Iron/sulfur (Fe/S) proteins are central to the functioning of cells in both prokaryotes and eukaryotes. Here, we show that the yhgI gene, which we renamed nfua, encodes a two-domain protein that is required for Fe/S biogenesis in Escherichia coli. The N-terminal domain resembles the so-called Fe/S A-type scaffold but, curiously, has lost the functionally important Cys residues. The C-terminal domain shares sequence identity with Nfu proteins. Mössbauer and UV-visible spectrocopic analyses revealed that, upon reconstitution, NfuA binds a [4Fe-4S] cluster. Moreover, NfuA can transfer this cluster to apo-aconitase. Mutagenesis studies indicated that the N- and C-terminal domains are important for NfuA function in vivo. Similarly, the functional importance of Cys residues present in the Nfu-like domain was demonstrated in vivo by introducing Cys→Ser mutations. In vivo investigations revealed that the nfua gene is important for E. coli to sustain oxidative stress and iron starvation. Also, combining nfua with either isc or suf mutations led to additive phenotypic deficiencies, indicating that NfuA is a bona fide new player in Isc- and Suf-dependent Fe/S biogenesis pathways. Taken together, these data demonstrate that NfuA intervenes in the maturation of apoproteins in E. coli, allowing them to acquire Fe/S clusters. By taking into account results from numerous previous transcriptomic studies that had suggested a link between NfuA and protein misfolding, we discuss the possibility that NfuA could act as a scaffold/chaperone for damaged Fe/S proteins.

Deciphering how Fe/S clusters are formed and inserted into apoproteins became a field of wide interest in both prokaryotes and eukaryotes. Seminal studies on the biosynthesis of the iron molybdenum cofactor contained in the Azotobacter vinelandii nitrogenase allowed Dean and co-workers to discover the Nif system (1, 2). It involves ~20 proteins among which three, NifS, NifU, and possibly IscANif, are dedicated to biogenesis of the nitrogenase Fe/S cluster (3, 4). Studies in Escherichia coli and a few other bacteria, including Synechococcus and Erwinia, led to the discovery of two additional systems, referred to as Suf and Isc, which allow Fe/S biogenesis and insertion in most, if not all, cellular Fe/S proteins (5–8). A fourth system, referred to as Csd, was described in E. coli, but its role in Fe/S biogenesis remains to be ascertained (9). Eukaryotic organisms contain an Isc system that locates in mitochondrion, while plants have in addition a chloroplastic Suf system (10, 11).

All of the Fe/S biogenesis systems mentioned above contain a cysteine desulfurase and at least one so-called scaffold protein. Cysteine desulfurases degrade l-cysteine to produce l-alanine and inorganic sulfur that eventually becomes part of a Fe/S cluster (9, 12, 13). Scaffolds are intermediate Fe/S assembly sites; by binding sulfur and iron they provide a structural and biochemical environment allowing formation of a Fe/S cluster that is eventually transferred to an apoprotein (3, 5, 6). The concept of scaffold was proposed to provide a solution to the problem of the toxicity of free iron and sulfide. The occurrence of a scaffold would also fit with the prediction that in vivo Fe/S clusters would not form spontaneously from free iron and sulfide as they could do in vitro. A scaffold was therefore defined as a protein that binds transiently Fe/S clusters, presumably using Cys residues as ligands, and transfers them to apotargets. The A. vinelandii NifU protein met with all these criteria and was concluded to be a scaffold (3, 14–17). Subsequently, NifU became a founder of the U-type family of scaffolds, although its special domain organization led to some confusion. In fact, NifU is organized in three domains, each able to bind a Fe/S cluster (14–19). The N-terminal domain, subsequently referred to as an IscU-type domain, contains one Fe/S cluster that is transiently formed and can be transferred to apoproteins (14–16). The central domain contains a Fe/S cluster that is stable, disqualifying this region to act as a scaffold
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(18). The C-terminal domain, subsequently referred to as the Nfu domain, contains an Fe/S cluster that is transferred to apoproteins (15, 16). Both the IscU and the Nfu domains can arise as single polypeptides (3, 5, 6, 20–24) and can act as scaffolds (3, 5, 20, 21).

Parallel investigations on Fe/S biogenesis allowed the discovery of another type of scaffold protein referred to as the A-type. Members of this family include IscA within the Isc system, SufA within the Suf system, and IscANif within the Nif system (3–6). Eukaryotic homologs entail mitochondrial ISA1 and ISA2 in yeast and plants, and a plastidic SufA-like (10, 11). Recently, we identified ErpA, which shares 30% sequence identity with IscA and SufA and has an essential function in E. coli (25). Evidence for Fe/S cluster binding and subsequent transfer to apoproteins was obtained for several IscA, SufA, and ErpA homologs originating from different organisms (3, 5, 6, 25). Structures of both the holo- and apoforms of IscA were obtained, and potential cysteine ligands were determined (26–28).

