ORIGINAL RESEARCH COMMUNICATION

Unraveling the Redox Properties of the Global Regulator FurA from *Anabaena* sp. PCC 7120: Disulfide Reductase Activity Based on Its CXXC Motifs

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Abstract

Cyanobacterial FurA works as a global regulator linking iron homeostasis to photosynthetic metabolism and the responses to different environmental stresses. Additionally, FurA modulates several genes involved in redox homeostasis and fulfills the characteristics of a heme-sensor protein whose interaction with this cofactor negatively affects its DNA binding ability. FurA from *Anabaena* PCC 7120 contains five cysteine residues, four of them arranged in two redox CXXC motifs. **Aims:** Our goals were to analyze in depth the putative contribution of these CXXC motifs in the redox properties of FurA and to identify potential interacting partners of this regulator. **Results:** Insulin reduction assays unravel that FurA exhibits disulfide reductase activity. Simultaneous presence of both CXXC signatures greatly enhances the reduction rate, although the redox motif containing Cys¹⁰¹ and Cys¹⁰⁴ seems a major contributor to this activity. Disulfide reductase activity was not detected in other ferric uptake regulator (Fur) proteins isolated from heterotrophic bacteria. *In vivo*, FurA presents different redox states involving intramolecular disulfide bonds when is partially oxidized. Redox potential values for CXXC motifs, –235 and –238 mV, are consistent with those reported for other proteins displaying disulfide reductase activity. Pull-down and two-hybrid assays unveil potential FurA interacting partners, namely phosphoribulokinase Alr4123, the hypothetical amidase-containing domain All1140 and the DNA-binding protein HU. **Innovation:** A novel biochemical activity of cyanobacterial FurA based on its cysteine arrangements and the identification of novel interacting partners are reported. **Conclusion:** The present study discloses a putative connection of FurA with the cyanobacterial redox-signaling pathway. *Antioxid. Redox Signal.* 20, 1396–1406.

Introduction

FERRIC UPTAKE REGULATOR (FUR) proteins are transcriptional regulators present in most prokaryotes. Initially, Fur was identified as the protein responsible for keeping iron homeostasis in *Salmonella enterica* Typhimurium (13), a process that is strictly regulated in all organisms. Despite its low bioavailability, iron can be a highly toxic element when absorbed in excessive amounts, leading to the production of highly reactive and damaging oxygen free radicals (58). To fulfill nutrient requirements avoiding iron toxicity, Fur usu-

Innovation

FurA is a global regulator in *Anabaena* which requires a reducing environment to optimally bind to DNA *in vitro*. In the present study we show that FurA exhibits a novel disulfide reductase activity, being the C₁₀₁XXC₁₀₄ motif the main responsible of this activity. Some FurA interacting partners have also been identified. These findings unveil an important link of this regulator with the redox-signaling networks of *Anabaena*.

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ally represses the transcription of several key genes involved in iron metabolism through its interaction with target DNA sequences named iron boxes (2). Fur proteins play a crucial role in cyanobacteria, whose iron requirements are around 10 times higher than in heterotrophic bacteria and imbalance of iron homeostasis readily ends up in oxidative stress and direct damage of the photosynthetic machinery (55).

In the cyanobacterium *Anabaena* PCC 7120, FurA is responsible for maintaining iron homeostasis and its coordination to the oxidative stress response (20–22, 43). FurA also participates in the control of several genes involved in nitrogen metabolism, like the master regulator NtcA (41, 42), and recent studies point to this protein as a heme-sensor in cyanobacteria (49). Phenotypic characterization of a FurA-overexpressing *Anabaena* strain, as well as the high representation of genes related to redox homeostasis as part of the FurA regulon (20, 21), indicates that, in addition to controlling iron metabolism, this regulator is critical for cyanobacterial defense against oxidative injuries. To have optimal binding of FurA to its DNA targets, both the availability of the metal co-repressor and a reducing environment are essential (27). Unlike other Fur proteins described in heterotrophic bacteria, FurA does not contain structural Zn²⁺ (26) and it exhibits five cysteine residues, four of them being arranged in two CXXC motifs (5). Site-directed mutagenesis and spectroscopic studies have shown that the CXXC sequence holding Cys¹⁴¹ and Cys¹⁴⁴ contains a heme-regulatory motif Cys-Pro, whose cysteine is involved in the coordination of this effector under aerobic conditions (49).

The CXXC motifs present in FurA are indeed a common signature of thiol-disulfide oxidoreductases. These canonical sequences are found in the active site of thioredoxins and thioredoxin-like proteins, being essential for their activity (51). Since the redox properties of the protein bearing the CXXC motif are modulated by the XX dipeptide located between the cysteines present in the active site, this signature has been described as a redox rheostat (9). In *Anabaena* FurA, both CXXC motifs contain one lysine residue preceding the terminal cysteine, allowing stabilization of the corresponding thiolates, a common feature of proteins acting as redox switches (4).

The functionality of many antioxidant proteins in cyanobacteria, as well as the basis of the redox control of the photosynthetic electron transport, relies on disulfide bond chemistry (39, 50). Among the main players involved in those processes, thioredoxins are some of the most extensively characterized proteins (19, 29). Thioredoxins are small disulfide-containing redox proteins that reduce disulfide bonds in other proteins through their CXXC motifs and are implicated in light-dependent enzyme regulation in all oxygen-evolving photosynthetic organisms, from cyanobacteria to higher plants (19). Thioredoxins can act not only as modulators of enzyme activity by reducing structurally important disulfide bonds in a target protein but also as reducing agents maintaining the redox homeostasis. Furthermore, direct interaction between thioredoxin and cyanobacterial response regulators such as PedR and Rre1 couples the thioredoxin-dependent redox status with changes in gene expression and signal transduction (30, 45). Together with other thiol-active proteins, such as glutaredoxins and peroxiredoxins, thioredoxins are highly represented in all cyanobacterial genomes (16, 39).

