5
Resolution (Å)	2.9 (3.00–2.90)
Total reflections observed	254737
Total unique reflections	40024
Completeness (%)	98.5 (99.5)
R_{merge}†	0.087 (0.369)
$I/\sigma(I)$	12.8 (4.7)

† $R_{merge} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i |\langle I_h \rangle|$.

Owing to the absence of Cys and Met residues, we are proceeding to solve the phase problem using a MAD protocol with crystals of a double mutant containing two SeMet residues. Two leucine residues at position 114 and 165 were substituted with methionine and the selenomethionine-containing protein has been expressed, purified and crystallized (Fig. 2*b*). Crystallographic studies are under way with L114,165SeMet SsuE to solve the three-dimensional structure by MAD phasing.

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