The yhgI gene is predicted to encode a protein-exhibiting sequence similarity with Nfu. Whether this is actually a Nfu-like protein was an important issue to investigate, because to date, E. coli is among the few organisms lacking such an activity. Moreover, in recent years, the yhgI gene has actually been proposed to be involved in a wide spectrum of different biological processes, ranging from gluconate metabolism (29) to nutritional competence and stress responses (30), rendering its actual role in E. coli uncertain. In this study, we provide evidence that Yhgl, which we renamed NfuA, is involved in Fe/S biogenesis, directly intervening in maturing apoprotein, and is required for E. coli to resist oxidative stress and iron starvation.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—E. coli strains were grown in Luria-Bertani (LB)-rich medium at 37 °C. Strains used in this study are listed in supplemental Table S1. Ampicillin (50 μg/ml) and kanamycin (25 μg/ml) were added when necessary. All chemicals were obtained from Sigma-Aldrich Chemical Co. or Fluka unless otherwise stated.

Plasmid Construction—The E. coli nfsA gene was amplified by PCR, with Y1 and Y2 primers, and ligated into the expression plasmid pET22b+ at the Ndel/Xhol sites, yielding the pETnfsA plasmid. By using Y5 and Y6 primers, the nfsA gene was ligated into the low copy vector pUC18 at the BamHI and HindIII sites, yielding the pUCnfsA plasmid. The pUCnfsA-Nt plasmid, allowing expression of the N-terminal domain of NfuA, was obtained by generating a PCR fragment with Y5 and Nty7 primers and ligating it between the BamHI and HindIII sites of the pUC18 plasmid. The pUCnfsA-Ct plasmid, allowing expression of the C-terminal domain of NfuA, was obtained by a similar strategy using Cty8 and Y6 primers. The A. vinelandii nfsA gene was amplified by PCR from the plasmid vector pDB1622 (D. Dean laboratory) by using the Nfu1 and Nfu2 primer pair. The resulting PCR fragment was ligated into the pUC18 vector, at the BamHI and HindIII sites, yielding the pUCnfsA plasmid. The substituted versions of NfuA were obtained by a two-step PCR mutagenesis method. The mutagenic primer pairs were designed (i) to be completely complementary to each other and (ii) to span the site of the substitution. To construct the NfuAC39S variant, two separate PCR reactions were performed with as template the pETnfsA plasmid and by using either the Y5/YC1mut2 pair or the YC1mut1/Y6 pair as primers. The full-length encoding sequence of the NfuAC39S variant was obtained in a second PCR reaction by combining the two previous PCR products and the Y5/Y6 primer pair. The final PCR product was digested with BamHI and HindIII and ligated into the pUC18 vector. The same procedure was followed to substitute each cysteine of NfuA. To construct plasmids allowing production of His-tagged versions of the NfuA cysteine variants, the same strategy was used except that the Y5/Y6 primer pair was replaced by the Y1/Y2 primer pair, and the final PCR product was ligated into the pET22b+ vector. All constructs were checked by DNA sequencing. DNA manipulations were performed in E. coli MG1655 using established standard protocols or under the conditions recommended by the manufacturer. All the primers used in this study are listed in supplemental Table S2.

Protein Purification—NfuA was obtained as follows. E. coli BL21(DE3)/pETnfsA was grown in LB medium containing 100 μg/ml ampicillin, at 37 °C. Protein expression was induced for 4 h by the addition of 0.5 mM isopropyl β-D-thiogalactoside (IPTG)3 to an exponentially growing culture. The bacterial pellet (10 g/6 liters) was resuspended in buffer A (0.1 M Tris-HCl, pH 8, 0.1 M NaCl) and sonicated before ultracentrifugation (90 min, 45,000 rpm, 4 °C). The supernatant was treated with DNase I and 10 mM MgCl2 at 4 °C for 45 min, and after centrifugation (30 min, 15,000 rpm, 4 °C), 800 mg of soluble proteins were loaded onto a 10-ml Ni-NTA affinity column (Qiagen) equilibrated with buffer A. The NfuA protein was eluted with buffer A containing 0.2 M imidazole. The NfuA-containing fractions were mixed with ammonium sulfate (60% saturation) for 1 h at 4 °C. After centrifugation (30 min, 15,000 rpm), the pellet was resuspended in buffer A and desalted onto a Nap-25 column (Amersham Biosciences). This protocol allowed for the purification of ~70 mg of NfuA from 800 mg of bacterial extracts. A similar procedure was used to purify NfuA variants. Aconitase (AcnA) was obtained from IPTG-induced cultures of JRG4004 strain according to a slightly modified version of the procedure reported by Jordan et al. (31). After the ammonium sulfate precipitation step, AcnA-enriched fractions were loaded on a phenyl-Sepharose column (Amersham Biosciences) equilibrated with buffer B (40 mM Tris-HCl, pH 8, 1.7 mM (NH4)2SO4). AcnA was eluted with a linear gradient of 1.7 to 0 mM (NH4)2SO4. Finally, after chromatography on a Superdex-200 column (Amersham Biosciences) in buffer C (40 mM Tris-HCl, pH 8, 5 mM DTT), 30 mg of AcnA protein was recovered (95% pure). Protein concentrations were measured by the method of Bradford using bovine serum albumin as a standard, which in the case of NfuA overestimates the concentration by a factor of 1.2, as determined by the quantitative amino acid analysis of purified NfuA.

