The ErpA/NfuA complex builds an oxidation-resistant Fe-S cluster delivery pathway

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Fe-S cluster–containing proteins occur in most organisms, wherein they assist in myriad processes from metabolism to DNA repair via gene expression and bioenergetic processes. Here, we used both in vitro and in vivo methods to investigate the capacity of the four Fe-S carriers, NfuA, SufA, ErpA, and IscA, to fulfill their targeting role under oxidative stress. Likewise, Fe-S clusters exhibited varying half-lives, depending on the carriers they were bound to; an NfuA-bound Fe-S cluster was more stable (t1/2 = 100 min) than those bound to SufA (t1/2 = 55 min), ErpA (t1/2 = 54 min), or IscA (t1/2 = 45 min). Surprisingly, the presence of NfuA further enhanced stability of the ErpA-bound cluster to t1/2 = 90 min. Using genetic and plasmon surface resonance analyses, we showed that NfuA and ErpA interacted directly with client proteins, whereas IscA or SufA did not. Moreover, NfuA and ErpA interacted with one another. Given all of these observations, we propose an architecture of the Fe-S delivery network in which ErpA is the last factor that delivers cluster directly to most if not all client proteins. NfuA is proposed to assist ErpA under severely unfavorable conditions. A comparison with the strategy employed in yeast and eukaryotes is discussed.

Fe-S clusters rank among the oldest cofactors, and they are surmised to have been key factors for life origin and evolution (1, 2). The first Fe-S proteins identified, by their electron paramagnetic signature, were those of mitochondrial respiratory complexes (3). In the following decades, biophysical and structural approaches revealed the great variety of Fe-S cluster proteins, whereas genomic analyses showed their widespread adoption in living organisms (4). Nowadays, in organisms, Fe-S cluster proteins participate in respiration, biosynthesis of cell building blocks and cofactors, central metabolism, gene regulation, tRNA modification, and DNA synthesis and repair (5).

In vitro studies revealed that, in favorable conditions, Fe-S clusters can form spontaneously on apoprotein clients (6, 7). However, in the 1990s, studies initiated on nitrogenase maturation revealed that in vivo Fe-S cluster assembly requires multiprotein systems: the NIF system dedicated to nitrogenase maturation and two general systems, ISC and SUF, which mature most, if not all, cellular Fe-S proteins (8–10). Components of the ISC and SUF systems are conserved throughout eukaryotes and prokaryotes. In eukaryotes, the ISC and SUF Fe-S machineries are located in mitochondria and chloroplasts, respectively (11, 12).

Fe-S cluster biogenesis proceeds in two steps, assembly and delivery. The former takes place on a scaffold protein, which allows sulfur and iron to meet and combine in an Fe-S cluster. Sulfur is produced via the catalytic degradation of L-cysteine by cysteine desulfurase; whereas the molecular source of iron remains elusive. The delivery relies on a series of Fe-S carriers that transport scaffold-bound clusters to the apo-clients. Numerous Fe-S carriers have been identified in both prokaryotes and eukaryotes. These include so-called A-type carriers (ATC),3 named IscA, SufA, and ErpA in prokaryotes and ISA1 and ISA2 in eukaryotes (13–17). Other carriers include the P-loop NTPases, (Ind1 in mitochondria, ApbC in Salmonella) (18–21), the monothiol glutaredoxins (Grx 5 in yeast and GrxD in E. coli) (22–24), and the highly conserved NFU-type proteins that have been shown to interact with target proteins (25–33).

Understanding how Fe-S clusters are delivered to the large number of functionally and structurally diverse Fe-S cluster proteins is a challenging task. Indeed, in most organisms, clients are actually a set of structurally and functionally diverse protein species, and it is difficult to think of a common maturation pathway shared by all of them. Moreover, Fe-S clusters...
are highly sensitive to environmental changes and, in particular, can be destabilized by reactive oxygen species (ROS), depending upon their solvent accessibility on the host protein (34–36). Last, Fe-S clusters arise under different structure (i.e. 2Fe-2S, 3Fe-4S, or 4Fe-4S) or in more complex association with other metal and cofactors (1). How organisms keep on inserting clusters in such a great diversity of substrates and conditions and whether all Fe-S proteins get their clusters delivered by the same set of factors is a daunting issue. Multiple parameters can be foreseen as controlling the delivery process: (i) genetically controlled level of delivery factors available in a given condition, (ii) affinity between delivery factors and apo-targets, and (iii) intrinsic biochemical features of delivery factors. Our previous study had illustrated how genetic regulation orchestrated the choice of routes Fe-S clusters take to reach essential enzymes, IspG/H, and the two transcriptional factors, IscR and NsrR, throughout fluctuating conditions (16, 37). The present study provides key findings on the two other parameters and provides us with an unprecedented view of Fe-S cluster trafficking and delivery.

