SufC hydrolyzes ATP and interacts with SufB from *Thermotoga maritima*

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Abstract Genetic experiments in bacteria have shown the *suf* operon is involved in iron homeostasis and the oxidative stress response. The *sufB* and *sufC* genes that always occur together in bacteria are also found in plants, and even the malaria parasite, associated with the plastid organelle. Although the *suf* operon is believed to encode an iron-dependent ABC-transporter there is no direct evidence. By immunolocalization we show here that SufB and SufC are associated with the membrane of *Escherichia coli*. We also present kinetic studies with a recombinant version of SufC from *Thermotoga maritima* that shows it is an ATPase and that it interacts with SufB in vitro. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: ABC-transporter; ATPase; *suf* operon; *Thermotoga maritima*; *Escherichia coli*

1. Introduction

The recently named *suf* (mobilization of sulfur) operon of *Escherichia coli* is involved in iron metabolism/assembly of [Fe–S] clusters/oxidative stress response [1–3]. The operon comprises six genes, *sufA*, *-B*, *-C*, *-D*, *-S* and *-E*, but only the function of *sufS* is known; it encodes a NifS ortholog for which a crystal structure has been solved [4]. SufA is an ortholog of iron-sulfur cluster (Isc)A, which together with IscS (NifS) and IscU (NifU) is involved in [Fe–S] cluster formation and repair [5,6]. Experiments with bacteria have shown the *suf* genes are induced under conditions of iron-limitation [1,2]. Moreover, *sufA*, *sufB* and *sufC* are strikingly up-regulated under conditions of oxidative stress [3].

Whilst representatives of the *suf* operon are found widely in the bacterial kingdom, the operon is rarely as complete as in *E. coli* and only *sufB* and *sufC* are invariably found together [7]. *sufB* corresponds to the hypothetical chloroplast frame *ycf24* found on the plastid genomes of red algae and the malaria parasite. In green plants it is found on the nuclear genome with a plastid targeting sequence [8]. *sufC* corresponds to *ycf16* on the plastid genome of red algae, but has translocated to the nucleus in both malaria and plants where it has acquired a putative plastid targeting sequence [7].

The *suf* operon in *E. coli* is annotated to specify an uncharacterized iron-regulated ABC-transporter, SufC having sequence similarity to the nucleotide-binding subunit of other ABC-transporters. We show here that both SufB and SufC are localized to the membrane of *E. coli*. In order to obtain soluble proteins for kinetic observations, we made recombinant versions from the thermophilic bacterium *Thermotoga maritima* and present the first biochemical evidence that SufC is an ATPase. We show that in addition to SufC's hydrolytic activity, it can interact with SufB.

2. Materials and methods

2.1. Recombinant proteins

sufC was amplified from *T. maritima* genomic DNA (ATCC®) by PCR (TaKaRa polymerase) using primers (GenoSys) based on the genomic sequence [9]. The cloned product was over-expressed from pET-28a (Novagen) with a C-terminal $(His)_6$-tag. Transformants in *E. coli*, BL21(DE3)pLysS, were induced with 1.0 mM IPTG for 3 h at 37°C and lysozyme-treated cells were disrupted by sonication in the presence of protease inhibitor cocktail III (Calbiochem). After centrifugation at 50 000 rpm, SufC was purified (Fig. 1) from the supernatant using the His-bind Kit (Novagen); desorption was in steps from 100 to 250 mM imidazole. Following dialysis against buffer A (50 mM Tris–HCl, pH 7.5, 100 mM KCl, 1 mM DTT, 2 mM $MgCl_2$, 1 mM EDTA) and concentration by pressure dialysis (Amicon), the protein (1–6 mg/ml) was frozen in liquid N_2 and stored at −80°C.

sufB was amplified and cloned into pET-28a as described for *sufC*. In this case over-expression gave an insoluble protein. Inclusion bodies were prepared [10], dissolved in 6 M urea/1× binding buffer, and absorbed on Ni^{2+}-resin under denaturing conditions, as described in the His-bind Kit (Novagen). The beads were washed in buffer containing 6 M urea and 20 mM imidazole and SufB was eluted in steps with 6× volumes of urea buffer containing 100–250 mM imidazole. The protein (Fig. 1) was re-folded by dialysis at 4°C against two 5 l changes of buffer A and stored frozen, as for SufC.

