

Mechanistic studies of the SufS–SufE cysteine desulfurase: evidence for sulfur transfer from SufS to SufE

Sandrine Ollagnier-de-Choudens^a, David Lascoux^b, Laurent Loiseau^c, Frédéric Barras^c,
Eric Forest^b, Marc Fontecave^{a,*}

^aLaboratoire de Chimie et Biochimie des Centres Rédox Biologiques, DBMS-CB, CEA/CNRS/Université Joseph Fourier, UMR 5047, 17 Avenue des Martyrs, 38054 Grenoble Cedex 09, France

^bLaboratoire de spectrométrie de masse des protéines, Institut de Biologie Structurale, CNRS/CEA/UJF, 41 rue J. Horowitz, 38027 Grenoble Cedex 1, France

^cLCB-CNRS, IBSM, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France

Received 12 September 2003; revised 20 October 2003; accepted 20 October 2003

First published online 3 November 2003

Edited by Hans Eklund

Abstract SufS is a cysteine desulfurase of the *suf* operon shown to be involved in iron–sulfur cluster biosynthesis under iron limitation and oxidative stress conditions. The enzyme catalyzes the conversion of L-cysteine to L-alanine and sulfide through the intermediate formation of a protein-bound cysteine persulfide in the active site. SufE, another component of the *suf* operon, has been previously shown to bind tightly to SufS and to drastically stimulate its cysteine desulfurase activity. Working with *Escherichia coli* proteins, we here demonstrate that a conserved cysteine residue in SufE at position 51 is essential for the SufS/SufE cysteine desulfurase activity. Mass spectrometry has been used to demonstrate (i) the ability of SufE to bind sulfur atoms on its cysteine 51 and (ii) the direct transfer of the sulfur atom from the cysteine persulfide of SufS to SufE. A reaction mechanism is proposed for this novel two-component cysteine desulfurase.

© 2003 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Key words: *suf* operon; Cysteine desulfurase; Persulfide; Sulfur transfer; Mass spectrometry

1. Introduction

Cysteine desulfurases are important enzymes of sulfur metabolism present in all living organisms. They are involved in the biosynthesis of a variety of sulfur-containing compounds such as thiamine, thionucleosides in tRNAs, molybdopterins and iron–sulfur clusters [1]. In *Escherichia coli* there are three cysteine desulfurases named IscS, SufS and CsdA [1,2]. They all are pyridoxal-phosphate-dependent enzymes which catalyze the decomposition of L-cysteine into L-alanine and sulfane sulfur. In all of them the active site contains a conserved catalytically essential cysteine residue, to which the sulfur atom of the cysteine substrate is transferred to generate alanine and a protein-bound persulfide intermediate [3,4]. This persulfide could then directly transfer its sulfur atom to target molecules or be reduced to release sulfide in solution.

SufS, a homodimeric protein, was first proposed to specifi-

cally serve for mobilization of selenium from selenocysteine. Indeed, it had been shown to display a relatively much weaker cysteine desulfurase activity, absolutely dependent on the presence of a cysteine at position 364 (in *E. coli*) in the active site [2,5]. We also observed a very poor cysteine desulfurase activity using the highly homologous SufS protein from *Erwinia chrysanthemi* [6].

However, we also discovered that SufS from *E. chrysanthemi* tightly interacts with another homodimeric protein named SufE and that, in complex with SufE, it displays a drastically enhanced cysteine desulfurase activity. The activity of the complex is abolished upon substitution of the conserved cysteine residue of SufS (Cys369) to serine [6]. *sufS* and *sufE* belong to the same *suf* operon which has been shown to play an important role in iron–sulfur cluster synthesis/repair and to provide a protective mechanism under stressful conditions such as oxidative stress and iron limitation [7–10].