Iron and Sulfide Binding to NfuA—The following procedure was carried out anaerobically in the glove box at 18 °C. The

3 The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; DTT, dithiothreitol; NTA, nitritotriacetic acid; AcnA, aconitase; qRT-PCR, quantitative real-time PCR.
purified NfuA was obtained in its apoprotein form. To reconstitute the protein, 0.7 mM apo-NfuA was mixed with 10 μM IscS and 8 mM 1-cysteine (Aldrich) in buffer A and 3 mM 57FeCl3 previously reduced with 2 mM DTT was then added to the solution leading to a 5 mM DTT final concentration. After 4 h of incubation, the protein was passed through a NAP-25 column (Amersham Biosciences) to remove unspecified bound iron and then concentrated on a Microcon concentrator (Amicon).

Spectroscopic Analyses—UV-visible absorption spectra were recorded with an Uvikon XL spectrophotometer (BioTek Instruments). Zero field 57Fe-Mössbauer spectra were recorded using a 400-μl cuvette with a spectrometer operating in constant acceleration mode using an Oxford cryostat that allowed temperatures from 1.5 to 300 K and a 57Co source in rhodium. Isomer shifts are reported relative to iron foil at room temperature.

Fe/S Cluster Transfer from NfuA to Apo-aconitase—All the following procedures were performed anaerobically in the glove box at 18 °C. Aconitase A, isolated in its apoform, was incubated in buffer D (buffer A containing 5 mM DTT) with a 3.5-fold molar excess of the reconstituted NfuA for 2 h. DTT was removed from the solution on a Nap-10 column equilibrated with buffer A. Proteins were then separated on a Ni-NTA affinity column (1 ml) equilibrated with buffer A. The aconitase was recovered in the flow-through fraction, as it did not contain a polyhistidine tag. NfuA was eluted with buffer A containing 0.2 M imidazole. The fractions containing pure aconitase, as judged from SDS-PAGE analysis, were concentrated on a Microcon device before analysis by UV-visible absorption spectroscopy and for metal content.

Construction of nfuA Mutant Strains—Deletion of the nfuA gene was performed by the method described by Datsenko and Wanner (32), using the strain BW25113, pKD4, and the Ymut-Up and YmutDown primers. The chromosomal deletion of the nfuA gene was confirmed by PCR analysis using primers complementary to the cassette and to regions flanking nfuA (P1inv, P2, bioH, and gntTiny primers). P1 transduction was used to move the ΔnfuA::kan mutation into MG1655 to create the strain SA001. The kanamycin cassette was then eliminated by site-specific recombination using the FLP plasmid pCP20 (33). The resulting ΔnfuA strain was named SA002. P1 transduction was also used to introduce the ΔnfuA::kan mutation into various mutant backgrounds (supplemental Table S1).

Identification of NfuA, a Unique Nfu-like Protein—The yhgl gene was identified by searching the E. coli chromosome for genes encoding putative Nfu-like proteins. BLAST analysis showed that the Yhgl-predicted protein has two domains (Fig. 1). The N-terminal domain shares 24–28% identity with the A-type Fe/S proteins of E. coli (i.e. IscA, SufA, and ErpA). Surprisingly, however, it does not possess the three conserved cysteine residues thought to act as Fe/S cluster ligands in A-type scaffolds proteins. The C-terminal domain of Yhgl exhibits 21% identity with the Nfu domain of NifU from A. vinelandii and contains the conserved CXXC motif. This as yet undescribed association between an A-type and a Nfu-type scaffold domain prompted us to further investigate the role of Yhgl. Evidence presented below support the conclusion that Yhgl is indeed involved in Fe/S biogenesis. Accordingly, the gene will be referred to as nfuA.

RESULTS

Identification of NfuA, a Unique Nfu-like Protein—Data from sequence analysis showed that NfuA has similarity to Fe/S-binding proteins. Therefore, we tested whether NfuA binds a Fe/S cluster. To

RNA Extraction—Total RNA was isolated from cell culture according to the following protocol. Cells in early exponential growth were treated for 20 min with 2,2′-dipyridyl (250 μM) or paraquat (250 μM). An aliquot of the bacterial cultures was centrifuged (5000 × g for 10 min) to pellet the cells. RNA was isolated by extraction, using the SV total RNA Isolation System (Promega). RNA samples were further treated with 10 units of RNase-free DNase (Roche Applied Science) in a 100-μl volume for 20 min, and then extracted using phenol/chloroform. Then, total RNA was precipitated using a standard ethanol precipitation. The RNA was resuspended in 50 μl of RNase-free water. The RNA concentration and integrity were assessed by optical density and by electrophoresis on a 1% agarose gel with Tris acetate-EDTA buffer. PCR was performed to assess DNA contamination. If necessary, DNase treatment was repeated to ensure that no detectable chromosomal DNA was in the RNA preparation.