2.2. ATP hydrolysis

All measurements were made in buffer A (see above) without EDTA at 20°C. The hydrolysis of ATP was monitored using a linked enzyme assay that coupled the formation of ADP to the oxidation of NADH. The solutions (total volume 0.25 ml) contained 0.16 mM NADH, 1 mM phosphoenolpyruvate, 50 U pyruvate kinase, 50 U lactate dehydrogenase and varying amounts of ATP. NADH absorbance was monitored at 340 nm and the rate of NADH oxidation was determined based on its extinction coefficient of 6220 M^{-1} cm^{-1}.

2.3. Fluorescence anisotropy

Fluorescence anisotropy measurements were made using an SLM 8000S fluorimeter operated in the 'T' format. Excitation was at 360 nm and emission monitored though KV 399 cut off filters. The fluorescent analogue 2′(3′)-*O*-*N*-methylanthaniloyl ATP (mantATP) was prepared as described previously [11].

2.4. Sedimentation equilibrium measurements

Sedimentation equilibrium measurements were made using a Beckman XLA analytical ultracentrifuge at 20°C. For each protein, three concentrations were centrifuged at three different speeds until equilibrium was reached when absorbance was monitored at 280 nm. The data were analyzed with Beckman XLA data analysis software.

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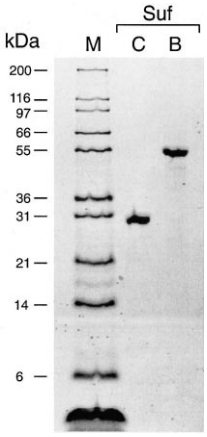

Fig. 1. SDS–PAGE of purified SufB and SufC of *T. maritima* stained with Coomassie blue.

2.5. Antibodies

BALB/C mice were immunized intraperitoneally with *T. maritima* recombinant proteins in Freund's complete adjuvant. Following two booster inoculations in incomplete adjuvant, antiserum was obtained by standard procedures.

2.6. Electron microscopy

For immunolocalization studies, *E. coli* (INVα F′) was grown for 3 h in the presence or absence of 30 μM $FeSO_4$ in M9 minimal medium, with glycerol as the energy source [2]. Washed cells were fixed in 1.2% paraformaldehyde and thin sections were incubated with mouse antiserum to SufB or SufC followed by gold-labelled rabbit anti-mouse IgG.

3. Results

3.1. Sequence predictions

SufB from *T. maritima* comprises 464 amino acids, calculated molecular weight ~51 899 Da (with Gly Arg Thr His_6 = 53 036). The predicted secondary structure of the C-terminal half has an extensive β-structure followed by three short terminal helices (Fig. 2). Fold recognition [12] gives a significant similarity score with β-solenoid proteins. The sequence does not predict an intrinsic membrane protein.

SufC from *T. maritima* comprises 246 amino acids, calculated molecular weight ~27 835 Da (with Ala Ala Ala Leu Glu His_6 = 29 113), with typical Walker A- and B-motifs (Fig. 2). The B-motif has a conserved Glu residue next to Asp, characteristic of the ATP-binding site of ABC-transporter proteins [13]. There is also a conserved His lying 30 residues downstream from the second Asp residue of the B-motif. With the program QUANTA, SufC was readily modelled in silico on to the co-ordinates of the α-carbon backbone of the ATP-binding subunit of the prokaryotic histidine permease [14].

3.2. Sedimentation equilibrium measurements

Sedimentation equilibrium measurements were made to define the oligomeric state of the purified recombinant proteins under the ionic strength conditions used for our kinetic studies. Equilibrium was attained at three appropriate speeds for each protein. Fig. 3A shows that SufB is well fitted to a single species model with a molecular weight of 381 561 Da. This compares to a monomeric molecular weight calculated from the amino acid sequence of 53 036 Da. It could be that the native protein is an oligomeric structure or that this is an artefact of the re-folding procedure. Fig. 3B shows that SufC is well fitted to a single species with a molecular weight of 32 012 Da. Since the calculated molecular weight is 29 113 Da (including the His-tag), SufC exists as a monomeric species under these conditions.