Other thiol-disulfide oxidoreductases include Dsb proteins, which regulate the formation of disulfide bonds in prokaryotes (25), as well as eukaryotic protein-disulfide isomerases,

whose versatility allows them to catalyze the formation, reduction, or isomerization of disulfide bonds depending on the redox environment and the characteristics of the substrate proteins (54). Disulfide bonds are important not only for the structural integrity of the proteins but also for the function of enzymes and transcription factors whose activities are controlled by thiol-disulfide exchange reactions (1, 10, 29). In cyanobacteria, the formation of disulfide bridges in the extracytoplasmic space requires the proper activity of the *SyndsbAB* gene product whose catalytic domains display significant similarity to proteins involved in disulfide bond formation in *Escherichia coli* and eukaryotes (56).

In the present study, the role of the CXXC motifs in the functionality of FurA from *Anabaena* has been investigated. We demonstrate that this regulator exhibits disulfide reductase activity, a property described for the first time in this superfamily of regulators. Besides, pull-down and two-hybrid assays evidence that FurA can interact with other potentially redox-responsive proteins, suggesting that, in addition to its iron-dependent regulatory activity, FurA might function as an important component of the cyanobacterial redox-signaling network.

Results

CXXC redox pairs in FurA confer disulfide reductase activity on the protein

FurA reductase activity was determined using the standard insulin reduction assay (28). To determine the contribution of each C₁₀₁VKC₁₀₄ and C₁₄₁PKC₁₄₄ motifs to the redox properties of *Anabaena* FurA, site-directed mutagenesis was performed to substitute cysteine residues into serine residues at positions 101, 104, and 133 (C101/104/133S) or 133, 141, and 144 (C133/141/144S), with the aim of maintaining each time only one CXXC motif in every recombinant variant. As controls, the C104/133/144S and C101/141/144S mutants were also tested. Additionally, a FurA mutant where all cysteines were replaced by serine residues (C101/104/133/141/144S) as well as a mixture lacking of FurA (insulin plus reduced dithiothreitol [DTT]) were used as negative controls.

Insulin disulfide reduction measurements show that FurA and the mutants containing at least one CXXC motif were able to catalyze insulin reduction compared to the negative controls (Fig. 1A). FurA displayed a higher rate of insulin reduction ($1.57 \pm 0.18 \Delta OD_{650} \cdot 10^{-3} / \text{min}^2$) than the different mutants. From those, the C133/141/144S variant was the most active ($0.55 \pm 0.04 \Delta OD_{650} \cdot 10^{-3} / \text{min}^2$), nearly 10-fold higher than that of the uncatalyzed control reaction (Fig. 1B and Table 1). Assays using the C101/104/133S FurA showed that this mutant was also capable of catalyzing the reduction of insulin but at a lower rate ($0.19 \pm 0.02 \Delta OD_{650} \cdot 10^{-3} / \text{min}^2$). Interestingly, a similar value ($0.13 \pm 0.02 \Delta OD_{650} \cdot 10^{-3} / \text{min}^2$) was obtained when the variant C104/133/144S was analyzed. The reductase activity in this mutant is consistent with the ability of Cys¹⁰¹ and Cys¹⁴¹ to form an intramolecular disulfide bond in the FurA C104/133/144S mutant (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars). These data suggest that cross-reactivity between cysteines in different CXXC motifs might contribute, at least in our *in vitro* experiments, to the overall reductase activity of FurA.

To determine whether this novel disulfide reductase activity of FurA from *Anabaena* was specific for the cyanobacterial

FIG. 1. Disulfide reductase activity of FurA mutant proteins. **(A)** Insulin reduction assays were performed in the reaction buffer containing 0.13 mM insulin, 50 mM Tris/HCl 7.5, 100 mM NaCl, 2 mM EDTA, and 10 μ M FurA proteins. Reactions were started by adding 0.33 mM DTT_{red}, and precipitation of reduced insulin was measured for 80 min at 25°C by the increase of OD at 650 nm. Buffer without protein and *Escherichia coli* DsbA (10 μ M) and DsbC (5 μ M) were used as negative or positive controls, respectively. **(B)** Disulfide reductase activity of FurA proteins.

protein or was a general feature of ferric uptake regulators, we purified the Fur proteins from the heterotrophic bacterial pathogens *Listeria monocytogenes* EGDe and *Helicobacter pylori* ATCC 26695. Both Fur orthologs were not capable of catalyzing the reduction of insulin (Supplementary Fig. S2),

TABLE 1. DISULFIDE REDUCTASE ACTIVITY OF FURA PROTEINS

Protein	$\Delta OD_{650} \cdot 10^{-3} / \text{min}^2$
DsbC	8.72 ± 0.69
FurA	1.57 ± 0.18
DsbA	0.90 ± 0.03
C133/141/144S	0.55 ± 0.04
C101/104/133S	0.19 ± 0.02
C104/133/144S	0.13 ± 0.02
C101/141/144S	0.01 ± 0.01
C101/104/133/141/144S	0.09 ± 0.01
Control	0.06 ± 0.01

The values of disulfide reductase activity shown by the tested proteins were calculated as previously described (44). Results are presented as the mean ± standard deviation of three independent experiments.

indicating that the occurrence of the disulfide reductase activity observed in the protein from *Anabaena* is not a common property of ferric uptake regulators.