Quantitative Real-time PCR—The nfuA mRNA level was measured by quantitative real-time PCR (qRT-PCR). Y1 and YRT2 primers were tested prior to qRT-PCR analysis to confirm that they resulted in a single amplicon of the proper size when genomic DNA was used as template. Total RNA (20 ng) was reverse-transcribed using SuperScript II with 100 ng of random primers according to the manufacturer’s specifications (Invitrogen). A series of cDNA dilutions, along with 150 nM Y1 and YRT2 primers, was then subjected to qRT-PCR using an Eppendorf Mestercycler® ep realplex and SYBR® Premix Ex Taq™, according to the manufacturer’s specifications (TaKaRa). Data analysis and normalization were performed with software supplied with the Mestercycler.

RESULTS

Identification of NfuA, a Unique Nfu-like Protein—The yhgl gene was identified by searching the E. coli chromosome for genes encoding putative Nfu-like proteins. BLAST analysis showed that the Yhgl-predicted protein has two domains (Fig. 1). The N-terminal domain shares 24–28% identity with the A-type Fe/S proteins of E. coli (i.e. IscA, SufA, and ErpA). Surprisingly, however, it does not possess the three conserved cysteine residues thought to act as Fe/S cluster ligands in A-type scaffolds proteins. The C-terminal domain of Yhgl exhibits 21% identity with the Nfu domain of NifU from A. vinelandii and contains the conserved CXXC motif. This as yet undescribed association between an A-type and a Nfu-type scaffold domain prompted us to further investigate the role of Yhgl. Evidence presented below support the conclusion that Yhgl is indeed involved in Fe/S biogenesis. Accordingly, the gene will be referred to as nfuA.

NfuA Binds a [4Fe-4S] Cluster—Data from sequence analysis showed that NfuA has similarity to Fe/S-binding proteins. Therefore, we tested whether NfuA binds a Fe/S cluster. To
characterize NfuA, large amounts of soluble C-terminal Histagged NfuA protein were purified from E. coli under aerobic conditions. The molecular mass of the purified protein was ~25 kDa, as estimated from SDS-PAGE electrophoresis (data not shown). This is in agreement with the theoretical mass of 22,062 Da deduced from the DNA sequence. The oligomerization state of NfuA was determined by gel filtration on Superdex 75. NfuA eluted in a major peak corresponding to a dimer; a very small peak corresponding to aggregated forms of larger size could also be observed (data not shown). As isolated, the NfuA protein was colorless and did not bind detectable iron and sulfide. Accordingly, the corresponding UV-visible spectrum displays no absorption band in addition to the tyrosine and tryptophan residue absorption at 280 nm. Thus, the ability of NfuA to bind a Fe/S cluster was tested after incubation under anaerobic conditions with 4–8 molar equivalents of both ferric iron and sulfide in the presence of DTT. Such a treatment yielded a protein that contained a maximum amount of 4.2 iron atoms and 2 sulfur atoms per polypeptide chain. The UV-visible spectrum of the reconstituted NfuA (holo-NfuA) displayed an absorption band at 420 nm and a shoulder at 320 nm, indicating the presence of a Fe/S cluster (supplemental Fig. S1). Mössbauer spectroscopy was used to characterize the nature of the iron sites present in holo-NfuA. The reconstituted protein was prepared with $^{57}$FeCl$_3$/DTT, and the sulfide was provided by the IscS-dependent cysteine desulfurization reaction. The Mössbauer spectrum recorded at 78 K is shown in Fig. 2. The spectrum mainly consists of one relatively broad and asymmetric central doublet and a much weaker one with the high energy peak at $\pm 2.8$ mms$^{-1}$. The spectrum was simulated assuming three doublets, labeled A, B, and C. Doublet A is characterized by $\delta = 0.45(2)$ mms$^{-1}$ and $\Delta E_Q = 1.13(3)$ mms$^{-1}$. Doublet A was retained in a spectrum recorded at 4.2 K in a large velocity range (Fig. 2). This observation combined with the characteristic Mössbauer parameters of doublet A strongly suggest that the corresponding species is a $S = 0$ [4Fe-4S]$^{2+}$ cluster. By taking into account the spectra at the two temperatures, we estimate that 60–70% of total iron corresponds to such [4Fe-4S]$^{2+}$ clusters in the present sample. For doublet B, the parameters are $\delta = 0.45(2)$ mms$^{-1}$ and $\Delta E_Q = 0.70(3)$ mms$^{-1}$. At 4.2 K, the doublet B is replaced by a broad background. This behavior indicates that doublet B belongs to a paramagnetic species of unknown origin at present. Doublet C is characterized by $\delta = 1.4(2)$ mms$^{-1}$ and $\Delta E_Q = 2.7(2)$ mms$^{-1}$, and these parameters are consistent with a high spin ferrous species in an octahedral environment comprising N/O donors. It accounts for roughly 6% of total iron and is attributed to impurities. All these data suggest that NfuA is able to bind a [4Fe-4S] cluster, presumably at the interface of two monomers. This cluster is unstable both in the presence of oxygen and upon anaerobic reduction (data not shown). No EPR signals could be detected under oxidative conditions. However, anaerobic addition of one equivalent of dithionite results in an EPR signal whose properties are characteristics for an $S = 1/2$ [4Fe-4S] cluster in the +1 oxidation state. This signal accounts for less than 10% of the total iron (supplemental Fig. S2). This cluster is unstable and rapidly degraded in the presence of an excess of dithionite.