We investigated the intrinsic capacity of IscA, SufA, ErpA, and NfuA to stabilize bound Fe-S cluster exposed to aerobic conditions. We proceeded by analyzing the contribution of each of them to the maturation of a series of Fe-S enzymes, including IspG and IspH, two essential Fe-S enzymes involved in the production of isoprenoids (38, 39). This provided us for the first time with a direct ranking of the Fe-S cluster carriers according to their intrinsic capacity to resist ROS potential damages. Another key finding was the observation of a privileged partnership between ErpA and NfuA, providing the cell with a new type of “hybrid” carrier, whose Fe-S cluster exhibited the highest level of resistance to ROS. Altogether, this allowed us to propose a model of the Fe-S cluster delivery network that predicts ErpA to be in charge of the maturation of most if not all Fe-S enzymes. Its closer association with NfuA appears as a new strategy for the cell to meet with transiently occurring destabilizing conditions.

Results

**NfuA is required for IspG/H maturation under oxidative stress**

Earlier, we found that growth of the *E. coli* ΔnfuA mutant on rich medium is severely affected in the presence of paraquat, a superoxide radical generator (25). Our previous analysis informed us that such a phenotype is probably due to an insufficient amount of isopentenylphosphate (IPP), due to a poor maturation of IspG and IspH, two Fe-S cluster–containing enzymes (17). Therefore, we tested whether the NfuA protein contributes to the Fe-S cluster delivery route toward maturation of IspG/H. For this, we introduced the eukaryotic IPP biosynthesis (Fe-S)–independent pathway, referred to as the mevalonate (MVA) pathway, in the ΔnfuA mutant and found that ectopic expression of this pathway was sufficient to rescue viability of the *nfuA* mutant (Fig. 1). This result indicated that NfuA is required for Fe-S cluster delivery to IspG/H under oxidative stress.

**NfuA interacts with ErpA**

To know whether NfuA cooperates with the ATC, we tested the interaction of NfuA with the ATC using the bacterial two-hybrid system. NfuA/ErpA interaction was observed as the BTH101 cells synthesizing the T18-NfuA and T25-ErpA hybrid proteins (pT18-NfuA and pT25-ErpA) exhibited β-gal activity (Fig. 2A). In contrast, no interaction was indicated between NfuA and IscA or between NfuA and SufA using this assay (Fig. 2A). ErpA/NfuA interaction was also tested by surface plasmon resonance (SPR) using apoproteins. Purified NfuA was immobilized onto a Biacore sensor CM5 chip, and ErpA was serially diluted and injected. The calculated dissociation constant ($K_d$) of the ErpA/NfuA interaction was determined to be $48.5 \pm 0.5 \, \mu M$ (Fig. 2B). In contrast, no interaction was observed between NfuA and SufA or IscA (data not shown). Taken together, these analyses revealed that NfuA and ErpA physically interact with each other and suggest that they might partner to form a discrete path within the Fe-S cluster delivery network.

**Unidirectional Fe-S cluster transfer from NfuA to ErpA**

To test whether Fe-S cluster transfer occurs between ErpA and NfuA, an *in vitro* test was used. Holo-NfuA contains a 4Fe-4S cluster per dimer, and ErpA is able to bind a 2Fe-2S cluster per monomer (17, 25, 26, 40). His-tagged holo-NfuA (1.2 ± 0.1 iron and 1 ± 0.2 sulfur/monomer; inset of Fig. 3A) was incubated, under anaerobic conditions, with one equivalent of untagged apo-ErpA, for 1 h. After separation by chromatography onto a Ni-NTA column, iron and sulfide contents of each protein were analyzed. ErpA contained 1 ± 0.1 iron and 0.7 ± 0.2 sulfur/monomer, and its visible spectrum was characteristic of a 2Fe-2S cluster protein with absorption bands at 420 and 320 nm. On the contrary, NfuA had lost its 420-nm cluster peak and contained less than 0.1 iron and sulfur/monomer (Fig. 3A). In a separate experiment, holo-ErpA was incubated with apo-NfuA, and the two entities were submitted to the same analyses as described above after separation. No modification either in iron/sulfide content or in spectrum was observed before and after co-incubation (Fig. 3B). Together, these analyses showed that, *in vitro*, a unidirectional Fe-S cluster transfer occurs from NfuA to ErpA.