Redox potential values of CXXC motifs in FurA are similar to the thiol-disulfide redox potential of the bacterial cytoplasm

Since the redox potentials of CXXC motifs are usually related to their *in vivo* functions, we sought to determine the reduction potential of both redox motifs in FurA. In our approach, DTT was used to modulate the redox state of the protein followed by a combination of thiol alkylation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to monitor the thiol redox state (31). The sequence of FurA contains five cysteine residues, which resulted in a complex pattern of bands upon electrophoretic analysis of the 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) modified wild-type protein (Supplementary Fig. S3). Consequently, and similar to earlier reports (37), we performed the assay with FurA triple cysteine mutants, which only contain one CXXC redox pair. Aliquots of the FurA

FIG. 2. Redox potentials of the FurA proteins. (A) Oxidized (OX) and reduced (RED) fractions of the proteins obtained by incubation with the indicated ratios $[DTT_{red}]/[DTT_{ox}]$ were subjected to thiol-trapping treatment with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. (B) Redox equilibrium constants were obtained by fitting the plotted data to nonlinear curves. Redox potential values were calculated by using the Nernst equation.

variants were incubated in redox buffers containing different ratios of reduced DTT (DTT_{red}) and oxidized DTT (DTT_{ox}), alkylated, and resolved by SDS-PAGE. The electrophoretic shifts under nonreducing conditions allowed the identification of the reduced fraction of the protein (R) in every aliquot (Fig. 2A). The equilibrium constants of redox reactions were obtained by plotting R against $[DTT_{red}]/[DTT_{ox}]$ (Fig. 2B), which resulted in K_{eq} values of $38.8 \cdot 10^{-3} \pm 0.43 \cdot 10^{-3}$ and $26.9 \cdot 10^{-3} \pm 0.27 \cdot 10^{-3}$ for C101/104/133S and C133/141/144S FurA variants, respectively. The corresponding redox potential values were calculated using the Nernst equation. Values of -235 and -238 mV were obtained for the redox pairs $C_{101}XXC_{104}$ and $C_{141}XXC_{144}$, respectively.

FurA forms disulfide bonds *in vivo*

For a protein to act as a disulfide reductase *in vivo*, its active site (CXXC motif) must be kept in the reduced state. To investigate the redox state of FurA in the cyanobacteria, we performed AMS trapping experiments according to Leichert and Jakob (40). Alkylation assays in wild-type *Anabaena* revealed that the majority of FurA contained a single reduced cysteine, as shown by the size shift of 540 Da of FurA in SDS-PAGE upon AMS alkylation. However, we could also detect an important fraction of FurA containing three reduced cysteines (size shift of 1620 Da), as well as fully reduced FurA (size shift of 2700 Da) (Fig. 3). In contrast with our previous *in vitro* results, where a large fraction of recombinant purified FurA formed dimers involving intermolecular disulfide bonds (26), the *in vivo* analysis shows only monomeric FurA. In spite of being considered a dimeric transcription factor, other Fur orthologs such as *Vibrio cholerae* Fur have also been detected as monomers in *Vibrio* cell extracts (61). This result clearly indicates the absence of intermolecular covalent bonds involving cysteines in

cyanobacteria and shows that, *in vivo*, FurA forms generally two and sometimes one intramolecular disulfide bond.

Identification of potential binding partners of FurA

Pull-down assays using crude extracts from *Anabaena* in the presence of DTT_{red} (Fig. 4), followed by matrix-assisted laser desorption/ionization-tandem mass spectrometry (MALDI-MS/MS) analyses allowed the identification of a number of potential *in vivo* FurA binding partners (Table 2). All1140, a 34 kDa hypothetical protein similar to N-acetylmuramoyl-L-alanine amidase previously found in the outer membrane fraction of *Anabaena* (46) specifically interacted with FurA-GST (Fig. 4, sample 1). From this sample, it was also identified Alr4123 (PRK), the phosphoribulokinase enzyme involved in CO_2 fixation, whose transcription is also controlled by FurA (41). Furthermore, a set of peptides corresponding to the DNA-binding

FIG. 3. *In vivo* redox state of FurA. *Anabaena* sp. PCC 7120 cultures were treated with 10% (v/v) trichloroacetic acid before being alkylated with 20 mM AMS. A control sample without AMS treatment was used. Western blotting analyses revealed several bands corresponding to FurA proteins with one (size shift of 540 Da), three (size shift of 1620 Da), or five (size shift of 2700 Da) AMS molecules.

FIG. 4. Glutathione-S-transferase (GST) pull-down assays to identify potential FurA targets. Purified FurA-GST fusion protein was incubated with a crude extract from *Anabaena*. Then, Glutathione (GSH)-Sepharose beads were added to recover FurA-GST with the potential interacting partners. SDS-PAGE and Coomassie staining revealed several bands, which were submitted to matrix-assisted laser desorption/ionization-tandem mass spectrometry (MALDI-MS/MS) analyses. Lane 1: molecular weight markers; lane 2: *Anabaena* crude extract control without adding any protein; lane 3: crude extract after adding FurA-GST; lane 4: control of crude extract with recombinant GST. Bands corresponding to FurA-GST and GST are indicated. Numbers from 1 to 3 in the gel point to samples from excised bands subjected to MALDI-MS/MS identification (1: All1140 and Alr4123; 2: Alr1691 and 3: Asr3935).

protein HU, a 10kDa FurA-regulated protein (Fig. 4, band 3) essential in heterocyst differentiation (35, 41), were identified. Finally, FurA was also found to interact with FurA-GST (Fig. 4, band 2). Indeed, it is well known that FurA is a transcriptional regulator that interacts with DNA as a homodimer (14).