In order to better understand the mechanism of this new type of cysteine desulfurase enzyme, consisting of a complex of two homodimeric proteins SufS and SufE, and the molecular basis for the stimulatory effects of SufE on SufS activity, we have prepared histidine-tagged SufS and SufE from *E. coli* in pure form. We made the working hypothesis that these effects were dependent on sulfur atom transfer from the active site persulfide of SufS to a conserved cysteine of SufE. Examination of SufE amino acid sequences for conserved cysteine residues that might be involved in this function revealed a highly conserved cysteine at position 51 (Fig. 1). We thus also investigated the properties of a SufE mutant in which this cysteine was substituted by serine. Using mass spectrometry we have indeed demonstrated (i) the ability of SufE to bind sulfur atoms on its cysteine 51 residue, (ii) the direct transfer of sulfane sulfur from SufS to cysteine 51 of SufE. A mechanism for cysteine desulfuration by the SufS/SufE system is proposed.

2. Materials and methods

2.1. Materials and plasmids

All chemicals were of reagent grade and obtained from Sigma-Aldrich Chemical Co. or Fluka unless otherwise stated. Cysteine was from Boehringer Mannheim.

E. coli strains were grown aerobically at 37°C in Luria–Bertani (LB) rich medium [11]. When necessary, antibiotics were added at the following concentration: 50 µg/ml ampicillin.

*Corresponding author. Fax: (33)-4 38 78 91 24.
E-mail address: mfontecave@cea.fr (M. Fontecave).

Abbreviations: DTT, dithiothreitol; PCR, polymerase chain reaction

Plasmids pET-Shis and pET-Ehis, encoding the His-tagged SufS and SufE proteins, respectively, were derived from pET22b+ (Novagen). *sufS* insert, obtained by polymerase chain reaction (PCR) amplification using oligonucleotides *NdeI*-5s/*XhoI*-5as (see below) and *NdeI*-*XhoI* digestion, was introduced into pET22b+ digested by the same enzymes, yielding pET-Shis. The same method was used for pET-Ehis with oligonucleotides *NdeI*-6s/*XhoI*-6as and *NdeI*-*XhoI* digestion. The oligonucleotides are: *NdeI*-5s, 5'-CCGCATATGATTTTTCGTCGACAAAGTG-3'; *XhoI*-5as, 5'-GTTCTCGAGTCCCATGCAACGGTGAATACG-3'; *NdeI*-6s, 5'-CCGCATA-TGGCTT-TATTGCCGG-3'; *XhoI*-6as, 5'-GTTCTCGAGGCTAAGTGCAGCGGCTTTGGC-3'.

Plasmid pET-E_{C51S}his was obtained as follows. Mutagenesis of *sufE* was performed in two steps. First, two complementary mutagenic oligonucleotides, 3'-sufE C51S and 5'-sufE C51S were each used in combination with an oligonucleotide complementary to the 5' end (*NdeI*-6s) and 3' end (*XhoI*-6as) of *sufE*, respectively. The mutagenic oligonucleotides were designed such as cysteine 51 is changed to a serine residue. Oligonucleotides 3'-sufE C51S and *NdeI*-6s in one hand and oligonucleotides 5'-sufE C51S and *XhoI*-6as in another hand yielded the DNA fragments *sufE1*, *sufE2*, respectively. The matrix used was pET-Ehis. In the second step *sufE1* and *sufE2* were hybridized and used as templates for PCR amplification with the pair of oligonucleotides *NdeI*-6s/*XhoI*-6as. The resulting *NdeI*-*XhoI* fragment was cloned into pET22b+ yielding the plasmid pET-E_{C51S}his. The oligonucleotides are: 3'-sufE C51S, 5'-CCACACCTGACTCTGGCTGCCCTGAATGC-3'; 5'-sufE C51S, 5'-AGCATT-CAGGGCAGCCAGAGTVAGGTGTGG-3'.

2.2. Purification of SufS, SufE and SufE_{C51S}

E. coli, BL21(DE3) cells were transformed with plasmids pET-Shis or pET-Ehis and expression was induced by adding 0.5 mM isopropyl β-D-thiogalactoside (IPTG) at A₆₀₀ = 0.5. After 4 h at 30°C (SufS) or 3 h at 37°C (SufE), the pellet obtained from a 500 ml (SufS) or 600 ml (SufE) culture was immediately resuspended in buffer A (100 mM Tris-HCl, 50 mM NaCl, pH 8). Cell disruption was obtained after two passages through a French press. After centrifugation at 12000 rpm for 30 min at 4°C, soluble proteins were loaded onto a 5 ml nickel Hi-trap column (Amersham Pharmacia Biotech). Elution was achieved with a 4–500 mM imidazole linear gradient in buffer A. Eluted fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie blue staining. The purified fractions were pooled and imidazole was removed using a BIOMAX-5K device (Millipore) equilibrated with buffer A. In the case of SufE, the resulting protein solution was loaded onto a Superdex-75 (Pharmacia Amersham Biotech) gel filtration column and elution was carried out at 4°C with buffer A, at 30 ml/h. Fractions containing pure protein were pooled and concentrated onto a BIO-MAX-5K. Samples were then aliquoted and stored at –80°C. Fast protein liquid chromatography (FPLC) gel filtration with an analytical Superdex-75 at a flow rate of 0.5 ml/min equilibrated with buffer A was used for size determination. A gel filtration calibration kit (molecular weight marker kit, Sigma) was used as molecular weight standards. The same purification procedure was used for SufE and SufE_{C51S}.

2.3. Cysteine and selenocysteine desulfurase activity assays

The activity was assayed from the amount of alanine formed from L-cysteine or L-selenocysteine. Selenocysteine solution was obtained after reduction of a 60 mM selenocysteine-HCl solution with 100 mM dithiothreitol (DTT). The standard reaction mixture in a final volume of 100 μl buffer B (0.1 M Tris-HCl pH 8, 30 mM KCl) contained 0.5 nmol SufS, 1 molar equivalent of SufE and 4 mM cysteine. The reaction was carried out at 37°C during 10 min and stopped by the addition of 10% (v/v) 1 M TCA. After centrifugation, the supernatant was analyzed for alanine production. For that procedure, the supernatant was dried on a speed-vac and the residue dissolved into 150 μl of a citrate buffer, pH 2.2. A ninhydrin derivative was generated and analyzed at 570 nm by high performance liquid chromatography (HPLC) on a 7300 Beckman apparatus working with an ion exchange column S101036 calibrated with pure amino acid standards according to a published procedure [12]. The cysteine desulfurase activity is in units (μmol alanine/min)/mg of protein. Protein concentration (by monomer) was determined by the method of Bradford standardized with bovine serum albumin [13].

2.4. Sulfur transfer assays

All the experiments were done under anaerobic conditions inside a glove box (< 2 ppm O₂, 18°C). Both SufS and SufE were pretreated with 10 mM DTT and then repurified by gel filtration prior to use. Sulfur transfer reactions were carried out at 37°C for 20 min with 100 μM SufS, 100 μM SufE (wild-type or mutant protein) and 4 mM cysteine in a final volume of 50 μl buffer C (50 mM Tris-HCl, pH 7.5). Reactions were initiated by the addition of cysteine and were stopped by centrifugation at 2000 × g through a size exclusion column (Micro Bio-spin 6 (Bio-Rad)). The spin column eluate was then analyzed by (ESI)-quadrupole mass spectrometry.

For the one turnover experiments, SufS (500 μM) was incubated with 4 mM cysteine for 30 min at 37°C in order to load the protein with sulfur and to generate the persulfide intermediate. Excess of cysteine was removed by desalting the protein over a Micro Bio-spin 6 column. The resulting protein (100 μM) was incubated with SufE (100 μM, wild-type or mutant protein) for different reaction times at 37°C in a final volume of 50 μl buffer C. The reaction was stopped by freezing in liquid nitrogen and proteins were analyzed by mass spectrometry.

2.5. Mass spectrometry

Mass spectra of proteins were obtained by ESI-mass spectrometry on a Q-TOF Micro mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray ion source, operating with a needle voltage of 3 kV. Sample cone and extraction voltages were 70 and 3.5 V, respectively. Samples were infused continuously at a 5 μl/min flow rate with a concentration between 400 and 900 nM in water/acetonitrile (1/1, v/v) with 0.2% formic acid. The mass spectra were recorded in the 700–1600 range of mass-to-charge ratio (*m/z*) with a 1 s scan time. A 1 μM solution of Glu-fibrinopeptide B was used to calibrate the instrument in the MS/MS mode. Spectra were acquired and processed with MassLinux 4.0 (Micromass).

3. Results

3.1. Purification and characterization of SufS, SufE and SufE_{C51S} from *E. coli*

The three proteins SufS, SufE and SufE_{C51S} were obtained in the C-terminal His-tagged forms easily purified using Ni-NTA columns. About 13.5 mg of SufS, in a more than 95% pure as judged by Coomassie blue stained SDS–PAGE were obtained from a 500 ml culture. The yields were significantly inferior for proteins SufE and SufE_{C51S} (1–1.2 mg from 600 ml culture). Further purification was achieved using an additional Superdex-75 gel filtration chromatography step. The latter experiment demonstrated that both wild-type and mutant SufE behaved each as a dimer of roughly 35 kDa in solution. Details of the purification procedures are described in Section 2.

SufS displays a large selenocysteine deselenase activity, leading to an efficient mobilization of selenium from selenocysteine (3.5 units/mg) (data not shown). In contrast, as shown in Table 1, it displays a very weak cysteine desulfurase activity which was greatly increased upon addition of SufE (about 30-fold). No stimulation of the selenocysteine deselenase activity could be observed (data not shown). Comparable effects were observed with the SufS and SufE proteins from *E. chrysanthemi* [6]. As in the case of *E. chrysanthemi* [6], SufE from *E. coli* has by itself no cysteine desulfurase activity (data not shown).

Finally, results in Table 1 clearly show that the mutated SufE_{C51S} protein has absolutely no effect on the cysteine desulfurase activity of SufS. This demonstrates the importance of cysteine 51 for SufE function. We checked, by using the yeast two-hybrid methodology, that SufE_{C51S} makes a complex with SufS in a way similar to wild-type SufE (data not shown).

cysteine (Fig. 3B). In Fig. 3B, a peak at 45 532.0 Da is observed in addition to the peak at 45 500.0 Da again assigned to the persulfide-containing form of SufS, generated by desulfuration of cysteine. Fig. 4 shows the mass spectrum of SufE before (Fig. 4A) and after (Fig. 4B, C) incubation with the persulfide-containing form of SufS. After a few seconds incubation (Fig. 4B) a major peak at 16 734.9 Da is observed, corresponding to the monomeric form of SufE, together with two other peaks at 16 767 and 16 800 Da corresponding to the addition of one and two sulfur atoms, respectively. Fig. 4C shows that after 5 min incubation SufE has the ability to bind additional sulfur atoms, up to four sulfur atoms (peaks at 16 832 and at 16 864 Da for three and four sulfur atoms incorporated, respectively). SufE mass spectra after 10 and 20 min reactions were identical to Fig. 4C showing that sulfur transfer is fast and complete after a few minutes. Incubation of SufE with sodium sulfide (2 mM final concentration) for 20 min did not result in sulfur binding to the protein (data not shown). These experiments suggest a fast and direct transfer of sulfur atoms from SufS persulfide intermediate to SufE.

4. Discussion

The experiments reported here provide new insights into the mechanism of cysteine desulfuration by the SufS/SufE system.

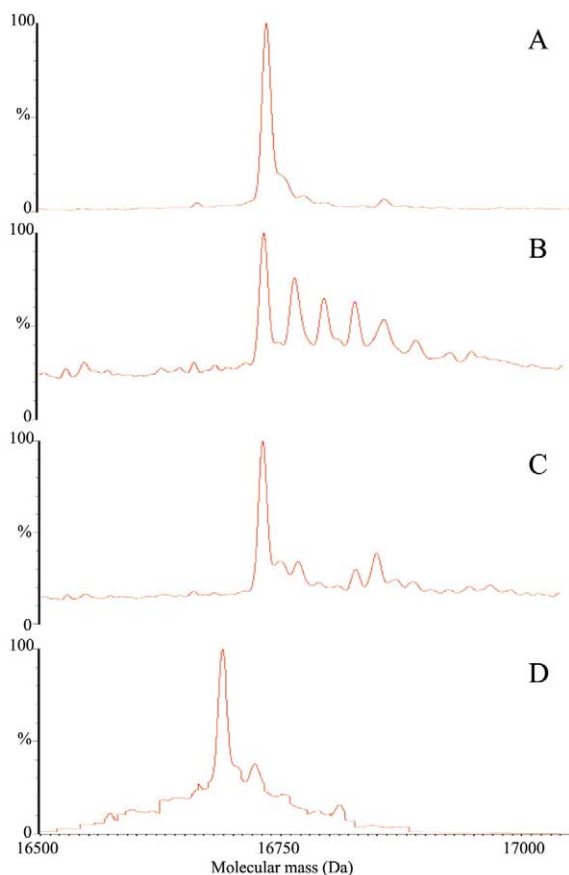


Fig. 2. Reconstructed ESI mass spectra of *E. coli* SufE. A: SufE (100 μ M). B: Reaction mixture containing SufS (100 μ M), SufE (100 μ M) and cysteine (4 mM) after 20 min reaction. C: Reaction mixture as in B after addition of DTT (10 mM). D: Reaction mixture containing SufS (100 μ M), SufE_{C51S} (100 μ M) and cysteine (4 mM) after 20 min reaction. Mass spectrometry analysis was achieved after dilution of the reaction mixtures (see Section 2).

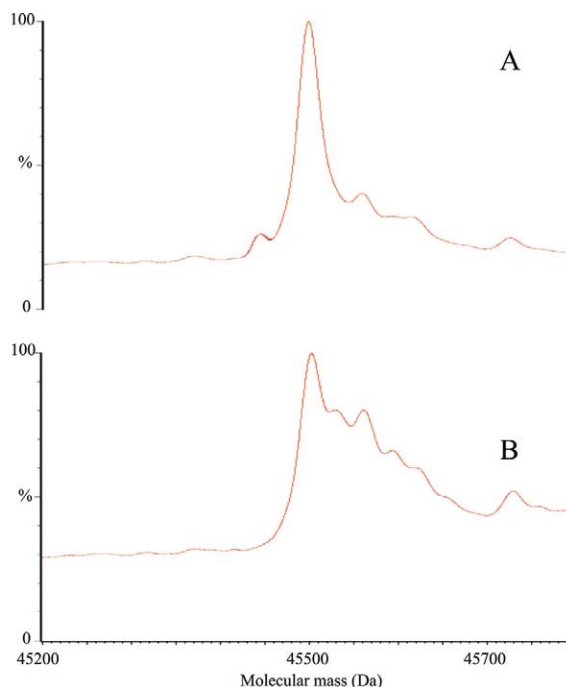
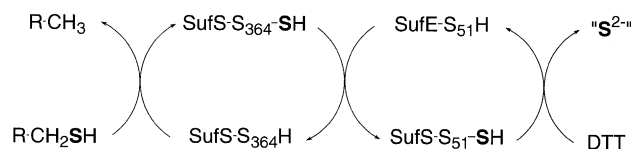


Fig. 3. Reconstructed ESI mass spectra of *E. coli* SufS (A) before and (B) after incubation of SufS (100 μ M) with 4 mM cysteine and desalting. Mass spectrometry analysis was achieved after dilution of the samples.

As previously shown with SufS from *E. chrysanthemi* [6], SufS from *E. coli* has a very weak cysteine desulfurase activity which is greatly increased upon addition of SufE. There are several possible explanations for the low activity of SufS. First, as shown by the three-dimensional structure of SufS [15] the distance between the critical active site cysteine residue (cysteine 364) and the PLP substrate Schiff base intermediate is too large to allow an efficient sulfur transfer to SufS. Alternatively, a low activity could result from the limited accessibility of the intermediate protein-bound persulfide and its slow decomposition to sulfide and cysteine by DTT which closes the catalytic cycle in the in vitro assay. A stable persulfide in SufS is consistent with the observation reported here of a peak at 45 532 Da in the mass spectrum of the protein treated with cysteine in the absence of DTT (Fig. 3B) and the slow decay of this peak upon treatment with DTT. It should be also noted that the enzyme form in which the active cysteine is in the persulfide state has been crystallized and its three-dimensional structure determined [16].

The mechanism of the reaction catalyzed by the SufS/SufE complex is depicted in Scheme 1.

In a first step, cysteine binds to SufS and transfers its sulfur atom to cysteine 364 to generate a persulfide on the protein. This is consistent with the following observations: (i) incubation of SufS with cysteine results in binding of a sulfur atom



Scheme 1. Cysteine desulfuration catalyzed by the SufS/SufE system. R-CH₂SH: cysteine, R-CH₃: alanine.

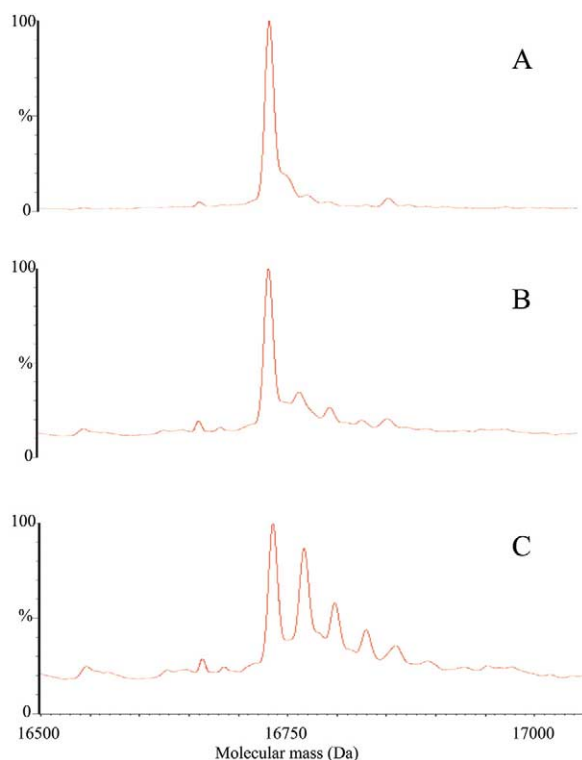


Fig. 4. Sulfur transfer from SufS to SufE. Reconstructed ESI mass spectra of *E. coli* SufE. A: SufE (100 μ M). Reaction mixture containing the persulfide-containing form of SufS (100 μ M), SufE (100 μ M) after a few seconds (B) and 5 min reaction (C). Mass spectrometry analysis was achieved after dilution of the reaction mixtures.

to the protein as shown by mass spectrometry (our results); (ii) the activity of the SufS/SufE-dependent reactions is abolished upon substitution of the critical cysteine of SufS to serine [6]; (iii) the K_m value for cysteine is the same in the SufS alone and in the SufS/SufE-dependent reactions [6]; (iv) SufE alone does not display a cysteine desulfurase activity [6].

In a second step, the sulfur atom of the persulfide in SufS is transferred to cysteine 51 of SufE presumably through a direct transpersulfuration reaction. This is consistent with the following observations: (i) in a one-turnover experiment in the absence of both substrate and DTT, the sulfur atom attached to SufS is transferred to SufE as shown by mass spectrometry; (ii) in the absence of the reducing agent DTT, incubation of SufS/SufE with cysteine results in the accumulation of several sulfur atoms (polysulfides) selectively in SufE (and not in SufS) which can be removed upon further treatment with DTT; (iii) this is abolished upon substitution of cysteine 51 of SufE to serine.

There are precedents of such transpersulfuration reaction: for example the transfer of sulfur from IscS to IscU, during iron–sulfur cluster biosynthesis, and to ThiI, during 4-thiouridine synthesis [14,17,18].

In the last step, sulfur is liberated upon reaction of SufE persulfide/polysulfide with an acceptor compound: DTT in the *in vitro* assay or presumably a physiological target compound *in vivo*.

The great acceleration of the cysteine desulfurase activity of the SufS/SufE complex is thus proposed to result from a fast sulfur transfer from SufS to a specific cysteine residue of SufE and a relatively increased accessibility of the sulfur atom in SufE as compared to SufS. Whether this also implies considerable conformational changes of both SufS and SufE which would occur upon binding of SufE to SufS is a possibility.

Further kinetic and structural studies are planned to better understand how the enzyme activity of this new type of cysteine desulfurase is tuned.

5. Note added in proof

The results of a study reported in a paper in press in the *Journal of Biological Chemistry* (F.W. Outten, M.J. Wood, F.M. Munoz and G. Storz) have led to similar conclusions.

Acknowledgements: We are grateful to J.P. Andrieu (LCM, IBS, Grenoble) for amino acid measurements.

References

- [1] Mihara, H. and Esaki, N. (2002) *Appl. Microbiol. Biotechnol.* 60, 12–23.
- [2] Mihara, H., Kurihara, T., Yoshimura, T. and Esaki, N. (2000) *J. Biochem.* 127, 559–567.
- [3] Zheng, L., White, R.H., Cash, V.L., Jack, R.F. and Dean, D.R. (1993) *Proc. Natl. Acad. Sci. USA* 90, 2754–2758.
- [4] Zheng, L., White, R.H., Cash, V.L. and Dean, D.R. (1994) *Biochemistry* 33, 4714–4720.
- [5] Mihara, H., Maeda, M., Fujii, T., Kurihara, T., Hata, Y. and Esaki, N. (1999) *J. Biol. Chem.* 274, 14768–14772.
- [6] Loiseau, L., Ollagnier-de Choudens, S., Nachin, L., Fontecave, M. and Barras, F. (2003) *J. Biol. Chem.* 278, 38352–38359.
- [7] Takahashi, Y. and Tokumoto, U. (2002) *J. Biol. Chem.* 277, 28380–28383.
- [8] Nachin, L., El Hassouni, M., Loiseau, L., Expert, D. and Barras, F. (2001) *Mol. Microbiol.* 39, 960–972.
- [9] Nachin, L., Loiseau, L., Expert, D. and Barras, F. (2003) *EMBO J.* 22, 427–437.
- [10] Zheng, M., Wang, X., Templeton, L.J., Smulski, D.R., laRossa, R.A. and Storz, G. (2001) *J. Bacteriol.* 183, 4562–4570.
- [11] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [12] Lutz, T., Westermann, B., Neupert, W. and Herrmann, J.M. (2001) *J. Mol. Biol.* 307, 815–825.
- [13] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [14] Smith, A.D., Agar, J.N., Johnson, K.A., Frazzton, J., Amster, J., Dean, D.R. and Johnson, M.K. (2001) *J. Am. Chem. Soc. USA* 123, 11103–11104.
- [15] Mihara, H., Fujii, T., Kato, S., Kurihara, T., Hata, Y. and Esaki, N. (2002) *J. Biochem.* 131, 679–685.
- [16] Lima, C.D. (2002) *J. Mol. Biol.* 315, 1199–1208.
- [17] Urbina, H.D., Silberg, J.J., Hoff, K.G. and Vickery, L.E. (2001) *J. Biol. Chem.* 276, 44521–44526.
- [18] Kambampati, R. and Lauhon, C.T. (2000) *J. Biol. Chem.* 275, 10727–10730.