**Stability of Fe-S cluster to oxidative damage varies with carrier identity**

*In vivo* studies showed that ErpA and NfuA are required for Fe-S cluster transfer under aerobicosis and oxidative stress, respectively. Therefore, we tested the stability of Fe-S clusters bound to NfuA and to ErpA when exposed to O$_2$. Before exposure to O$_2$, reconstituted holo-NfuA contained 1.4 ± 0.2 iron molecules per monomer and displayed a 4Fe-4S cluster-char-
characteristic UV-visible spectrum in agreement with published data (40) (Fig. 4A). The degradation of the Fe-S cluster was monitored by the absorbance variation at 420 nm as a function of time when diluted into oxygenated buffer containing a controlled amount of O\textsubscript{2}. Under these conditions, the half-life of the NfuA Fe-S cluster was 100 ± 10 min (Fig. 4A). Holo-ErpA exhibited a 2Fe-2S cluster-characteristic UV-visible spectrum (Fig. 4B). Upon controlled O\textsubscript{2} exposure, half-life of its cluster was 54 ± 4 min (Fig. 4B). We also tested the stability of the Fe-S cluster of two other ATC, IscA and SufA, under the same experimental conditions and found half-lives of 45 ± 3 and 55 ± 7 min, respectively (Fig. 4C). All together, these results showed that Fe-S clusters bound to diverse carriers exhibit different capacities to resist oxidative damage with the following decreasing stability order: NfuA > ErpA = SufA > IscA.

The NfuA/ErpA-bound cluster has an increased stability

Last, we tested whether stability of the ErpA bound Fe-S cluster was modified in the presence of NfuA. Both apo-NfuA and holo-ErpA proteins were mixed and exposed to O\textsubscript{2}, as above. Half-life of the Fe-S cluster bound to ErpA reached 90 ± 6 min (i.e. an enhancement by 2-fold as compared with Fe-S bound to ErpA alone) (Fig. 5). Thus, our results indicated that the presence of NfuA led to increased stability of the ErpA-bound Fe-S cluster toward O\textsubscript{2}.

NfuA and ErpA interact directly with the client proteins IspG and IspH

SPR was used to test the interaction between IspG/H and the Fe-S carriers. For the experiments performed with IspG, serially diluted IspG was injected into sensor chips coated with NfuA.
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![Figure 3. Unidirectional Fe-S cluster transfer between NfuA and ErpA.](image)

**Figure 3. Unidirectional Fe-S cluster transfer between NfuA and ErpA.** A, the apo-ErpA (150 μM) was first pre-reduced with 5 mM DTT. After removal of the latter, holo-NfuA apo, the Fe-S cluster donor, and the apo-ErpA proteins were mixed together in 0.1 M Tris-HCl, pH 8, 50 mM KCl in a molar ratio calculated to give one iron and one sulfur atom per ErpA monomer. After separation on the Ni-NTA column, the UV-visible spectra of the wash fraction containing ErpA (solid line) and the eluate fraction (dashed line) containing NfuA were recorded. B, for transfer from holo-ErpA to apo-NfuA, apo-NfuA (150 μM) was mixed together with the holo-ErpA in a molar ratio calculated to give two iron and two sulfur atoms per NfuA monomer in a buffer of 0.1 M Tris-HCl, pH 8, 50 mM KCl, 5 mM DTT. After removal of DTT and separation onto a Ni-NTA column, the UV-visible spectra of the wash fraction containing ErpA (solid line) and the eluate fraction (dashed line) containing NfuA were recorded. The insets show holo- and apoproteins, ErpA (solid line) and NfuA (dashed line), before the reaction.

or with ErpA. The sensorgrams showed binding of IspG to both NfuA and ErpA (Fig. 6). Global evaluation using the 1:1 Langmuir binding model yielded $K_d$ values of 22.5 ± 0.5 μM for the IspG/ErpA interaction and of 30 ± 2 μM for the IspG/NfuA interaction.

For the experiments performed with IspH, serially diluted NfuA or ErpA was injected into IspH-coated sensor chips. Although sensorgrams showed binding of NfuA and ErpA to IspH, the maximum response, was unattainable, preventing determination of a $K_d$ value (data not shown). We also used a bacterial two-hybrid approach to detect interactions between NfuA, ErpA, and IspH. We showed that the BTH101 cells synthesizing T18-IspH and T25-NfuA hybrid proteins exhibited β-gal activity, indicating an IspH/NfuA interaction (Fig. 7A). In addition, the two-hybrid system allowed us to show that the N-terminal domain of NfuA was sufficient to mediate the interaction with IspH (Fig. 7B). An interaction, albeit weak, was also observed between IspH and ErpA (Fig. 7A). In contrast, no interaction was observed between IspH and SufA or IscA (Fig. 7A). Collectively, these results indicated that NfuA and ErpA are able to interact directly with the client IspG and IspH proteins.

**Both ErpA and NfuA are required for the maturation of aconitase B and the respiratory complexes I and II**

Our previous study revealed a role of NfuA in the maturation of AcnB and the respiratory complex I (Fig. S1) (40). We then asked whether ErpA participates in the maturation of these Fe-S cluster-containing proteins.