To confirm those results, the proteins detected by pull-down were cloned and expressed in *E. coli* to be tested in a bacterial two-hybrid (BACTH) assay using FurA as a bait (33). Figure 5 shows that as expected, FurA interacts with another

FurA monomer *in vivo*. The BACTH assays also confirmed the interaction of FurA with the HU protein, as well as with All1140. However, we could not detect the interaction between FurA and PRK *in vivo*. This could be due to the heterologous *E. coli* expression system not being suited for the production of the functional form of PRK, capable of interacting with FurA as it occurs in cyanobacterial crude extracts. Indeed, PRK is a photosynthetic enzyme that requires different cofactors to be fully active, which might not be readily available in *E. coli*. Furthermore, instability or misfolding of high-molecular mass proteins like PRK has also been reported as a potential drawback of this technique (3).

Notably, FurA binding partners HU and All1140 have been localized in the outer membrane fraction of *Anabaena*, and FurA itself has been annotated as cell wall binding protein in the CyanoBase (<http://genome.kazusa.or.jp/cyanobase/Anabaena/genes/all1691>) and UniProt database (<http://uniprot.org/uniprot/Q7A2F4>). To confirm whether FurA is present in the cell wall fraction of *Anabaena*, localization assays were performed using a protoplast suspension and supernatant containing the outer membrane and the peptidoglycan fractions. Western blot analysis using polyclonal antibodies raised against FurA recombinant protein (26) showed that in *Anabaena*, wild-type FurA entirely localizes in the protoplasts (Fig. 6).

FurA does not bind glutathione

S-Glutathionylation plays a critical role in the regulation of signaling mediators under basal conditions, as well as in the protection against oxidative or nitrosative stresses (18, 32). In cyanobacteria, glutathione (GSH) is beneficial during acclimation to environmental and redox perturbations and essential during extreme oxidative stress (8). Although the glutathionylated proteome in cyanobacteria remains to be characterized, in other photosynthetic organisms many of the proteins that have found to be glutathionylated are thiorodoxins and other proteins involved in redox homeostasis exhibiting CXXC motifs (18). To evaluate whether glutathionylation might contribute to the redox-regulate FurA activity,

TABLE 2. POTENTIAL FURA TARGETS IDENTIFIED BY THE PULL-DOWN ASSAY IN ANABAENA

Protein	Gene	GenBank/UniProtKB accession number	Peptide sequences identified by MALDI-MS/MS	Coverage
All1140	<i>all1140</i>	GI:17130486/Q8YXS1	SGSHLFVLR ALGHEVIPCKPDR IAKPVLDIILKGYFNR LPSTPVSPVDEEQNIDTSILR GLTGKLPSTPVSPVDEEQNIDTSILR	21%
HU	<i>asr3935</i>	GI:17231427/P05514	VPFASAGKLF	11%
PRK	<i>alr4123</i>	GI:17131442/Q8YPR9	VYLAPPENLR FVIIIEGLGYSTR	11%
FurA	<i>all1691</i>	GI:17229183/Q7A2F4	AELNERGWR CNSTIEFKNDSILK LEADGEGISLSTIYR LEADGEGISLSTIYRTLK EGFHLLDCQMTHAVCPKCQR TAQKEGFHLLDCQMTHAVCPK ETILHIFQELPQGEHLSAEDLYHR EELGEGHKHYEINQYPHHHHHLICVK	78%

Anabaena proteins identified by MALDI-MS/MS after the band excision from the gel in the GST pull-down assays. All1140: putative N-acetylmuramoyl-L-amidase; HU: Asr3935, DNA-binding protein; PRK: Alr4123, phosphoribulokinase; FurA: All1691, ferric uptake regulator. MALDI-MS/MS, matrix-assisted laser desorption/ionization-tandem mass spectrometry; GST, glutathione-S-transferase.

FIG. 5. Bacterial two-hybrid assays. *E. coli* BTH101 blue colonies in LB X-Gal plates indicate strong positive interaction of FurA with All1140, HU, and FurA, whereas no interaction of FurA with phosphoribulokinase (PRK) could be detected (white colonies). Positive (+) and negative (−) interaction controls are also indicated. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

a potential interaction of biotin-conjugated GSH (BioGEE) and FurA has been analyzed. After incubation of different aliquots of reduced FurA with or without H₂O₂ and treatment with BioGEE, no signal was detected by Western blot analysis in samples corresponding to FurA, indicating that FurA could not be glutathionylated under the tested conditions (Fig. 7).

Discussion

FurA works as an iron-dependent global transcriptional regulator in the cyanobacterium *Anabaena* sp. PCC 7120. In the present study, we describe for the first time a novel disulfide-reductase activity for a Fur family member. This finding suggests that FurA may play a dual role in this cyanobacteria, functioning not only as transcriptional regulator of a plethora of genes involved in different functional categories but also in other processes involving dithiol/disulfide exchanges, such as redox signaling or maintenance of the redox homeostasis in *Anabaena*.

FIG. 6. Subcellular localization of FurA *in vivo*. Fraction of spherical protoplasts and supernatant containing cell wall proteins from *Anabaena* wild type were resolved by SDS-PAGE and western blotting analysis. Lane 1: cell wall fraction; lane 2: protoplast fraction; lane 3: lysozyme control.