![Mössbauer spectra of holo-NfuA](image1)

**FIGURE 2. Mössbauer spectra of holo-NfuA.** Mössbauer spectra recorded at 78 K and 4.2 K of 0.7 mM Holo-NfuA. Solid lines above the experimental 78 K spectrum are theoretical simulations corresponding to doublets A, B, and C as described in the text. A theoretical curve corresponding to doublet A is superimposed on the experimental spectrum of 4.2 K.

NfuA Is Able to Transfer Clusters to Apo-aconitase in Vitro—We then tested whether NfuA could insert Fe/S clusters into apoproteins. The AcnA protein was incubated anaerobically with a 3.5-fold molar excess of reconstituted NfuA. After 2 h of reaction, AcnA was separated from NfuA on a Ni-NTA column and further analyzed by UV-visible spectrometry as well as for its enzymatic activity. This preparation was found to be active (supplemental Fig. S3), indicating that transfer of a [4Fe-4S] cluster has occurred. This is supported by the observation of an absorption band at 420 nm, characteristic of the holo-AcnA (supplemental Fig. S3). The iron and sulfur content in the AcnA preparation was 3 iron and 3 sulfur atoms per monomer. This is likely attributed to incomplete cluster transfer rather than the presence of a 3 Fe-bound form of AcnA, because no evidence for the presence of $S = 1/2$ [3Fe-4S]$^{2+}$ clusters could be obtained by EPR spectroscopy (data not shown). Together these results showed that aconitase can be activated by building [4Fe-4S] clusters during reaction with holo-NfuA.

The *nfuA* Gene Is Essential for Growth under Oxidative Stress and Iron Starvation—To investigate the physiological role of NfuA in *E. coli*, the *nfuA* gene was deleted, yielding the strain referred to as SA002. Early on, this gene was proposed to be related to gluconate metabolism, and was referred to as gntY, but we found that the SA002 strain exhibited no defect in gluconate metabolism (data not shown) (29). In contrast, in the...
presence of paraquat, a superoxide generator, the SA002 strain was severely affected in its ability to grow on rich-medium plates (Fig. 3A). Moreover, in the presence of 2,2'-dipyridyl, a strong iron chelator, the SA002 strain formed colonies much smaller than those of the wild-type strain (Fig. 3B). In contrast, growth was unaltered by addition of hydrogen peroxide (data not shown). In complementation experiments, the pUCnufA plasmid was able to restore the growth of the SA002 strain in the presence of either paraquat (Fig. 3C) or 2,2'-dipyridyl (data not shown), indicating that the growth defect was due to the deletion of nufA. These results showed that NufA is required for E. coli to grow in oxidative stress and iron starvation.

A. vinelandii contains a NufA protein that exhibits 52% identity with NufA from E. coli. To test whether A. vinelandii and E. coli NufA are functionally homologous, we carried out complementation tests. The pUCnufA-Av plasmid, encoding the NufA protein of A. vinelandii, was introduced into the SA002 strain. The SA002/pUCnufA-Av strain was able to grow on an LB plate containing either paraquat (Fig. 3C) or 2,2'-dipyridyl (data not shown). These results suggested that A. vinelandii NufA and E. coli NufA proteins perform similar functions in vivo.

IscR-mediated Induction of nufA Expression by Oxidative Stress and Iron Starvation—To know whether nufA expression is regulated by oxidative stress and iron depletion, qRT-PCR was performed. The wild-type strain (MG1655) was grown in LB until the early exponential phase and then exposed for 20 min to either paraquat (250 μM) or 2,2'-dipyridyl (250 μM). Total RNA was subsequently extracted, and the mRNA level of nufA was monitored. The results obtained in the presence of paraquat or 2,2'-dipyridyl showed a ~2-fold increase in the nufA mRNA level when compared with untreated cells (supplemental Fig. S4). Thus, the expression of nufA is induced during a superoxide stress and under iron depletion conditions. Recently, the nufA gene was found to belong to the IscR regulon (34). To investigate whether IscR was responsible for paraquat and 2,2'-dipyridyl induction of nufA expression, the same experiments as described above were conducted in a ΔiscR background. Induction ratios for both conditions were close to one (supplemental Fig. S4). This result indicated that the induction of the nufA gene expression by iron starvation and oxidative stress was mediated by IscR.