First, we assayed the AcnB activity in the conditional mutant in which the expression of the erpA gene was under arabinose induction and glucose repression referred to as LL401 (ara, erpA), in which the ΔacnA mutation has been introduced (BP721) (17). ErpA-depleted cells were obtained after 3.5–4 h of growth in glucose-supplemented medium (Fig. 8). In the ErpA-depleted cells, aconitase activity was decreased by 80% when compared with the ErpA-replete cells grown in the presence of arabinose, whereas AcnB was detected at an identical level in both ErpA-replete and ErpA-depleted cells (Fig. 8). The NfuA protein level was slightly increased in ErpA-depleted cells (Fig. S2). SPR was used to investigate the interaction between ErpA and AcnB. Serially diluted ErpA (0–270 μM) was injected into sensor chips coated with AcnB (Fig. S3A). The $K_d$ value for the ErpA/AcnB interaction was 50 ± 5 μM. We also used SPR experiments to quantify the previously shown NfuA/AcnB interaction (40). Serially diluted NfuA (0–160 μM) injected into sensor chips coated with AcnB yielded a $K_d$ value of 29 ± 3 μM (Fig. S3B).

We then tested whether ErpA was also required for the maturation of complex I. ErpA-depleted cells of the LL401 strain exhibited a drastic decrease (70%) for complex I activity when compared with ErpA-replete cells grown in the presence of arabinose (Fig. 8).

Last, we tested the contribution of ErpA and NfuA to the maturation of the respiratory complex II (Sdh). When grown in glucose, the Sdh activity of the LL401 strain was found to be drastically reduced (85%) (Fig. 8). In the nfuA mutant, the Sdh activity was decreased by 35% (Fig. 9). Collectively, all of these data indicate that maturation of the respiratory complexes I and II is with the assistance of both ErpA and NfuA under aerobic conditions and that maturation of AcnB requires ErpA under aerobic conditions and NfuA under oxidative stress conditions.

**ErpA interacts with IscA and SufA**

Using the Biacore experiment, we tested whether ErpA can physically interact with the ATC of the ISC and SUF machineries, IscA and SufA, respectively. ErpA was immobilized onto a Biacore sensor CM5 chip, and IscA and SufA were serially diluted and injected. A dose-response curve was obtained, where an increase in response units was observed with increasing concentrations of IscA (0–200 μM) and SufA (0–600 μM), indicating that ErpA interacted with IscA and SufA (Fig. 10). The sensorgrams indicated that IscA and SufA were released in a short time without the necessity of chip regeneration. The calculated dissociation
constant ($K_d$) of the ErpA/IscA and ErpA/SufA interactions was determined to be $40 \pm 3$ and $90 \pm 2 \mu M$, respectively. These results indicated that ErpA is able to interact with IscA and SufA.

**Multicopy suppression of the nfuA growth defect by erpA**

The results above showed that NfuA and ErpA contribute to the maturation of the same set of enzymes. Therefore, we tested whether NfuA and ErpA are functionally redundant *in vivo*. For this, we made use of a multicopy-based suppression approach. A pBAD derivative plasmid expressing *erpA* (pBAD-*erpA*) suppressed paraquat sensitivity of the *nfuA* mutant (Fig. 11). In contrast, increased *nfuA* gene dosage failed to suppress nonviability caused by *erpA* mutation under aerobiosis (Fig. 12). Last, pBAD-*iscA* and pBAD-*sufA*, plasmids expressing *iscA* and *sufA*, respectively, failed to suppress *ΔnfuA* mutant sensitivity to paraquat (Fig. 11). Altogether, these data showed that ErpA overproduction overcomes the need of NfuA for IspG/H maturation but that the converse is not true.

**Discussion**

Interest in Fe-S cluster biology keeps expanding as one realizes how much these cofactors are central to multiple issues, from basic knowledge to molecular medicine, antibiotic resistance, and biotechnological applications (41, 42). In most organisms, several dozen structurally and functionally diverse client protein species need to acquire an Fe-S cluster for func-
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Figure 5. NfuA stabilizes the Fe–S cluster of ErpA. An equimolar amount of apo-NfuA was added to reconstituted ErpA, and the mixture was incubated for 1 h inside the glove box before being injected into a cuvette containing 100 μl of oxygenated Tris buffer (28.4 nmol of O2) to give a ratio of 1:20 for amount iron/O2. Right, UV-visible spectra every 20 min (0–160 min); left, representation of the decay of A420 as a function of time (∆A420 = A420 sample − A420 blank) (left).