FIG. 7. Analysis of glutathionylation of FurA *in vitro*. Prereduced FurA was treated or not with H₂O₂ before being incubated with biotinylated GSH ethyl ester (BioGEE). Western blotting analysis was performed to detect biotinylated GSH, using GST as positive control in the assay.

Interestingly, this novel redox activity appears to be unique to Fur proteins displaying features similar to *Anabaena* FurA since no disulfide reductase activity was detected in *Listeria* or *Helicobacter* Fur homologs. Crystal structures of *Helicobacter* and *Campylobacter* Fur proteins confirmed that its two CXXC motifs are involved in zinc binding in a ZnS₄ metal site (7, 12). LmFur has not yet been structurally characterized; nevertheless, a structural model based on the Fur family member *Bacillus subtilis* PerR suggests that the four cysteine residues in both CXXC motifs of LmFur are probably also involved in metal binding (15). Conversely to those Fur homologs, *Anabaena* FurA has been shown to contain neither zinc nor other structural metals (26). Metal ion coordination could prevent CXXC cysteine residues of HpFur and LmFur from reacting with insulin or other substrates, whereas in *Anabaena*, FurA cysteines might be available to catalyze this disulfide reductase reaction.

Disulfide redox potential of thiol-disulfide oxidoreductase enzymes generally varies between −95 and −330 mV, whereas structural disulfides generally have much lower values (62). The standard redox potentials for both CXXC pairs of FurA are consistent with redox active nonstructural disulfides. Since the redox potential of a highly oxidizing protein like *E. coli* DsbA is −121 mV and that of reductant *E. coli* thioredoxin is −270 mV (51), *Anabaena* FurA could be classified as a reductant protein with proven ability to reduce disulfide bridges. The values calculated for FurA are similar to that described for the bacterial cytoplasm, which is assumed to be between −220 and −240 mV (62). Consequently, cysteine residues in CXXC motifs present in FurA may be responsive to slight changes affecting cytosolic redox homeostasis, sensing specific, still unidentified, redox signals. For instance, heme-binding to the cysteine-proline regulatory motif involving Cys¹⁴¹ recently reported (49) might be related to cyanobacterial signal management and transduction. In addition, since a reduced form of FurA is required for an *in vitro* optimal DNA-binding (26) and an active photosynthetic electron chain is required for the transcription of the *furA* ortholog in cyanobacteria (43), we cannot exclude a light-driven modulation of the activity of FurA itself. Thus, cell redox status would not only influence the activity of FurA as a transcriptional regulator by tuning its interaction with DNA but would also determine the redox state of the cysteines involved in disulfide exchange. Furthermore, the occurrence of different forms of FurA *in vivo* indicates that it may react forming a new disulfide bond or reducing a preformed one,

depending on environmental conditions, such as light intensity or the level of oxidative stress. This hypothesis is in good agreement with the similar potential values obtained for both CXXC motifs that would enable the protein to reversibly respond to the appropriate signals. Our results point to the C₁₀₁XXC₁₀₄ pair as the major contributor to the FurA reductase activity, although the simultaneous presence of both CXXC motifs greatly enhances disulfide reduction rate. According to most known Fur structures (7, 12), the proximity of both cysteine motifs, as well as the absence of structural Zn²⁺ in *Anabaena* FurA, would eventually elicit electron transfer between cysteines belonging to different CXXC arrangements. This is supported by the slight disulfide reductase activity displayed by the C104/133/144S variant (Table 1) and evidences that all the cysteines present in the CXXC signatures are redox active. With the aim to identify the FurA reducing partner, its ability to interact with other cyanobacterial electron carriers such as reduced plastocyanin and thioredoxin was checked using cross-linking experiments. However, the formation of the complex necessary for electron transfer could not be detected in any of those cases (data not shown). In contrast, glutathione-S-transferase (GST) pull-down and two-hybrid assays revealed that FurA is able to interact in the cell with several potential partners *in vivo*. Among them, the hypothetical protein All1140 that contains an Ami₃ domain, related to N-acetylmuramoyl-L-alanine amidase activity, and two peptidoglycan binding domains, according to Pfam database. Additionally, a CXXC-derived motif (S₆₁QRC₆₄) followed by a predicted α -helix points to All1140 as a putative candidate redox protein (17). N-acetylmuramoyl-L-alanine amidases are enzymes that hydrolyze the amide bond between L-alanine and N-acetylmuramoyl residues in bacterial peptidoglycan (60). Surprisingly, analysis of known and predicted protein interactions of All1140 using STRING software retrieved Alr1690, the peptidoglycan binding protein encoded tail-to-tail to the *furA* gene, as a predicted target of All1140. Both *all1140* and *alr1690* gene products were found in the outer-membrane fraction of *Anabaena* (46). Furthermore, according to the STRING database, some microorganisms such as *B. halodurans* and *Oceanobacillus iheyensis* possess a unique protein containing the N-terminal region similar to Alr1690 and the C-terminal domain similar to All1140. Fur has also been related to the control of amidase activity in *H. pylori*, where differentially regulates two enzymes with amidase or formamidase activities. The *amiE* gene coding for an amidase is repressed at transcriptional level by a direct interaction of Fur with its promoter region, whereas the formamidase activity of AmiF is inhibited by Fur at enzymatic level through an unknown mechanism (59). Regarding All1140, *Anabaena* FurA does not interact with the promoter region of *all1140* (data not shown), but our pull-down experiments might suggest that the modulation of All1140 amidase activity could occur through protein-protein interactions. Another FurA-interacting protein identified in our pull-down assays was the DNA-binding protein HU, encoded by the *hanA* (*asr3935*) gene. This homodimeric protein containing a bacterial histone-like domain is also essential for heterocyst differentiation, albeit it is not present in these differentiated cells (35, 47). HU protein has been recently detected as a cell surface-associated protein in *Anabaena* (63) and has been proposed to play a role in controlling cell wall assembly, as it occurs in mycobacteria (34). In