3.3. SufC is an ATPase and interacts with SufB

Hydrolysis of ATP was determined from the linked enzyme system, described in Section 2. The rate of hydrolysis by SufC was hyperbolically dependent on ATP concentration (Fig. 4). Fitting the data to the Michaelis–Menten equation gave a K_m of 45 μM and V_{max} of 12 μM min^{-1} that corresponds to a k_{cat} of 2.4 min^{-1}.

TM1369 (SufB)

MMERLIIDDSRFNFVPKVKTAYKAPPGLDEKLIMEISRAKDEPEWMLKHRLESLKVFNEW
HNPRFGVDISGLDLGKIVSYIKPDAKKSTSWEEVPEEVKEAFDKLGIPEAERKYLAGVGA
QLDSEIVYQNMKKELEKMGVIFLDMESAVREYPDLVKKYFMKLVPITDHKFAALHGAIWS
GGTFLYVPAGVKIPMPLQAYFLMSNPGMGQFEHTIIVAEEGSEVTFIEGCSAPRYNIINL
HAGMVEIYVKKGAKVKYLTIQNWSKNTYNLNTKRSIVDEEGSMTWVSGSLGSQKTMLYPM
TILKGKGARAESMSITYAGPGQHMDTGSKVVHLAPYTSSIVSAKSISLGGGWAFYRGLLK
ITKEAVKSKASVECAALMLDNRSKSDTVPIIEVETDRADVGHEARIGRIGEDQIFYLMSR
GLSEQEAKAMIVKGFVEPVVKELPFEYAVELNKLLELEIEKSIG

TM1368 (SufC)

Walker A
MLRIVNLHAKLRDEDKEILKGVNLEIEKGEVHVLMGPNGSGKSTLANVIMGNPRYIVTEG
****
DIVFEGNSIKDLPPNERAKLGIMMTFQNPYEVEGVKLSQFLITAHRRIHGEDKNYLELRK
ABC signature Walker B
ELEETAEKLGLDKDFLERYLNVGFSGGEKKRSEILQSLFLRPKLLILDEIDSGLDVDALR
*************** **********
LIANLIARLNEEGVTLLIITHYKRLLDHLKRIDKVHVYVDGRIVTSGGPELADEIEEKGY
*
SLEGVR

Fig. 2. Primary sequence and secondary structure predictions based on position-specific scoring matrices [20] for SufB and SufC of *T. maritima* (dark shading = α-helices; light shading = β-strand). Motifs are indicated, as is a conserved His residues (*).

The fluorescence anisotropy of a solution of 1 μM mantATP was measured and had a value of 0.025. SufC was then added to a final concentration of 15 μM and the fluorescence anisotropy was measured after 30 s, when binding was complete but before significant hydrolysis occurred. A value of 0.122 was obtained. When the measurement was repeated in the presence of 15 μM SufB, the fluorescence anisotropy increased to 0.165. Addition of SufB alone to mantATP resulted in a negligible increase in fluorescence anisotropy. These results show that SufB can interact with SufC as reflected in the larger anisotropy increase of SufC–mantATP in the presence of SufB. However, at saturating concentrations of ATP (500 μM), SufB had little, if any, effect on the steady-state rate of ATP hydrolysis by SufC.

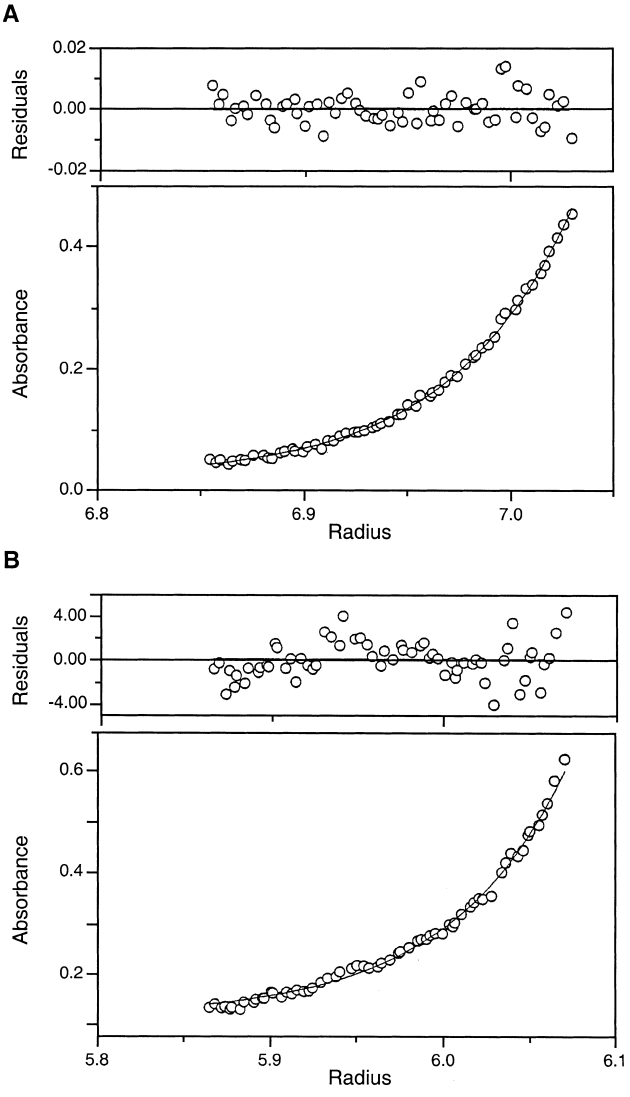

Fig. 3. Sedimentation equilibrium measurements of SufB and SufC from *T. maritima*. A: SufB was allowed to reach equilibrium at 4200, 5000 and 7500 rpm. The data were globally fitted to a single species model using a calculated partial specific volume of 0.746. The solid line is the best fit of the data giving a molecular weight of 381 561 Da. The data and residuals shown are from the 7500 rpm measurements. B: SufC was allowed to reach equilibrium at 15 000, 18 000 and 26 000 rpm. The data were globally fitted to a single species model using a calculated partial specific volume of 0.748. The solid line is the best fit of the data giving a molecular weight of 32 012 Da. The data and residuals shown are from 26000 rpm measurements.

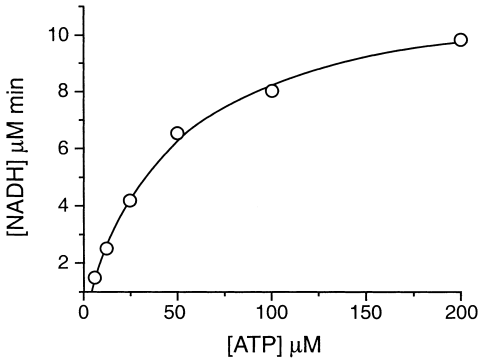

Fig. 4. The concentration dependence of the steady-state rate of hydrolysis of ATP by SufC. The line shows the best fit of the data to the Michaelis–Menten equation giving a K_m of 45 μM and V_{max} of 12 μM min^{-1}.

3.4. Localization of SufC in E. coli

Mouse antibodies to SufB and SufC from *T. maritima* cross-reacted in Western blots with the corresponding *E. coli* proteins (not shown). Electron micrographic sections of *E. coli* grown in minimal medium, with or without 30 μM $FeSO_4$, were examined with immunogold-labelled antibodies (Fig. 5). The considerable shrinkage of the bacterial cytosol found in the preparation is assumed to be a fixation artefact rather than damage due to growth in minimal medium. Immunogold labelling was confined largely to the membrane ($P = < 0.001$)

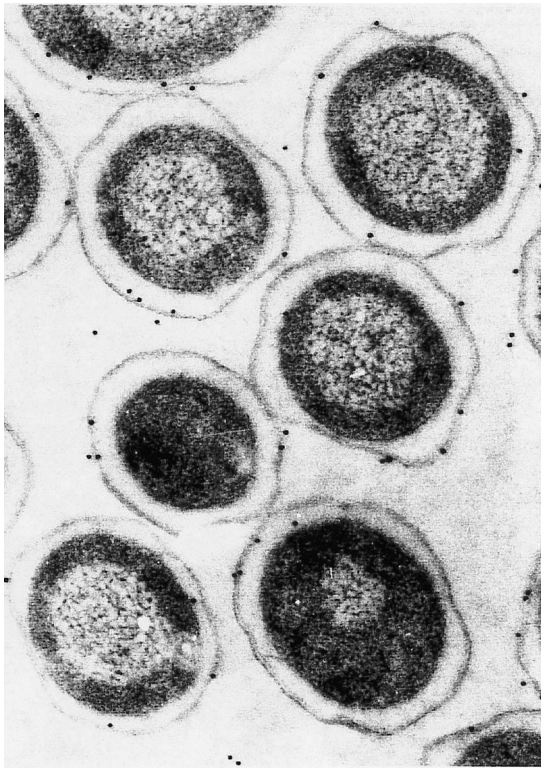

Fig. 5. Electron micrographic section showing immunogold labelling of SufB in *E. coli* grown in iron-deficient minimal medium. Magnification × 67 500.

(Fig. 4). Under iron-limiting conditions the number of gold particles decorating the membrane increased ($\sim$1.5×) for both Suf proteins.

4. Discussion

Sequence and genetic analyses predict that the bacterial *suf* operon specifies components of an iron-dependent ABC-transporter. We have looked for direct evidence, both morphological and biochemical. By immunogold localization we found SufB and SufC associated with the membrane of *E. coli*. This contrasts with previous immunolocalization studies of these two proteins in plant tissues. Whilst SufB was associated with the chloroplast inner envelope in *Arabidopsis thaliana* [8], SufC was localized in the plastid stroma and not the membranes of the diatom *Odontella sinensis* [15].

The sequence of SufB predicts it is not an intrinsic membrane protein, despite its association with bacterial and plastid membranes. Although SufB might be a peripheral membrane protein, it failed to cluster with 'single nucleotide binding fold', non-intrinsic proteins of the ABC superfamily of *A. thaliana* (acronym Nap1) [16]. Our results do not support the model put forward by Møller et al. [8] in which SufB (acronym atABC1) was designated the ATPase component of an inner membrane transporter in the chloroplast of *A. thaliana*. This role can more likely be ascribed to SufC, as we have shown here that SufC from *T. maritima* is an ATPase, confirming the likelihood that it is the nucleotide binding subunit of an ABC-transporter. We also suggest the elevated levels of protoporphinogen IX described by Møller et al. in their *laf6* mutant (*sufB* deleted) might be attributed to a disturbance of co-ordinated heme biosynthesis and iron homeostasis, as has been reported for the bacterium *Bradyrhizobium japonicum* [17].

In bacteria, *sufB* and *sufC* always occur together. This is true also in plants where the two genes are present either in the plastid or in the nucleus, the products being directed to the organelle in the latter case. Although the two proteins could still be the subunits of a transporter, uncoupled functions need not necessarily be discounted as the *suf* operon is often deficient in some of its components [7]. Moreover, proteins of cyanobacterial origin can alter their functions in plastids: for example, the *Synechocystis* protein SynToc75 has switched from a secretory role in the bacterial outer membrane to part of the import machinery (translocon) [18].

In conclusion, we have confirmed that SufB and SufC are induced under conditions of iron limitation in *E. coli* and have shown that SufC of *T. maritima* is an ATPase that can interact with SufB in vitro. Whilst our studies with bacteria support the conjecture that SufB and SufC might interact as subunits of an iron-dependent ABC transporter, further work is needed to elucidate their role in plastids. Our suggestion [7] that SufB and SufC are required for conversion of apoferredoxin to the holo [Fe–S] protein in the stroma of plastids [7,19] is consistent with the linkage of SufB and SufC to iron homeostasis and the oxidative stress response, but remains untested.

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