Stabilizing. The diversity of clients, the diversity of the clusters, and the fluctuating environmental conditions under which Fe–S cluster trafficking takes place make the question of the delivery a most challenging issue. Here we show that synthesizing different carriers can afford different ROS resistance environments for transported Fe–S clusters, hence delivery circuits better adapted to sustain oxidative stress. We show that enhanced resistance to ROS can also be achieved by interaction between Fe–S carriers. We propose a model in which, right after the assembly step, the Fe–S trafficking network diversifies in multiple branches, which eventually converge toward ErpA. This unprecedented view of Fe–S cluster trafficking and delivery is discussed compared with the eukaryotic situation.

The present study allowed us to quantitate ROS resistance capacity of a cluster bound to NfuA, ErpA, SufA, and IscA carriers. Likewise, NfuA- and SufA-carried clusters showed the highest resistance to ROS. This is fully consistent with the genetically based view of SufA and NfuA as stress-responding factors and predicted to be better adapted to deliver clusters under oxidative stress conditions (16, 25, 26). NfuA results from the fusion between an NFU domain, in the C-terminal region, and, in the N-terminal region, a degenerated ATC domain lacking the Fe–S cluster-liganding cysteine residues (25, 26, 40). NfuA forms a dimer and binds a 4Fe-4S cluster in its NFU domain, probably at the dimer interface (25, 26, 40). Then it is possible that the cluster stability of NfuA is related to the four cysteinyl ligation and/or shielding by the dimer formation.

An unsuspected association between NfuA and ErpA was observed both by SPR and two-hybrid–based methods. Interestingly, the presence of NfuA led to increased stability of the ErpA-bound Fe–S cluster toward O2. An attractive hypothesis is that the ErpA/NfuA association resulted in an enhanced stability of the ErpA-bound cluster as compared with when bound to ErpA alone. Next, biochemical and structural studies will aim to test this hypothesis and investigate the molecular basis for the enhanced ROS resistance procured by NfuA. In particular, we will investigate whether apo-forms of NfuA stabilize the ErpA-bound cluster via its Cys-reactive residues and whether the A-type Fe–S carriers, IscA and SufA, might also exert the same effect.

Our previous phylogenetic studies permitted us to classify ATC into two different families. ErpA and IscA/SufA were classified into the ATC-I and -II families, respectively (16). Members of the ATC-I family were predicted to partnership with the apo-targets, whereas the ATC-II members were thought to be connected to scaffolds (16). The present bacterial two-hybrid and SPR-based investigations fully support the phylogenetically based functional prediction. Direct interactions were observed between apo-targets (IspG/H and AcnB) and ErpA, but not IscA and/or SufA (data not shown). Taken together with our previous genetic analysis, this observation confirms the view that scaffold-bound Fe–S clusters are transferred to ATC-II (IscA or SufA), which transfers them to ErpA, which delivers them to the apo-targets. In mitochondria, once assembled by the core biogenesis machinery, 2Fe–2S clusters reach a heteromeric platform, the ISCA1-ISCA2-Iba57 complex, which converts them into 4Fe-4S before targeting them to a so-called dedicated factor, which in turn allows maturation of cellular Fe–S proteins (15, 43–45). Mammalian ISCA1/2 proteins contain 2Fe-2S clusters. Heterodimer ISCA1-ISCA2 can assemble a 4Fe-4S cluster in the presence of GRX5, implying that the heterodimeric complex is the functional unit for 4Fe-4S cluster formation before their transfer to specific targets (45). ISCA1 is an ATC-II, like IscA and SufA, whereas ISCA2 is an ATC-I, like ErpA. In essence, therefore, the complex ISCA1-ISCA2 resembles the partnership IscA/ErpA or SufA/ErpA discussed above except that no stable bacterial ATC-II–ATC-I complex has ever been isolated. Note, however, that in mice, despite forming a complex, ISCA1 and ISCA2 are able to carry out separate tasks, presumably depending upon the substrates and the conditions (46).

Both NfuA and ErpA exhibit related affinity for Fe–S client proteins; the Kd values of the ErpA/IspG (23 μM) and NfuA/IspG (30 μM) interactions are within a similar range as those for the interaction between AcmB and ErpA (50 μM) or NfuA (29 μM). A Kd value of 44 μM was reported for the interaction of NfuA with MiaB, involved in tRNA modification (32). Thus,
like ErpA, NfuA appears to have all features required to interact directly with targets and could be positioned at the ultimate step within the Fe-S cluster delivery process. If direct transfer between NfuA and target proteins can occur \textit{in vivo}, its contribution appears very modest as compared with the contribution of ErpA. Hence, lack or depletion of ErpA caused a stronger phenotype than the lack of NfuA, as shown here by a drop in complex I and II activities by over 70 and 85\%, whereas an \textit{nfuA} mutant exhibited a 2-fold reduction in complex I and complex II activities. Similarly, aconitase B activity was down by 80\% in ErpA-depleted cells, whereas it was only slightly altered under stress conditions in cells lacking NfuA. Also, \textit{erpA} was able to act as a multicopy suppressor of \textit{nfuA}, whereas the reverse was not true. We thus favor the hypothesis that in \textit{E. coli}, NfuA acts conjointly with ErpA, by providing it an Fe-S cluster, rather than being an ultimate Fe-S donor for target proteins (Fig. 13).

The yeast and human Nfu1 and the \textit{E. coli} NfuA proteins share a conserved NFU-type C-terminal domain that binds a 4Fe-4S cluster. Interestingly, as NfuA, the yeast and human Nfu1 were found associated with the ISA proteins (33, 46). In yeast, defects exhibited by \textit{nfu1} mutation were of much lesser extent than those caused by mutations in the ISA complex

Figure 6. IspG interacts with NfuA and ErpA. Shown is surface plasmon resonance analysis of IspG to the Fe-S cluster carrier proteins NfuA and ErpA. A, serially diluted IspG (0 (red), 4.69 $\mu$M (green), 9.38 $\mu$M (blue), 18.75 $\mu$M (pink), 37.5 $\mu$M (cyan), 75 $\mu$M (yellow), and 150 $\mu$M (gray)) in HBS buffer was injected at a flow rate of 10 $\mu$L/min through flow cells with the immobilized NfuA on a CM5 chip. The injection of IspG (4.69 $\mu$M) was run in duplicate. B, serially diluted IspG (0 (red), 0.61 $\mu$M (green), 1.21 $\mu$M (blue), 2.44 $\mu$M (pink), 4.88 $\mu$M (cyan), 9.75 $\mu$M (yellow), 19.5 $\mu$M (gray), 39 $\mu$M (purple), and 78 $\mu$M (light green)) in Tris buffer was injected at a flow rate of 10 $\mu$L/min through flow cells with the immobilized ErpA on a CM5 chip. Inset, affinity curve of IspG flowing on NfuA (A) or on ErpA (B). The experiments were run in duplicate. RU, response units.
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In conclusion, independently of the organisms considered and of the type of Fe-S client proteins to be matured, a similar overall strategy has been retained throughout evolution. Despite the multitude of apoprotein clients waiting for their clusters, cells evolved a handful of carriers, which they either synthesize at appropriate levels under a set of conditions or combine in different higher-order organization, from homodimers to heterotetramers, each possibility providing a better solution to adapt to the diversity of client proteins and growth conditions. This study pinpoints how diversifying carriers and combining different carriers constitute two strategies that the cell has evolved to yield multiple delivery pathways, in particular a robust ROS-resistant delivery network.

Experimental procedures

Strains and growth conditions

Strains used in this study are listed in Table 1. The ΔacnA::kan KEIO mutation was introduced by P1 transduction (48). E. coli strains were grown in Luria–Bertani (LB) rich medium at 37 °C. Solid medium contained 1.5% agar. Ampicillin was used at 50 μg/ml. Arabinose (0.2%) and mevalonate (1 mM) were added when required.

Paraquat sensitivity test

The paraquat sensitivity test was performed on overnight cultures that were diluted in sterile PBS and directly spotted onto LB plates containing 100 μM paraquat (25). The plates were incubated overnight at 37 °C before growth was scored.

Plasmid construction

Construction of plasmids pT18-NfuA, pT25-NfuA, and pT25-ATC* was described previously (40). Plasmids pT25-ErpA, pT25-IscA, pT25-SufA, and pT18-IspH were constructed by, first, PCR amplification from the MG1655 chromosomal DNA using 2HErpAup/2HErpAdo, 2HIscAup/2HIscAdo, 2HSufAup/2HSufAdo, and 2HIspHup/2HIspHdo primer pairs. The erpA-, ispH-containing PCR fragments were digested by EcoRI/PstI enzymes, and the iscA-, sufA-containing PCR fragments were digested by the PstI/KpnI enzymes. All of the constructs obtained by PCR fragment cloning were checked by DNA sequencing.

Protein purification

Apo-NfuA, -ErpA, -SufA, and -IscA were obtained as described previously (25, 49). Aconitase B was purified as described for aconitase A (50). Purification of IspG-His was performed as described (17), and IspH was a gift of M. Seeman (Institut de Chimie, Strasbourg, France).

Surface plasmon resonance analysis

The binding studies were performed using a Biacore™ T200 instrument and CM5 sensor chips. Proteins were covalently immobilized onto the chip using a standard primary amine-coupling procedure. The same amine-coupling procedure, but without any protein, was performed on the reference channel as a blank to subtract any nonspecific binding signal. The analytes, dissolved in running Tris buffer (50 mM Tris, 150 mM NaCl, pH...
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7,4) or HBS buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% Tween, pH 7.4), were serially diluted to various concentrations with the running buffer. According to their kinetics of association and dissociation, they were injected for 50–125 s during the association phase at a constant flow rate of 10 μl/min at 25 °C. The dissociations were subsequently followed for 75–250 s at the same flow rate. For each cycle, the sensor surface was regenerated with 10 mM Gly-HCl buffer (pH 2.0) when the data collection was finished. To calculate the rate and affinity constants, the results from the sensograms were fit globally with BIAcore 3000 analysis software (BIAevaluation version 4.1) that proposes 1:1 Langmuir binding modes.

Bacterial two-hybrid technique

We used the adenylate cyclase-based two-hybrid technique (51). DNA inserts encoding the proteins of interest were obtained by PCR and were cloned into pUT18C and pKT25 plasmids. After co-electroporation of the BTH101 strain with the two plasmids expressing the hybrid proteins, plates were incubated at 30 °C for 2 days. Three milliliters of LB medium supplemented with antibiotics and isopropyl β-D-galactopyranoside at the recommended concentration (51) were inoculated and incubated overnight at 30 °C. β-Galactosidase activity was determined in Miller units.

Figure 8. Aconitase B and respiratory complex I and II activities are compromised in ErpA-depleted cells. Strains were grown overnight in LB supplemented with arabinose. Fresh LB medium with 0.2% glucose (Glu) or arabinose (Ara) was inoculated. Samples were taken and analyzed when cells reached late-exponential phase (3.5–4 h of growth). A, aconitase activity was assayed using the arap:erpA ΔacnA strain (BP721). B, immunoblot analysis was performed on glucose- or arabinose-grown cells of the BP721 strain, using antibodies raised against AcnB (top) and ErpA (bottom); identical amounts of total proteins were loaded. Extract of the ΔacnA ΔacnB double mutant (BP444) was used as a negative control for immunoblotting using antibodies raised against AcnB. The molecular mass (kDa) of the proteins used in the molecular weight size marker (MW) is indicated. D-NADH oxidase activity (C) and succinate dehydrogenase (D) activities were assayed from the LL401 strain (arap:erpA) strains. The experiments were run in triplicate, and the S.E. values are shown (error bars).

Figure 9. NfuA is required for respiratory complex II activity. Shown is the effect of a ΔnfuA mutation on SDH activity. Succinate dehydrogenase activity was assayed from MG1655 (WT) and MG1655 ΔnfuA. The experiments were run at least in triplicate, and the S.E. values are shown (error bars).
Figure 10. ErpA interacts with IscA and SufA. In surface plasmon resonance experiments, serially diluted IscA (0–200 μM) (A) and SufA (0–600 μM) (B), in Tris buffer, were injected at a flow rate of 10 μl/min through flow cells with the immobilized ErpA on a CMS chip. Inset, affinity curve of IscA (A) or SufA (B) flowing on ErpA. The experiment was run in duplicate. RU, response units.

Figure 11. Multicopy suppression of the nfuA growth defect during oxidative stress by erpA. WT (MG1655) and ΔnfuA strains transformed with the pBAD24 control vector or the pBAD-ΔnfuA, pBAD-ΔiscA, pBAD-ΔsufA, or pBAD-ΔerpA plasmid were spotted on LB medium plates containing ampicillin and arabinose, supplemented (right) or not (left) with 100 μM paraquat. Growth was analyzed after overnight incubation at 37 °C. Each spot represents a 10-fold serial dilution.

Figure 12. NfuA does not suppress lethality of the conditional erpA mutant. The LL401 araA:erpA/pUC18 (top), LL401 araA:erpA/pUC-erpA (middle), and LL401 araA:erpA/pUC-nfuA (bottom) strains were grown on LB medium plates supplemented with 0.2% arabinose (left) or 0.2% glucose (right).
In vitro Fe-S reconstitution

NfuA and the three ATC were reconstituted as described (25, 49). The purified proteins were obtained primarily in the apo-form and were reconstituted anaerobically in the glove box at 18 °C as follows. 0.08 mM protein was mixed with 5 mM DTT, 10 μM IscS, 1 mM L-cysteine, and 0.2 mM ferrous iron in 50 mM Tris, pH 8, 50 mM KCl. After a 90-min incubation, samples were passed over a desalting column and concentrated when needed.

Cluster transfer

All of the following procedures were performed anaerobically in the glove box at 18 °C.

Fe-S cluster transfer from holo-ErpA to apo-NfuA—Apo-NfuAHis (75 μM) was mixed for 1 h with holo-ErpA (reconstituted ErpA, as described above) in buffer A (0.1 M Tris–HCl, pH 8, 50 mM KCl). After a 90-min incubation, samples were equilibrated with buffer B (0.1 M Tris–HCl, pH 8, 0.1 M NaCl) before separation of the proteins on a Ni-NTA affinity column (1 ml) equilibrated with the same buffer. Fe-S donor proteins, which do not contain a polyhistidine tag, were recovered in the flow-through and wash fractions, whereas NfuAHis was eluted with buffer B containing 0.2 M imidazole and desalted over a Nap-10 column to remove imidazole. Each fraction (wash and elution fractions) was analyzed for its iron content, and a UV-visible spectrum was recorded.

Fe-S cluster transfer from holo-NfuA to apo-ErpA—Apo-ErpA was first pre-reduced with 5 mM DTT for 10 min. DTT was removed off a MicroBio-Spin 6 chromatography column. Reduced ErpA (150 μM) and holo-NfuAHis were mixed together in buffer B in a molar ratio allowing for the provision of one iron and one sulfur atom per ErpA monomer. After a 1-h incubation, proteins were separated on the Ni-NTA affinity column and analyzed as described above.

Protein analysis

Protein concentrations were measured by the method of Bradford using BSA as a standard. Iron and sulfide quantifications were carried out as described previously (53, 54). UV-visible spectra were recorded on a Cary Bio (Varian) spectrophotometer.

Fe-S cluster stability toward oxygen

To control the amount of oxygen that would react with the reconstituted protein Fe-S cluster, the experiment was performed using a cuvette containing 100 μl of a solution (0.1 M Tris, 0.1 M KCl, pH 8) equilibrated with air outside the glove box. At 20 °C and at 760 mm Hg, this solution contains 284 μM oxygen (55). The cuvette was sealed with a septum and intro-
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duced into the glove box. A controlled amount of the reconstituted proteins was introduced into the cuvette using a Hamilton syringe to give a ratio of 1:20 for amount of iron/O2. The kinetics of degradation of the Fe-S cluster was followed by UV-visible spectroscopy.

Enzymatic activity

Aconitase activity—Strains were grown in LB at 37 °C to an A600 of 0.8. The cells were harvested; washed with 50 ml of 50 mM Tris-HCl, pH 7.5, buffer; frozen in liquid nitrogen; and stored at −80 °C. The cell pellets were transferred to a Coy anaerobic chamber (90% N2, 10% H2) and resuspended in anaerobic 50 mM Tris-HCl, pH 7.5 (0.4% of the culture volume). Cells were lysed using a French press and centrifuged at 16,000 × g for 5 min. Supernatant was immediately frozen in liquid nitrogen. In the Coy anaerobic chamber, cell extracts containing 100 mg of protein were added to 50 mM Tris-HCl, pH 7.5, 0.6 mM MnCl2, 30 mM citrate, 0.2 mM NADP+, 1.7 units of isocitrate dehydrogenase in a 1-ml final volume. Aconitase activity was assayed by following the formation of NADPH in the coupled assay as an increase in absorbance at 340 nm (56).

NADH dehydrogenase activity—NADH dehydrogenase activity was adapted from a method described previously (57). Briefly, cells were harvested by centrifugation; resuspended in 50 mM phosphate buffer, pH 7.5; and lysed using a French press and centrifuged at 7700 J. Biol. Chem. for 5 min. Supernatant was immediately frozen in liquid nitrogen. NADH activity was assayed on the pellet fraction resuspended in 50 mM phosphate buffer, pH 7.5, 0.6 mM MnCl2, 30 mM citrate, 0.2 mM NADP+, 1.7 units of isocitrate dehydrogenase in a 1-ml final volume. Aconitase activity was assayed by following the formation of NADPH in the coupled assay as an increase in absorbance at 340 nm (56).

Succinate dehydrogenase activity—Cells were harvested by centrifugation; resuspended in 50 mM phosphate buffer, pH 7.5; and lysed using a French press. Following centrifugation (11,000 r.p.m. for 15 min at 4 °C), the supernatant was submitted to ultracentrifugation (45,000 r.p.m. for 2 h at 4 °C). SDH activity was assayed on the pellet fraction resuspended in 50 mM phosphate buffer, pH 7.5. Samples were preincubated for 30 min at 30 °C in 4 mM succinate, 1 mM KCN, 50 mM phosphate buffer, pH 7.5. The assay was performed by adding dichlorophenolindophenol (100 mM) and phenazine ethosulfate (1 mM) as substrate and by following A340.

Immunoblotting

Equal quantities of proteins were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membrane filter was incubated with appropriate antibodies (anti-AcnB, anti-ErpA) diluted 1:2000. Immunoblots were developed by using horseradish peroxidase–conjugated goat anti-rabbit antibody, followed by chemiluminescence detection.

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