addition to establishing protein-protein interactions, in *Anabaena*, FurA directly represses the expression of the *hanA* gene *in vitro* (41). Finally, pull-down assays suggests that FurA interacts with the PRK enzyme, encoded by *prk* (*alr4123*) gene. This result could not be confirmed by the two-hybrid experiments, probably due to the cofactor requirements and high-molecular mass of this enzyme that may hinder its correct folding in a heterologous nonphotosynthetic system, compromising its ability to interact with potential binding partners. In higher plants, PRK proteins are regulated by a ferredoxin-thioredoxin system under light/dark conditions (6). In cyanobacteria, these enzymes are also redox-regulated *in vitro*, but they are not reduced by thioredoxin, despite conserving the consensus cysteine residues (36). Further work to address molecular and catalytic interactions of FurA with their partners, as well as the identification of a potential FurA reducing partner is under way.

Our data indicate that despite being annotated as a cell wall binding protein in the Cyanobase, FurA localizes in the cytoplasmic fraction of *Anabaena*. Furthermore, several bioinformatics tools predict a cytosolic localization for All1140 despite the presence of peptidoglycan-binding domains in its sequence. Therefore, it can be speculated that FurA could interact in the cytoplasm with All1140 and the DNA-binding protein HU, stopping them from localizing in the outer membrane until the right, unknown signal is detected. FurA would then be released from the complexes allowing the transport of All1140 and HU to the outer membrane to participate in cell wall assembly during cell division or heterocyst development. Localization of FurA at the membrane in some environmental conditions cannot be excluded, although further work is needed to validate these hypotheses.

In summary, our results suggest a novel biochemical function of cyanobacterial FurA based on its disulfide reductase activity. In addition to iron-dependent transcriptional regulation, FurA can potentially work as a redox sensor, taking part in cyanobacterial signaling networks.

Materials and Methods

Bacterial strains and culture conditions

E. coli DH5 α was used for general cloning procedures, and the BL21 (DE3) strain was used for protein expression and purification. Both strains were cultured at 37°C on solid or liquid Luria Bertani medium supplemented with 50 μ g·ml⁻¹ kanamycin sulphate (Sigma-Aldrich). *E. coli cya*⁻ strain BTH101 was used for the BACTH assay and handled according to the instructions provided (Euromedex). *Anabaena* sp. PCC 7120 was photoautotrophically grown to mid-log phase in the BG11 medium (53) at 30°C, using an incubator shaker at 120 rpm with white light.

Genetic manipulations and protein purification

The *furA* gene (*all1691*) from *Anabaena* sp. PCC 7120 was inserted into the pET28a vector (Novagen; Merck) as previously described (5) to generate the pET28a-*furA* construct using the oligonucleotides FurA-Fw (ATTACCATGGCTGTCTACAC) and FurA-Rv (CTTGTAAGCTTACCAGGTGC). Several *furA* variants were generated by site-directed mutagenesis (Mutagenex), using pET28a-*furA* mutant vectors containing single cysteine to serine substitutions. The correct DNA sequences of

pET28a-*furA* mutant constructs were confirmed by Mutagenex. Recombinant expression and purification of the resulting FurA wild-type and mutant proteins were performed as previously described and obtained free of heme (48). Purification of the FurA batch used in the disulfide reductase assays was performed in 50 mM Tris/HCl, 10% glycerol, 0.1 M KCl buffer at pH 8 using a Heparin Sepharose 6 Fast Flow (GE Healthcare) affinity chromatography to minimize the precipitation of the pure protein. Purified FurA was eluted in the same buffer using a KCl gradient from 0.1 to 0.7 M. The wild-type form of FurA was also expressed and purified as a GST fusion protein. To this aim, the *furA* gene from *Anabaena* sp. PCC 7120 was amplified by PCR using *Pfu*-DNA polymerase (Promega) and the oligonucleotides FurAGST-Fw (CGGGATCCATGACTGTCTACA CAAATACTTC) and FurAGST-Rv (ACGCGTCGACCTAA AGTGGCATGAGCG). The PCR fragment was cloned into *Bam*HI-*Sall* sites of the pGEX4T-3 vector (GE Healthcare). The construct pGEX4T-3-*furA* was verified by DNA sequencing. Expression of recombinant proteins was induced by adding 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG; Ap-lichem) for 3 h at 30°C. Harvested cells were resuspended in buffer A (PBS [10 mM sodium phosphate buffer, pH 7.3, 140 mM NaCl, and 2.7 mM KCl] containing 10 mM reduced DTT [DTT_{red}; Sigma]) and 1 mM phenylmethylsulfonyl fluoride (Sigma). Cells were immediately sonicated in an ice-water bath and centrifuged at 48,000g for 60 min at 4°C. The soluble cell lysate was incubated with pre-equilibrated GSH-Sepharose resin (GE Healthcare). After washing with buffer A, FurA-GST protein was eluted with 50 mM Tris/HCl pH 8 containing 30 mM reduced GSH (Sigma) and concentrated by centrifugation in 10 kDa Centricon devices (Millipore). GST protein used as control through this work was expressed from pGEX4T-3 vector and purified by GSH-affinity chromatography similar to FurA-GST. Fur homologs from *L. monocytogenes* EGD-e (LmFur) and *H. pylori* ATCC 26695 (HpFur) were produced as N-terminally histidine-tagged proteins. Both *fur* genes (*lmo01956* from *Listeria* and *hp1027* from *Helicobacter*) were PCR amplified using the oligonucleotides LmFur-Fw (GGAGG AACCCATATGGAAGG) and LmFur-Rv (GCAATTCAGTC AAGCTTTTTG) for LmFur and HpFur-Fw (GGGAATTCAT ATGAAAAGATTAGAAAC) and HpFur-Rv (CCCAAGCTT TTAACATTCACTCTCTTGGC) for HpFur and cloned into the *Nde*I-*Hind*III sites of the pET-28a vector. *Listeria* and *Helicobacter* Fur recombinant proteins were expressed and purified as previously described (11, 15).