Functional Analysis of the NfuA Protein—The NfuA protein exhibits the unique feature of resulting from the fusion of two scaffold-related domains: an A-type at the N terminus and a NfuA-type at the C terminus. To investigate the functional importance of the two domains in vivo, recombinant plasmids that carried the separate N- or C-terminal domains of NfuA were constructed and introduced into the SA002 strain (Fig. 4A). Neither the N- nor the C-terminal domains of NfuA restored SA002 growth onto 2,2'-dipyridyl or paraquat-containing plates (Fig. 4B and data not shown). These results indicated that both domains are important for NfuA to fulfill its function in vivo.

NfuA possesses two cysteine residues, Cys-39 and Cys-44, in the N-terminal domain and two cysteine residues in the C-terminal domain, Cys-149 and Cys-152. To determine the contribution of these cysteine residues to the function of

FIGURE 3. NfuA is required for E. coli to grow under oxidative stress and iron starvation. A and B, wild-type (MG1655) and ΔnufA (SA002) strains were grown overnight at 37 °C in LB medium. Cultures were diluted, and 5 μl were directly spotted onto LB medium plates containing either 100 μM paraquat (A) or 250 μM 2,2'-dipyridyl (B). C, MG1655 and SA002 strains transformed with the pUC18 control vector, the pUCnufA, or the pUCnufA-Av plasmids were spotted on LB medium plates containing ampicillin, 100 μg/ml paraquat, and 0.2 mM IPTG. Growth was analyzed after overnight incubation at 37 °C. Each spot represents a 10-fold serial dilution.

FIGURE 4. Both N- and C-terminal domains and the two conserved cysteine residues are essential for NfuA function. A, schematic representation of the different NfuA constructs tested for complementation: the wild-type NfuA protein, NfuA derivatives with single cysteine to serine substitution (positions Cys-39, Cys-44, Cys-149, and Cys-152), the N-terminal domain of NfuA (amino acids 1–105), and the C-terminal region of NfuA (amino acids 101–191). B, complementation analysis of the ΔnufA mutant strain. The MG1655/pUC18 strain and the ΔnufA (SA002) strain, carrying the vector control pUC18, the pUCnufA, pUCnufA-C39S, pUCnufA-C44S, pUCnufA-C149S, pUCnufA-C152S, pUCnufA-Nt, or pUCnufA-Ct, were grown overnight in LB. 5 μl of serial dilutions of the overnight cultures were spotted on LB medium plates containing 100 μM paraquat. Plates were incubated overnight at 37 °C.
NfuA, each cysteine residue was individually substituted by a serine residue. The plasmids pUCnfuA-C39S, pUCnfuA-C44S, pUCnfuA-C149S, and pUCnfuA-C152S, allowing production of four cysteine variants of NfuA, were tested for their abilities to complement the phenotypes of the SA002 strain (Fig. 4A). Both the pUCnfuA-C149S and pUCnfuA-C152S plasmids failed to complement the growth defect of the SA002 strain in the presence of paraquat (Fig. 4B) or 2,2′-dipyridyl (data not shown). In contrast, the pUCnfuA-C39S and pUCnfuA-C44S plasmids were able to complement these growth defects under stress conditions. These results showed that Cys-149 and Cys-152, the two conserved cysteine residues of the Nfu domain of NfuA, are functionally important in vivo, while Cys-39 and Cys-44, the cysteine residues of the N-terminal domain are dispensable.

The cysteine variants of NfuA, NfuA-C39S, NfuA-C149S, and NfuA-C152S were purified to homogeneity. On a Superdex 75 column, all NfuA variants eluted in a major peak corresponding to a dimer, similar to what was observed with the wild-type NfuA (data not shown). In addition, similar solubility, stability, and proteinase K sensitivity were observed between the different variants and the wild-type protein (data not shown), indicating that the introduced mutations did not cause important structural modifications. As isolated, the NfuA variants were colorless and did not contain any iron and sulfide (<0.1 iron and sulfur per polypeptide chain). Thus, the NfuA variants were incubated with 4–8 molar excess of both ferrous iron and sulfide, under anaerobic and reducing conditions. Surprisingly, such treatment yielded proteins that contained amounts of iron and sulfide similar to those found in the wild type (3.5–4 equiv. iron and 1.8–2 equiv. sulfur per polypeptide chain). These results indicated that substitution of a single Cys residue is insufficient to eliminate Fe/S cluster binding in vitro.

**Gene Interaction between nfuA and the isc/suf Genes**—To identify a potential link between NfuA and the Fe/S cluster biogenesis systems known in E. coli, we tested whether a nfuA mutation would exert an additive phenotypic defect when combined with isc or suf mutations. Mutations in the isc system have been reported to yield a reduced growth rate, with the exception of iscA (35, 36). The nfuA mutation did not cause changes in growth parameters on its own (Table 1 and supplemental Fig. S5). The introduction of the nfuA mutation in the iscA, iscUA, iscB, or fdx backgrounds, and the resulting strains tested for their ability to grow in the presence of 2,2′-dipyridyl. Additive effects were observed in all cases (Fig. 5). Collectively, these results indicated that NfuA is connected to the Isc and Suf Fe/S cluster biogenesis systems.

**DISCUSSION**

In this article, we report the identification and the characterization of a new Fe/S protein of E. coli, which we named NfuA. NfuA presents the unique structural feature resulting from a fusion between a Nfu-type and an A-type domain. Both biochemical and genetic analyses demonstrate that NfuA is involved in Fe/S biogenesis under severe conditions such as iron starvation or oxidative stress.

That NfuA could bind a Fe/S cluster was demonstrated by Mössbauer spectroscopy. The results showed that NfuA was able to bind a [4Fe-4S]2+ cluster, and this conclusion was in agreement with data from the UV-visible absorption spectrum. Assays for iron/sulfide content indicated that holo-NfuA contained 2 sulfur and 4.2 iron atoms per monomer. This excess of iron with regard to sulfide was reproducible. An attempt to remove it by treatment with EDTA, an iron chelator, was successful, but in that case the Fe/S cluster was partially degraded as indicated by Mössbauer spectroscopy (data not shown). Further experiments to determine whether this additional iron reflects some adventitious binding or is biologically relevant, are under way.

NfuA contains two domains, each containing two Cys residues. Surprisingly, the N-terminal domain that shares sequence identity with A-type scaffolds does not possess the three cons-
**Escherichia coli NfuA**

served cysteine residues shown to be essential for the function of these Fe/S proteins (25). Moreover, mutation of the two N-terminal nonconserved Cys-39 and Cys-44 residues had no effect on in vivo activity, as measured by their ability to complement sensitivity to oxidative stress and iron starvation. In contrast, Cys-149 and Cys-152 residues from the C-terminal domain were expected to be functionally important as they are strictly conserved in all members of the Nfu family. Accordingly, we showed them to be essential for NfuA function in vivo.

A likely hypothesis is then that they act as ligands of the [4Fe-4S] cluster. However, in vitro characterization failed to support this view as all Cys-to-Ser variants constructed retained the ability to bind iron and sulfide. For diverse Fe/S proteins, such as anaerobic ribonucleotide reductase, ErpA and SufA, single cysteine mutations have already been showed to be insufficient to prevent Fe/S binding in vitro (37). To explain the fact that the cysteine variants NfuA<sub>C149S</sub> and NfuA<sub>C152S</sub> were not functional in vivo, one possibility is that the properties of the clusters, such as their stability, might have changed enough to impact on the function in vivo. More spectroscopic analyses and reactivity studies would be needed to test this hypothesis. However, we cannot rule out the hypothesis that the Cys-149 and Cys-152 residues may play another function in vivo. Interestingly, the Cys-149 and Cys-152 residues form a CXXC motif, and such a motif is essential for the activity of most proteins of the thioredoxin superfamily and some zinc finger proteins (38, 39). Collectively, our results demonstrate that Cys residues from the C-terminal domain are essential for NfuA to carry out its function in vivo. That these residues act as ligands for a [4Fe-4S] cluster is a good possibility, but discrepancies between in vivo and in vitro investigations will have to be resolved before such a conclusion is firmly established.

In vitro, NfuA exhibits properties expected for a protein participating in Fe/S biogenesis, of which the most compelling is that it could transfer its Fe/S cluster to the apoprotein of aconitase. Importantly, the aconitase form obtained upon incubation with NfuA had regained wild type catalytic activity, indicating that its [4Fe-4S] cluster was correctly assembled. Moreover, we noticed that NfuA shared some features with Nfu- and A-type scaffolds such as: (i) as-isolated NfuA did not contain iron and sulfide indicating that the cluster was labile; (ii) NfuA Fe/S cluster was partly degraded upon exposure to the iron-chelating reagent EDTA. Altogether these observations suggest that NfuA acts as a scaffold protein. However, the fact that an iron- and sulfide-containing form of NfuA could not be purified from cells might call for caution, and leaves open the possibility that NfuA actually intervenes in between a scaffold and an apotarget, acting as a Fe/S transporter.

Our in vivo analysis was most revealing in attributing a central role for NfuA in Fe/S biogenesis. The most convincing in vivo observation is that the extent of the phenotypic defects added up when combining mutations in the *nfuA* gene and mutations in either the *isc* genes or the *suf* genes. In a pure genetic sense, these data indicated that NfuA participates in the Isc- and/or Suf-dependent Fe/S biogenesis pathways. It is quite remarkable that NfuA might act in conjunction with two systems, Isc and Suf, which are supposed to work under different conditions. Such a possibility positions NfuA as a key factor used by the cell for Fe/S biogenesis, regardless of the environmental conditions, and, presumably, regardless of which system, Suf or Isc, is being used. An implication is that aptargets matured by NfuA are required under a wide spectrum of conditions for *E. coli* to grow properly.

The *nfuA* gene was found to belong to the IscR regulon (34). IscR is a Fe/S repressor that was shown to act as a transcriptional repressor of *nfuA* (34). Here, use of qRT-PCR allowed us to observe that *nfuA* gene was induced 2-fold by exposure to 2,2′-dipyridyl or paraquat. A hypothesis was that oxidative stress and iron starvation diminish the intracellular concentration of holo-IscR and rather favor the occurrence of apo-IscR, a form that would no longer bind the *nfuA* promoter. This hypothesis was supported by our observation that 2,2′-dipyridyl and paraquat induction of *nfuA* gene expression was found to be IscR-dependent.

As mentioned in the Introduction, the *nfuA* gene was identified in a series of quite different transcriptomic analyses. Considering these studies in conjunction with those collected here leads us to predict a most interesting role for NfuA in Fe/S protein folding. One study identified *nfuA* as belonging to a set of genes responding to translational misfolding (40). Included in this set of genes were most chaperones and heat-shock-responding proteins. In another study, *nfuA* gene expression was found to increase in the presence of kanamycin (41). Kanamycin binds irreversibly to the 30 S ribosomal subunit, inhibiting protein synthesis, and causing misreading of mRNA and presumably translational misfolding. Interestingly, the addition of kanamycin to *E. coli* has been shown to result in a pattern of protein expression similar to those induced by heat shock (41). Finally, *nfuA* was identified as a member of the σ<sub>32</sub>-dependent heat shock regulon (42). Thus, all of these transcriptomic studies point to a potential link between misfolding and NfuA. Considering this connection within our proposal that NfuA acts as a maturing factor of Fe/S proteins opens the exciting possibility that NfuA would be endowed with a chaperone/repair function for damaged Fe/S proteins. Evidently, the fact that NfuA is required for *E. coli* to survive under oxidative stress fits perfectly in this perspective, because such a condition is well known to lead to protein damage. Moreover, it is important to mention that, recently, the *nfuA* gene was found to be required for nutritional competence in *E. coli*, and as a matter of fact considered as a *com* gene (30). Although we have no speculative thoughts about the potential role of a Fe/S protein in the transformation process, it might be of interest to emphasize that in this study the *nfuA* mutant was found severely altered in its survival ability (30), again pointing to a general stress cellular role.

The structural organization of NfuA might be of interest to consider from an evolutionary point of view. Indeed, NfuA seems to have arisen from two events, a fusion between A-type and Nfu scaffolds, and the loss of functionally essential Cys residues in the N-terminal A-type domain. A similar scenario seems to have occurred in the evolution of other protein species: (i) the *Rhodobacter sphaeroides* Q3J011 protein is a fusion

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*S. Ollagnier-de Choudens, unpublished data.*
between IscU-type and Nfu-type scaffold domains, in which the IscU domain no longer contains the conserved functionally essential Cys residues (data not shown), (ii) Arabidopsis thaliana NFU1–3 and the Oryza sativa OsCsnfu-1A protein contain two Nfu domains (I and II), whereas domain II does not contain the conserved cysteine residues (24, 41). Our present characterization of NfuA showed that the two domains are important for function and that the Fe/S binding activity is very likely to be carried out by the Nfu-like domain. If that is the case, what is the function of the N-terminal A-type domain in NfuA and, by extension, what is the function of those domains that have lost their functional Cys residues? If one assumes that these domains used to be acting as scaffolds, we can infer that they exhibited both the ability to interact with apoprotein and to transfer their Fe/S cluster. Subsequently, a proposal is that these Cys-lacking domains kept the ability to interact with apoproteins. In this view, it is worth mentioning that mixing the O. sativa OsCsnfu-1A protein with a target apoprotein, led to a chemical shift change in the Cys-lacking domain, suggesting that it interacts with the target (43). Whether these Cys-lacking ancient scaffold domains have been retained and used throughout evolution to mediate protein-protein interaction is an exciting hypothesis currently under investigation.

In summary, we have identified a new player, which we named NfuA, involved in Fe/S biogenesis in E. coli. In a parallel study, a similar role was shown for the NfuA protein of A. vinelandii (44). NfuA seems to have arisen from the fusion of two scaffold proteins, an A-type and a Nfu-type, with concomitant loss of the scaffold function of the A-type domain. While it is still unclear whether NfuA acts as a scaffold or as a Fe/S transporter, our results show that it helps in maturing apotargets. Moreover, its role in survival under oxidative stress and iron depletion, in conjunction with a series of transcriptomic analyses from others, leads us to envision a general role for NfuA in Fe/S protein folding under stress conditions.

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Sandra Angelini, Catherine Gerez, Sandrine Ollagnier-de Choudens, Yiannis Sanakis, Marc Fontecave, Frédéric Barras and Béatrice Py

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