E. coli K-12 DsbA and DsbC proteins were obtained as C-terminally octa-histidine tagged proteins from pET42b::*dsbA* and pET42b::*dsbC* plasmids, respectively. Expression of the recombinant proteins was performed by the auto-induction method (57) and periplasmic proteins were isolated by cold osmotic-shock (24). Recombinant DsbA and DsbC were purified by nickel-affinity chromatography using Chelating Sepharose Fast Flow (GE Healthcare). Fractions containing DsbA or DsbC were pooled and dialyzed against 25 mM 4-(2-hydroxyethyl)-1-piperazine-N-2-ethanesulfonic acid (Hepes)-NaOH, 150 mM NaCl, pH 7.

AMS trapping of reduced thiols

FurA proteins (25 μ M) were incubated for 3 h at 30°C in a redox buffer containing 100 mM oxidized DTT (*trans*-4,5-dihydroxy-1,2-dithane, DTT_{ox}; Sigma) and 1 μ M or 10 mM

DTT_{red} to obtain oxidized or reduced FurA proteins, respectively. Reactions were stopped by adding 5% (v/v) trichloroacetic acid (TCA; Sigma), and proteins were precipitated by centrifugation at 13,300g for 10 min at 4°C. After washing pellets with cold acetone, air-dried protein pellets were resuspended in 10 μ l thiol-modification buffer containing 50 mM Tris/HCl pH 7.5, 1% (w/v) SDS (Sigma), and 20 mM AMS, disodium salt (Molecular Probes, Life Technologies Ltd.). Proteins in nonreducing Laemmli buffer (38) were resolved on 12.5% (w/v) polyacrylamide (30:0.8 [w/w] acrylamide/bisacrylamide) gels by SDS-PAGE and stained with Coomassie Blue R-250 (31).

For *in vivo* trapping experiments, mid-log cultures of wild-type *Anabaena* were incubated with a final concentration of 10% (v/v) TCA for 30 min on ice to avoid artificial thiol-disulfide modifications (40). After centrifugation at 8000g for 10 min at 4°C, pellets were washed with cold acetone and divided into two aliquots. One of them was resuspended in 25 μ l thiol-modification buffer and the other sample in the same buffer solution without the thiol-modifying agent AMS as control. Both samples were incubated for 2 h and subjected to 12.5% SDS-PAGE under nonreducing conditions and revealed by Western blotting analysis as previously described (20). In addition, samples of *in vitro* fully reduced and oxidized FurA were used as controls to compare the electrophoretic shifts of the distinct redox states of the protein.

Determination of redox potential

The redox potential values of FurA proteins were determined as previously described (31) with some modifications. Briefly, FurA proteins (40 μ M) were incubated for 3 h at 30°C in a water solution containing 100 mM DTT_{ox} and variable concentration of DTT_{red} (10 μ M to 2 mM). After incubation, proteins were precipitated with a final concentration of 5% (v/v) TCA and collected by centrifugation at 13,300g for 10 min at 4°C. Protein precipitates were washed with cold acetone and treated with thiol-modification buffer containing AMS. Proteins in nonreducing Laemmli buffer were resolved by 12.5% SDS-PAGE and stained with Coomassie Blue R-250. Intensities of bands were determined using the NIH ImageJ software (52). The equilibrium constant K_{eq} and the standard redox potential were calculated as previously described (23), taking the value of the standard redox potential of DTT of -312 mV as reference (31). A three-state model was used to analyze data from C101/104/133S as earlier reported (23), assuming that the intermediate band between the oxidized and the reduced protein corresponds to a putative DTT adduct.

In vitro disulfide reductase activity

The ability of the proteins to catalyze the reduction of insulin mediated by DTT_{red} was assessed as previously described (28). Aggregation of insulin free chain is determined by measuring the light scattering corresponding to the optical density (OD) at 650 nm. The thiol-disulfide oxidoreductases DsbA and DsbC proteins from *E. coli* were used as controls in the assay. As uncatalyzed negative control, a mixture of insulin plus DTT_{red} was used. The reaction mixtures were freshly prepared with a final concentration of 0.13 mM insulin (Sigma) in a reaction buffer containing 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, and 2 mM EDTA. Variable concentrations (5–10 μ M) of the tested protein were added to the cuvette

before the addition of a final concentration of 0.33 mM DTT_{red} to start the reactions. Precipitation of the reduced insulin chains was monitored at 25°C by for 80 min in a Cary 100 UV-Visible Spectrophotometer (Varian). The disulfide reductase activity was calculated as previously described (44).

Identification of FurA interacting partners

For GST pull-down assays, *Anabaena* sp. PCC 7120 were harvested at mid-log phase by centrifugation at 8000g for 10 min at 4°C and washed twice with cold buffer A. Cells were resuspended in 2.1 ml cold buffer A and sonicated before centrifugation at 16,000g for 10 min at 4°C. Soluble cell lysate was divided into three equal volumes. One aliquot was incubated with 400 µg FurA-GST, whereas 400 µg of purified GST was added to the second sample as a nonspecific interaction control and the last aliquot was untreated. All the mixes were incubated in a rotating wheel for 3 h at 4°C. After adding 40 µl pre-equilibrated GSH-Sepharose resin to each sample, incubation continued for 2 h at 4°C. Samples were centrifuged at 700g for 5 min, and the beads were washed five times with buffer A. Proteins were eluted with 35 µl buffer A and boiled for 5 min with Laemmli buffer. The bound proteins were resolved by 15% SDS-PAGE and stained with Coomassie Blue R-250. After band excision and in-gel tryptic digestion, protein identification was performed by MALDI-MS/MS at the Proteome Core Facility of Instituto Aragonés de Ciencias de la Salud (IACS), a member of ProteoRed (30).

Two-hybrid assays (BACTH) were performed according to Karimova *et al.* (33) using the kit provided by Euromedex and used following the manufacturer's recommendations. The *furA* (*all1691*), *all1140*, *asr3935* (*hanA*), and *alr4123* (*prk*) genes from *Anabaena* sp. PCC 7120 were amplified by PCR and cloned into the *KpnI* and *BamHI* sites of pUT18, pUT18C, pKT25, and pKNT25 vectors, using *E. coli* XL-1 Blue cells for cloning procedures. Termocompetent *E. coli* BTH101 cells were prepared and co-transformed by the heat-shock method with the different combinations of plasmids. Transformants (blue-positive or white-negative colonies) were selected on LB agar supplemented with ampicillin, kanamycin, 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (Sigma), and IPTG at 30°C for 48–72 h. *E. coli* BTH101 transformants obtained with provided pKT25-*zip* and pUT18C-*zip* or with pKT25 and pUT18C served as positive or negative controls for complementation.

Subcellular localization of FurA

Wild-type *Anabaena* sp. PCC 7120 cells were collected by centrifugation at 5000g for 10 min at 4°C. The cell pellets were washed with 30 mM Hepes pH 7, 400 mM sucrose, and 1 mM EDTA. After centrifugation, cells were resuspended in 500 µl washing buffer containing 1 mg/ml lysozyme (Sigma) and incubated for 30–60 min at 30°C. Spherical protoplasts in the absence of cell walls were pelleted by centrifugation at 5000g for 10 min at 4°C, and then, they were resuspended in 500 µl of double-distilled water. The supernatant solution containing cell wall proteins was collected. Both samples were subjected to 12% SDS-PAGE and western blotting analyses. A sample containing only lysozyme in washing buffer was used as control in the assay (64).

Analysis of biotinylated GSH ethyl ester interaction

Proteins (12.5 µg) were fully reduced by incubating with 25 mM DTT_{red} for 1 h at room temperature. Nonreacted DTT_{red} was removed using Nanosep centrifugal devices (Pall Corporation). Different aliquots were treated with or without 1 mM hydrogen peroxide (H₂O₂; Panreac) for 10 min, and then, they were incubated with 666 µM biotinylated GSH ethyl ester (BioGEE, a kind gift from Dr. F. Pallardó) for 1 h. Detection of biotinylated GSH was performed by electrophoresis under nonreducing conditions and western blotting analysis using streptavidin-linked horseradish peroxidase (HRP; Pierce, Thermo Fisher Scientific, Inc.) antibody. Free sites were blocked with 0.5% (w/v) bovine serum albumine (Sigma). Recombinant GST protein was used as positive control.

Bioinformatics tools

Protein–protein interactions were analyzed using STRING 9.0 database (<http://string-db.org/>), whereas SMART 7.0 was used to search the modular architecture of individual proteins (<http://smart.embl-heidelberg.de/>). Physicochemical properties of the proteins were deduced using the ProtParam tool (<http://web.expasy.org/protparam/>) from ExPaSy database. SignalP 4.0 Server (www.cbs.dtu.dk/services/SignalP/) was used to predict the presence and location of signal peptides and for subcellular location prediction PSORTb v3.0.2 (www.psort.org/psortb/) as well as SubLoc v1.0 (www.bioinfo.tsinghua.edu.cn/SubLoc/) were run.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

- AMS = 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid
 BACTH = bacterial two-hybrid assay
 BioGEE = biotinylated glutathione ethyl ester
 Cys = cysteine
 Dsb = disulfide bond protein
 DTT_{red} = reduced DTT, dithiothreitol
 DTT_{ox} = oxidized DTT, *trans*-4,5-dihydroxy-1,2-dithiane
 Fur = ferric uptake regulator
 GSH = glutathione
 GST = glutathione-S-transferase
 HpFur = *Helicobacter pylori* Fur
 IPTG = isopropyl β-D-thiogalactopyranoside
 K_{eq} = equilibrium constant
 LmFur = *Listeria monocytogenes* Fur
 MALDI-MS/MS = matrix-assisted laser desorption/ionization-tandem mass spectroscopy
 OD = optical density
 OX = oxidized protein(s)
 PAGE = polyacrylamide gel electrophoresis
 PRK = phosphoribulokinase
 R = fraction of reduced proteins
 RED = reduced protein(s)
 SDS = sodium dodecyl sulfate
 TCA = trichloroacetic acid
 X-Gal = 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside