Biochemical analysis of the recombinant Fur (ferric uptake regulator) protein from *Anabaena* PCC 7119: factors affecting its oligomerization state

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Fur (ferric uptake regulator) protein is a DNA-binding protein which regulates iron-responsive genes. Recombinant Fur from the nitrogen-fixing cyanobacterium *Anabaena* PCC 7119 has been purified and characterized, and polyclonal antibodies obtained. The experimental data show that Fur from *Anabaena* dimerizes in solution with the involvement of disulphide bridges. Cross-linking experiments and MALDI-TOF (matrix-assisted laser desorption/ionization time of flight) MS also show several oligomerization states of Fur, and the equilibrium of these forms depends on protein concentration and ionic strength. In intact recombinant Fur, four cysteine residues out of five were inert towards DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)], and their modification required sodium borohydride. Metal analysis and electrospray ionization MS revealed that neither zinc nor other metals are present in this Fur protein. Purified recombinant Fur bound to its own promoter in gel-shift assays. Fur was shown to be a constitutive protein in *Anabaena* cells, with no significant difference in its expression in cells grown under iron-sufficient compared with iron-deficient conditions.

Key words: cyanobacteria, DNA binding, iron stress.

INTRODUCTION

Even though iron is a very abundant element in Nature, iron deficiency is one of the most common stresses for microorganisms that limits growth. However, an excess of iron is toxic because of its ability to catalyse Fenton reactions. Consequently, iron uptake must be carefully regulated. The main molecular switches for the regulation of iron metabolism in bacteria have been identified as regulatory proteins that recognize specific DNA sequences: Fur (ferric uptake regulator) and DtxR. Iron-dependent Fur regulation has been exhaustively studied in *Escherichia coli*, where the *fur* gene encodes a 17 kDa DNA-binding protein [1]. The current model proposes that, when complexed to ferrous ions, a dimer of Fur binds to a specific DNA sequence (known as the Fur box) located in iron-responsive gene promoters and affecting gene expression either directly or indirectly, causing a cascade of negative and positive regulatory responses [2,3]. Fur exhibits DNA-binding activity in the presence of other bivalent metal ions, and although it has been proposed by some authors that Fur does not bind DNA in the absence of such metals [1,4], there are abundant contradictory data in the literature describing that Fur binds to these promoters even in the absence of metals [5,6]. The precise mechanism of how Fur binds the DNA remains unsolved, and the method of interaction of Fur with the different metal ions, as well as the interaction of the metal–Fur complex with its DNA target, are not yet clarified.

The Fur protein from *E. coli* was first purified in 1988 [7], and sequences of Fur homologues from at least 93 other bacteria are available in the databases to date. However, little is known about the structural features of the protein [5]. Members of the family of Fur proteins range in molecular mass between 14 and 20 kDa, and have histidine-rich regions that are highly conserved. Fur from *E. coli* is a dimeric protein with an N-terminal DNA recognition domain and a C-terminal metal-binding domain that is involved in dimerization [8,9]. Interestingly, Fur family members show regions with certain identity with the bed-finger patterns, a newly described motif in eukaryotic DNA-binding proteins [10]. In *E. coli*, Fur binds one Fe^{2+} and one Zn^{2+} per monomer [11,12], and binding seems to involve a cluster of conserved histidines and two cysteines in the C-terminal domain [11,13]. Fur from *Vibrio anguillarum* also shows conservation of a structural Zn^{2+} ion [14]. A second, weakly bound molecule of Zn^{2+} per molecule of Fur monomer has been described for the *E. coli* protein [12]. However, the metal ion binding site in Fur is still a controversial topic.

A large number of genes involved in iron acquisition have been described as being Fur-controlled. In addition, some genes not directly related to iron metabolism are controlled by Fur, such as some involved in acid shock response, oxidative stress or the well described production of toxins and other virulence factors [15].

In cyanobacteria, *fur* homologue genes have been identified in *Synechococcus* [16], *Synechocystis* [17] and *Anabaena* [18]. Fur boxes have been described in the promoter sequences of cyanobacterial genes such as *isiAB* [19] and *fur* [18], but the role of the master regulator Fur in cyanobacteria is still unexplored.

The IronEx II experiment performed in the Equatorial Pacific [20] demonstrated unequivocally that iron does limit primary production of the phytoplankton, with important environmental implications [21]. Moreover, uncontrolled cyanobacterial blooms often result in the introduction of toxins and carcinogenic compounds into the trophic chain. Extensive proliferations of cyanobacteria are often related to changes in iron availability, and have significant effects on the environment and public health [21,22]. Therefore understanding the mechanism of action of Fur, the regulator of iron metabolism, will give new insight into the control of cyanobacterial populations in different habitats. This knowledge requires an exhaustive study of the properties of Fur itself.

Abbreviations used: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; ESI-MS, electrospray ionization MS; Fur, ferric uptake regulator; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight.

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In this work, we report the purification and an extensive study of the properties of Fur from *Anabaena*, the first member of the Fur family to be purified and characterized from cyanobacteria. To date, the only extensive biochemical study of a Fur protein was limited to the *E. coli* protein. The study of the oligomerization of Fur may have important functional implications, as is the case with other DNA-binding proteins, and these results will be helpful in producing homogeneous preparations of Fur that will facilitate structural studies.

MATERIALS AND METHODS

Strains and growth conditions

Anabaena PCC 7119 was obtained from the Pasteur Culture Collection. Cloning and overexpression of the *fur* gene was performed as previously described by Bes et al. [18]. *Nco*I and *Hin*dIII restriction sites were generated at the 5′ and 3′ ends of the *fur* open reading frame by PCR. After digestion with these enzymes and purification of the DNA using the GFX-PCR purification kit (Amersham Pharmacia), the amplified *fur* gene was ligated into the *Nco*I and *Hin*dIII sites of the expression vector pET28a(+) (Novagen). Cultures were grown from a fresh transformant at 37 °C in Luria–Bertani medium supplemented with 30 μg/ml kanamycin sulphate. Once the absorbance at 600 nm reached 0.7–0.9, isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM. Cells were harvested after 2–3 h of incubation. *Anabaena* sp. PCC 7119 was grown in BG11 medium [23] at 22 °C. Cells were cultured in iron-replete concentrations (30 μM) or in iron-deficient medium (0.5 μM iron).

Purification of recombinant Fur from *Anabaena* sp. PCC 7119

A typical purification procedure started with 25–30 g of recombinant *E. coli* cell paste, which was resuspended in 2.4 ml/g 50 mM Tris/HCl, pH 8, containing 10 % glycerol and final concentrations of protease inhibitors of 1 mM PMSF, 1 μg/ml pepstatin, 1 mM benzamidine and 2 μg/ml leupeptin.

The cell suspension was disrupted by sonication with a Branson sonifier for 3 min (five pulses of 45 s at 45 W). Lysates were clarified twice by centrifugation at 30000 ***g*** for 20 min each time. The resulting supernatant was applied to a heparin–Sepharose 6 Fast Flow (Amersham-Pharmacia) column (2.5 cm × 30 cm) equilibrated in 50 mM Tris/HCl, pH 8, 0.1 M KCl and 10 % glycerol at 4 °C. The column was washed with 10 vol. of the equilibration buffer, and Fur was eluted with a linear gradient of 0.1–0.7 M KCl in 50 mM Tris/HCl, pH 8, containing 10 % glycerol. Fur eluted at approx. 0.4 M KCl. Fur-containing fractions were pooled and applied to a Zn–iminodiacetate column (Chelating Sepharose Fast Flow; Amersham Pharmacia; 1.8 cm × 20 cm). The metal affinity column was equilibrated as indicated by the manufacturer using 3 vol. of 0.25 M $ZnSO_4$, washed with 5–10 vol. of water, followed by 10 vol. of 50 mM Tris/HCl, pH 8, containing 0.4 M KCl and 10 % glycerol. The column was then washed with 5 vol. of 0.5 M $(NH_4)_2SO_4$ in 50 mM Tris/HCl (pH 8)/10 % glycerol. After a second wash with 3 vol. of 35 mM glycine in 50 mM Tris/HCl (pH 8)/10 % glycerol, Fur was eluted with a linear gradient of 0–1 M imidazole in 50 mM Tris/HCl (pH 8)/10 % glycerol. Fractions were analysed by SDS/15 %-PAGE. Fur eluted at approx. 0.5 M imidazole. The pure fractions were pooled and dialysed against 10 mM acetic acid/acetate, pH 4, containing 10 % glycerol. The samples were frozen in liquid nitrogen and stored at −80 °C.

Alternatively, Fur protein was purified using nickel in the iminodiacetate column instead of zinc. In this case, 3 vol. of 0.25 M $NiSO_4$ was used in the metal equilibration step. *E. coli* Fur protein was also purified according to [7].

Immunological methods

Polyclonal antibodies against *Anabaena* Fur were obtained using protein in a 0.15 M NaCl solution mixed with 1:1 (v/v) Freund's adjuvant (1 ml, final volume). A 100 μg sample of Fur was injected subcutaneously into New Zealand White rabbits in five aliquots of 200 μl. A second immunization was performed after 4 weeks, using 75 μg of Fur in the same conditions but using incomplete Freund's adjuvant. Serum was obtained after 1 week, and tested using the Ouchterlony method [24].

SDS/PAGE, Western blotting and isoelectric focusing

SDS/PAGE was performed as described by Laemmli [25], using 15 % (w/v) polyacrylamide [30:0.8 (w/w) acrylamide/bisacrylamide] gels. Precasted Novex™ 8–20 % SDS/PAGE gels were also used. Determination of the pI of Fur was performed using a Phast System from Amersham Pharmacia, according to the manufacturer's instructions. Immunoblotting was performed as described by Towbin et al. [26]. Chemical cross-linking experiments were performed in 10 mM acetic acetate buffer, pH 4, using purified Fur protein. The reaction mixture was incubated for 30 min at room temperature with 0.5 % glutaraldehyde. Cross-linked samples were boiled for 5 min under standard denaturating conditions for SDS/PAGE, and the samples were analysed in 15 % gels. Fur protein was concentrated when indicated using an Amicon Stirred Ultrafiltration Cell (Millipore).

Analytical methods

Total protein was quantified using the bicinchoninic acid method (BCA™ Protein Assay Reagent Kit; Pierce). Fur was quantified spectrophotometrically on the basis of the molar absorption coefficient calculated in the present study. The molar absorption coefficient at 276 nm was determined based on the protein quantification of pure samples. The theoretical value of the molar absorption coefficient was also calculated based on the numbers of Trp, Tyr and Cys residues [27].

For elemental analysis, glassware was rinsed several times with 0.1 M HNO_3 and then with MilliQ-grade water. The metal/protein stoichiometry study was carried out according to Althaus et al. [12]. Analysis was performed using Fur samples obtained from the different purification procedures, some of which were incubated with 4 equivalents of $ZnCl_2$ and dialysed extensively using 2 mM ammonium acetate, pH 4, or 2 mM ammonium bicarbonate, pH 8. When indicated, an aliquot of Fur was pretreated with 100 mM metal-free EDTA. Metal analysis was performed by inductively coupled plasma MS in a PerkinElmer ELAN 6000 instrument (Servicios Científico-Técnicos, Barcelona University, Spain).

The free thiol content of Fur preparations was determined by using a 10-fold molar excess of Ellman's reagent, DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)], under denaturing conditions [28]. The total thiol content was confirmed in the presence of 8 M urea and excess $NaBH_4$ [29].

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS was performed on a Voyager-DE-RP device (Applied Biosystems; Servicios Científico-Técnicos of Barcelona University). Sinapinic acid was used as matrix, and BSA and

cytochrome *c* were used as standard proteins. For the experimental analysis, 5.5 μM Fur was mixed with sinapinic acid [10 mg/ml in water/acetonitrile (1:1, v/v) containing 0.3 % trifluoroacetic acid], using a 1:6 ratio. Electrospray ionization MS (ESI-MS) was also performed at the Servicios Científico-Técnicos at Barcelona Universtiy, using 20 μM Fur in 2 mM ammonium acetate, pH 4. Myoglobin was used as standard, with an estimated error of $\pm$2.9 Da. When Fur was tested at pH 8, 75 μM Fur in 2 mM ammonium bicarbonate, pH 8, was used.

Gel filtration on an FPLC Superose-12 column (Amersham Pharmacia) was also carried out. The protein was eluted at a flow rate of 0.5 ml/min using 10 mM acetic acid/acetate, pH 4, or 50 mM Tris/HCl, pH 8, containing, in both cases, 2 mM EDTA, 1 mM dithiothreitol (DTT) and variable concentrations of NaCl (from 100 to 800 mM).

Protein–DNA gel retardation

Binding assays were carried out as described in [18] using a modified binding buffer which contained 10 mM Bis-Tris, pH 7.5, 40 mM KCl, 0.1 mM $MnCl_2$, 0.5 mg/ml BSA and 15 % glycerol. In the assays at pH 4, 10 mM sodium acetate/acetic acid was used instead of Bis-Tris. DNA fragments to be used in electrophoretic mobility band shift assays were obtained by PCR and purified further using the GFX PCR DNA and Gel Band Purification kits (Amersham Pharmacia). To demonstrate the specificity of the DNA-binding activity of the Fur protein to its own promoter, reactions were carried out in the presence of several concentrations of a non-specific competitor DNA from the fourth exon of the human *apoE* gene (results not shown).

Absorption spectral studies

These were carried out using UV–visible double-beam Kontron Uvikon 860 or 942 spectrophotometers.

NMR studies

NMR samples were prepared up to a final volume of 450 μl by using Amicon concentration cells. Small amounts of neat deuterated acetic acid were added to give a final pH of 4; 50 μl of ^2H_2O was also added to allow for lock detection. The pH was measured at the beginning and end of every experiment using a Russell glass electrode; no differences between the two measurements were found. The solution was centrifuged briefly to remove insoluble protein and then transferred to a 5 mm NMR tube. Spectra were recorded on a Bruker AMX-600 spectrometer, working at a ^1H frequency of 600.13 MHz, and at 25 °C. 3-(Trimethylsilyl)propionic-2,2,3,3-$^2H_4$ (sodium salt) was used as an external chemical shift reference. The protein concentration was approx. 125 μM. One-dimensional spectra were acquired using 16 K data points, with water presaturation, averaged over 1000 scans and using a 7801.69 Hz spectral width (13 p.p.m.). Spectra were processed in an SGI workstation and baseline-corrected using Bruker software.

RESULTS AND DISCUSSION

Purification of recombinant *Anabaena* Fur and biochemical characterization

The described purification method is based on two chromatographic steps: a heparin–Sepharose column, and a chelating Sepharose column to which Fur binds by means of metal affinity interactions. It is noticeable that the histidine-rich motif present in Fur from *Anabaena* gives this protein high affinity for metals. Therefore, since the extra His-tag provided by pET28a(+) was not necessary for purification, the *fur* gene was cloned using the *Nco*I and *Hin*dIII sites in order to avoid unnecessary modifications of the Fur protein which could alter its behaviour compared with the native form. Alternative purification procedures not involving a heparin matrix were tried, but in all of them it was necessary to remove nucleic acids prior to any other chromatographic step, in order to avoid heterogeneity in Fur distribution during the purification procedure. Different nucleic acid precipitating agents, such as streptomycin sulphate and poly(ethyleneimine), were assayed in crude extracts of *E. coli* containing recombinant *Anabaena* Fur, but large amounts of Fur were lost. Metal affinity chromatography was performed preferentially with zinc, since the use of nickel resulted in a lower yield of pure Fur, due to the increase in impurities in several Fur-containing fractions. Imidazole was preferred as eluent over histidine due to the low solubility of the latter in the elution buffer at 4 °C. Once purified, Fur protein from *Anabaena* could not be stored concentrated at neutral or basic pH, due to marked protein precipitation.

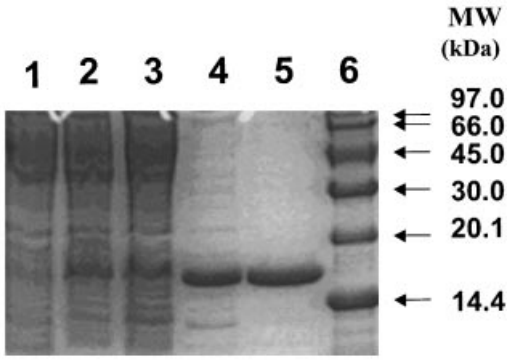

Figure 1 Purification of Fur from *Anabaena* sp. PCC 7119 expressed in *E. coli*, monitored by SDS/PAGE

Lane 1, soluble *E. coli* extract without the expression plasmid; lane 2, crude extract from transformed cells after induction with isopropyl β-D-thiogalactoside; lane 3, soluble crude extract after sonication and clarification; lane 4, Fur-containing fractions after the heparin–Sepharose column; lane 5, Fur-containing fractions after the metal affinity (Zn-iminodiacetate) column; lane 6, molecular-mass markers.

The purity of the sample was estimated as > 99 %. Figure 1 shows the progression of the purification on SDS/15 %-PAGE, where fractions after the zinc affinity column (lane 5) showed a single band corresponding to a molecular mass of 17 kDa. The N-terminal sequence of this protein was determined previously, and corresponded to the predicted sequence of recombinant Fur protein [18]. Isoelectric focusing gave two main isoforms (results not shown), with estimated pIs of 6.0 and 6.4 respectively; the calculated theoretical pI is 6.9.

The obtained molar absorption coefficient at 276 nm, 13760 $M^{-1} \cdot cm^{-1}$, was comparable with the theoretical value deduced from the protein sequence, i.e. 12940 $M^{-1} \cdot cm^{-1}$ assuming all possible cysteine residues are half-cystines. This theoretical method is consistent with globular proteins, with an error of 5–10 % [27]. Fur from *Anabaena* has a single Trp, five Tyr and five Cys residues, with a low molar absorption coefficient in the near-UV range.

Although cysteine residues have been postulated to play a key role in metal and DNA binding [30], little information exists concerning their oxidation state and reactivity. The *Anabaena* Fur protein has five cysteine residues in its sequence, as deduced from the nucleotide sequence, three of which are close to the

C-terminus. In the obtained recombinant Fur, none of the cysteines were reactive to DTNB. After unfolding with 8 M urea or 6 M guanidinium chloride, four out of five cysteines were inert towards DTNB, even in the presence of EDTA, which would remove metals. Complete modification of the five cysteines of *Anabaena* Fur by DTNB requires unfolding of the protein and the presence of the strong disulphide reducing agent sodium borohydride. The SH oxidation state has been studied in *Vibrio anguillarum* Fur, and in this protein all five cysteines were accessible to chemical modification by DTNB. In *E. coli* Fur, three or four cysteines [7,12] have been found to be accessible, and Cys-92 and Cys-95 have been shown to be essential for protein activity both *in vivo* and *in vitro* [31]. However, when aligning known Fur sequences (http://wwwbioq.unizar.es/departamento/investigacion/Fur.htm), we observe that some members of the family do not conserve these cysteine residues, or even do not have any cysteines in their sequence (Fur from *Pseudomonas putida* [32]; Swiss-Prot databank entry O68563). The DNA-binding properties of *Pseudomonas* Fur, even though it possesses either one cysteine or none, are well documented [33], so it seems that the presence of cysteines may be not essential for the activity of all Fur proteins.

One-dimensional NMR experiments at pH 4 and 25 °C indicated that the recombinant Fur was folded, but also aggregated at the concentrations used for NMR (125 μM). The spectra showed up-field shifted signals at 0.3, 0.4 and 0.5 p.p.m. (spectrum not shown); these signals probably belong to methyl groups of residues involved in tertiary interactions. In the amide region, the amide protons were in the range 7.5–9.00 p.p.m., also suggesting the presence of tertiary structure. However, these signals in either the methyl or the amide regions were broader than those expected for a monomeric protein of this size. Thus we can conclude that under these conditions at pH 4 Fur is folded, although we cannot discern whether that structure is due to intra- or inter-molecular interactions.

Recombinant *Anabaena* Fur protein does not contain zinc or other metals

The determination of the total Fe, Zn, Mn, Cr, Co, Ni, Cu, Mg and Cd contents in Fur revealed that no metals were present in significant amounts in the samples of purified recombinant Fur protein, either at pH 4 or at pH 8. An attempt to reconstitute Fur with $ZnCl_2$ also gave negative results. Duplicate EDTA-treated samples gave the same values. This was confirmed by very accurate ESI-MS, which gave a molecular mass of exactly that calculated from the amino acid composition (estimated error lower than ± 3 Da). ESI-MS determinations were performed either with Fur dialysed against 10 mM acetic acid/acetate buffer, pH 4, or with a sample of Fur maintained at pH 8. The published results for the three Fur proteins investigated to date with regard to their zinc content, i.e. those from *E. coli* [12], *Bacillus subtilis* [6] and *Vibrio anguillarum* [14], suggested that the presence of structural zinc is a characteristic of the Fur family. In our case, it seems that this is not a feature of *Anabaena* Fur, although possible artefactual loss of zinc during the purification of the protein cannot be ruled out. The absence of Zn was also found in samples of recombinant *Anabaena* Fur protein obtained using nickel as the affinity metal instead of zinc, while *E. coli* Fur, used as control, contained 1.92 mol of zinc/mol of protein.

Oligomeric state of the Fur protein

The oligomeric state of the recombinant Fur protein was studied by SDS/PAGE in the presence and absence of reducing agents. Figure 2 shows that the monomer was the only detectable form

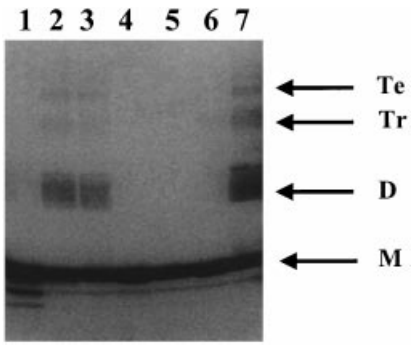

Figure 2 SDS/PAGE (8–20% gels) of purified Fur protein showing the oligomerization of Fur in the presence and absence of SH-modifying reagents

Portions of 7 μg from a 70 μM Fur solution were used. Lane 1, 5 mM DTT and 15 mM $MnCl_2$; lane 2, 15 mM $MnCl_2$; lane 3, Fur without additives; lane 4, 5 mM DTT; lane 5, 50 mM DTT; lane 6, 10 mM 2-mercaptoethanol; lane 7, 15 mM diamide. Arrows indicate the estimated molecular masses of the oligomers: M, monomer; D, dimer; Tr, trimer; Te, tetramer.

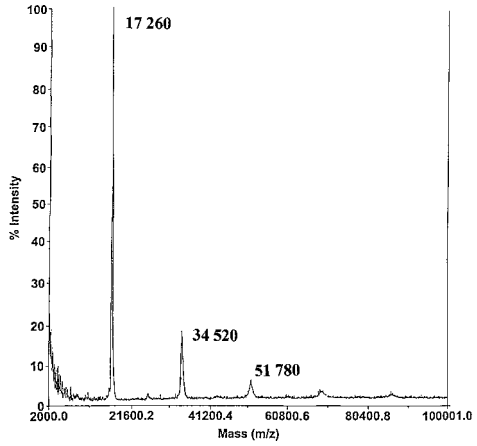

Figure 3 MALDI-TOF MS spectrum of Fur protein (5.5 μM)

when DTT or 2-mercaptoethanol was present (lanes 4–6), while in absence of reducing agents a dimer was also present (lane 3), although the monomeric form was predominant. The presence of metal (Mn) did not change the oligomerization pattern, with only monomer in the presence of DTT (lane 1) and monomer plus dimer in absence of the reducing agent (lane 2). It is interesting to note that, when Fur was incubated with Mn and DTT simultaneously (lane 1), the appearance of low-molecular-mass products indicated increased Fur lysis. The presence of an oxidizing agent, diamide [azodicarboxylic acid bis(dimethylamide)], promoted high-molecular-mass forms (lane 7), with detectable trimer and tetramer bands.

Gel filtration experiments were difficult to carry out with the *Anabaena* Fur protein, because of its low UV absorbance and uncontrolled precipitation into the gel matrix. Due to very poor protein recovery, Superose-12 FPLC gel filtration is unsuitable for oligomerization studies with this protein.

MALDI-TOF MS showed several oligomerization states of Fur, with the monomeric form being predominant (Figure 3). It is interesting to note that, even though the Fur concentration was very low (5.5 μM), appreciable relative amounts of dimeric and trimeric forms were present, and even some Fur tetramer and

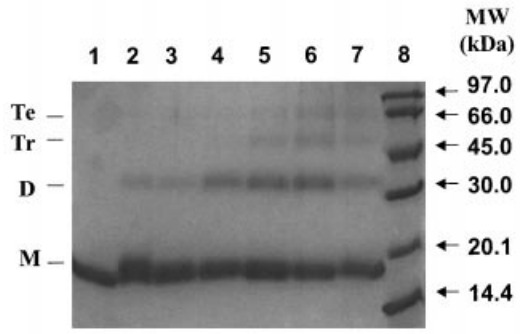

Figure 4 Cross-linking of Fur at different concentrations

Fur aliquots of increasing concentration were treated with 0.5% glutaraldehyde; 10 μg of each sample was analysed by SDS/15%-PAGE. Cross-linked samples were boiled in the presence of 2-mercaptoethanol. Lane 1, untreated Fur, as a negative control; lane 2, 15 μM Fur; lane 3, 50 μM; lane 4, 100 μM; lane 5, 250 μM; lane 6, 500 μM. In order to investigate if the oligomerization was reversible, 500 μM Fur was diluted and treated with glutaraldehyde. Also, 10 μg of Fur was applied to the gel. Lane 7, 500 μM Fur diluted to 15 μM; lane 8, molecular-mass standards. The estimated molecular masses of the oligomers are indicated: M, monomer; D, dimer; Tr, trimer; Te, tetramer.

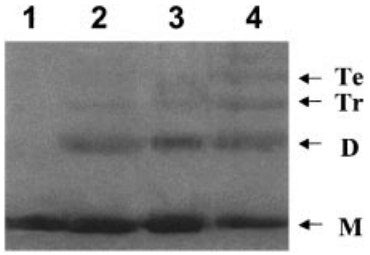

Figure 5 Effect of ionic strength on the *in vitro* oligomerization of Fur

Cross-linking experiments with glutaraldehyde using 300 μM *Anabaena* Fur were performed in the presence of different amounts of NaCl. Portions of 7 μg of protein were analysed by SDS/15%-PAGE. Cross-linked samples were boiled in the presence of 2-mercaptoethanol. Lane 1, Fur not treated with glutaraldehyde; lane 2, 300 μM Fur without salt; lane 3, 300 μM Fur with 20 mM NaCl; lane 4, 300 μM Fur with 500 mM NaCl. Arrows indicate the estimated molecular masses of the oligomers: M, monomer; D, dimer; Tr, trimer; Te, tetramer.

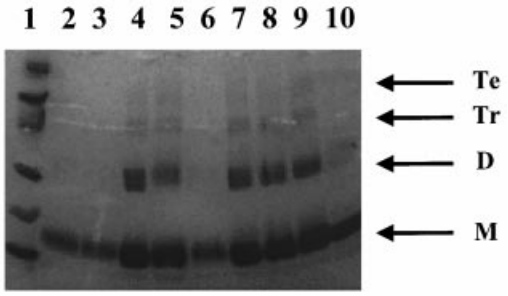

Figure 6 Effect of thiol oxidation on Fur oligomerization

Cross-linking experiments with glutaraldehyde using 140 μM *Anabaena* Fur were performed in the presence of different SH-modifying reagents. Portions of 9 μg of Fur were analysed by SDS/PAGE (8–20% gels). Cross-linked samples were boiled in the presence of 2-mercaptoethanol. Lane 1, molecular-mass standards (14.4, 20.1, 30, 45, 66 and 97 kDa); lane 2, Fur incubated with 5 mM $MnCl_2$, treated with 50 mM DTT and cross-linking agent; lane 3, Fur incubated with 50 mM DTT, treated with 5 mM $MnCl_2$ and cross-linking agent; lanes 4–9, cross-linked Fur in the presence of 15 mM diamide (lane 4), 30 mM 2-mercaptoethanol (lane 5), 50 mM DTT (lane 6), 3 mM DTT (lane 7), 0.1 mM DTT (lane 8), and without SH reagent (lane 9); lane 10, untreated Fur. Arrows indicate the estimated molecular masses of the oligomers: M, monomer; D, dimer; Tr, trimer; Te, tetramer.

pentamer. Only the monomeric form was detected when a similar experiment was performed in the presence of 1 mM DTT (spectrum not shown). ESI-MS of the monomer gave a calculated mass of 17259.6$\pm$3 Da, identical with that calculated from its amino acid composition (17259.9 Da).

Another approach to studying the aggregation state of Fur was the use of glutaraldehyde as cross-linking agent. The cross-linking experiment traps oligomers that are normally in a dynamic equilibrium with the monomer, showing the state of the Fur protein in each particular condition. SDS/PAGE analysis of cross-linked Fur samples (Figure 4) revealed monomeric (17 kDa), dimeric (34.5 kDa), trimeric and tetrameric forms, as well as at least four bands of higher molecular mass corresponding to multimeric forms, even though oligomers higher than the dimeric form were not observed by SDS/PAGE in the absence of reducing agents (cf. Figure 2), when no glutaraldehyde was used. A tendency to oligomerize seems to be a common feature of DNA-binding proteins [34–36], although the functional implications of this phenomenon *in vivo* are not yet established. Oligomerization of Fur on its binding sites has already been observed by electron microscopy [37], and such protein–protein interactions, together with the presence of repetitive Fur binding sites, have been proposed as a feature that could explain how a relatively simple protein controls a complex regulon in a gradual fashion, with a wide range of responses in each specific case [15]. Figure 4 shows that the proportions of the oligomers are dependent on the concentration of Fur in the incubation mixture (lanes 2–6). When the Fur concentration is increased, the bands corresponding to oligomers of high molecular mass also increase. Oligomerization is not totally reversible. Lane 7 in Figure 4 shows cross-linking experiments performed using a diluted Fur sample from a 500 μM solution. The oligomers of high molecular mass persisted when an equivalent concentration of Fur was used compared with that in lane 2. Even though cross-linking experiments do not give a quantitative estimation of the actual quaternary structure of Fur, it is interesting to note the relatively lower amount of the dimer compared with the monomer in the gels.

In order to determine if the concentration of glutaraldehyde influences the oligomerization pattern, increasing glutaraldehyde concentrations were tested with 60 μM Fur, but no significant changes in oligomer distribution were found (results not shown). The time course of chemical cross-linking (1, 2, 5 and 30 min) also revealed that the oligomerization pattern did not change during the incubation period, ruling out the occurrence of consecutive aggregation events (results not shown).

Ionic strength also influences the oligomerization of Fur, promoting the appearance of cross-linked high-molecular-mass species (Figure 5, lanes 3 and 4). This suggests the involvement of hydrophobic interactions in the oligomerization of Fur.

The SH-reducing agents DTT and 2-mercaptoethanol, and the SH-oxidizing reagent diamide, were tested in cross-linking experiments. Figure 6 shows the oligomerization patterns of treated samples. At low DTT concentrations (lanes 7 and 8), the oligomerization pattern was similar to that of non-reduced Fur (lane 9), showing the presence of high-order oligomers. When DTT was present at higher concentrations (lane 6), only monomer was visible, with a decrease in band intensity. 2-Mercaptoethanol at 30 mM did not prevent the occurrence of oligomerization when Fur was cross-linked with glutaraldehyde (lane 5). Diamide-treated Fur gave the same oligomerization pattern as the

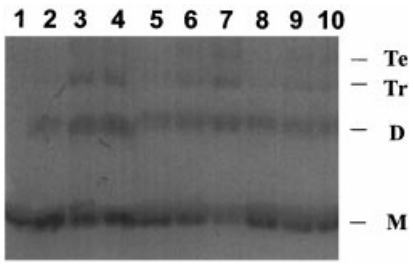

Figure 7 Influence of metals on the oligomerization of Fur

Cross-linking experiments with glutaraldehyde using 300 μM *Anabaena* Fur were performed in the presence of different metals (1 mM). Portions of 7 μg of Fur were analysed by SDS/15%-PAGE. Cross-linked samples were boiled in the presence of 2-mercaptoethanol. Lane 1, untreated Fur; lane 2, Fur treated with glutaraldehyde but without metal; lane 3, Zn^{2+}; lane 4, Zn^{2+} plus 1 mM DTT; lane 5, Ca^{2+}; lane 6, Fe^{2+} plus 0.1 mM ascorbate; lane 7, Fe^{3+}; lane 8, Co^{2+}; lane 9, Mn^{2+}; lane 10, Ni^{2+}. The estimated molecular masses of the oligomers are indicated: M, monomer; D, dimer; Tr, trimer; Te, tetramer.

non-treated protein (lane 4). The effect of Mn^{2+} on the redox state of Fur was investigated, and an identical pattern of oligomerization was obtained when $MnCl_2$ was added to Fur treated with 50 mM DTT (lane 3) or when Fur previously incubated with $MnCl_2$ was reduced under the same conditions (lane 2).

Freezing/thawing to promote oligomerization has been studied in other proteins [38], showing that this treatment can affect the distribution of oligomer sizes. This is an important practical point for the correct handling and storage of Fur samples. However, the oligomerization pattern of the Fur protein from *Anabaena* did not change after storage at -20 °C or -80 °C, or after two consecutive freezing/thawing steps (results not shown).

The presence of metals in the cross-linking incubation mixture appeared to promote the formation of high-molecular-mass bands, i.e. of oligomers (Figure 7). Metals were added to the incubation mixture at 1 mM, only 3-fold in excess compared with the Fur concentration. Figure 7 shows the effects of Zn^{2+} (lanes 3 and 4), Fe^{2+} (lanes 6 and 7), Co^{2+} (lane 8), Mn^{2+} (lane 9) and Ni^{2+} (lane 10). The physiological significance of oligomerization, with possible implications for modulated responses as a function of co-repressor concentration, will be an important aspect to consider. Previously, Althaus et al. [12] found differences in oligomerization state depending on the zinc content. Calcium (lane 5), which is not a co-repressor, was also tested as a negative control; no difference compared with glutaraldehyde-treated Fur in the absence of metals was found.

Recombinant Fur from *Anabaena* is active in binding a target promoter

The ability of recombinant Fur from *Anabaena* PCC 7119 to bind iron boxes was tested by electrophoretic mobility shift assays. Figure 8 shows the binding of Fur to its own promoter in the presence of a fragment of exon IV from the human *apoE* gene, as non-specific competitor DNA. The pattern of band retardation obtained suggests the presence of multiple DNA–protein complexes along the promoter binding sites. DNA-binding assays showed the ability of the protein to bind the iron boxes present in its own promoter. This result is in good accordance with the data obtained by NMR, which indicated that the recombinant protein is folded under our storage conditions. Both the partially purified Fur, which was obtained from the heparin column at pH 8 (Figure 8, lane 2), and the homogeneous protein after the chelating Sepharose column, which was stored

Figure 8 Electrophoretic mobility shift assays of the *fur* promoter in the presence of Fur and a fragment of human *apoE* (non-specific competitor DNA)

The reaction was carried out at room temperature in a binding buffer containing 10 mM Bis-Tris, pH 7.5, 40 mM KCl, 1 mM DTT, 0.1 mg/ml BSA, 10 mM $MnCl_2$ and 50% glycerol. Equimolar amounts of 349 bp and 228 bp DNA fragments containing the *fur* promoter and from human *apoE* respectively were incubated for 30 min after the addition of Fur. Lane 1, free DNAs; lane 2, DNA binding to partially purified Fur obtained after the heparin column; lane 3, binding of purified Fur obtained after the metal-affinity column.

at pH 4 (lane 3), bound the *fur* promoter under the assay conditions. The *in vitro* activity of the purified recombinant protein indicates its functionality and suitability for further regulatory and biophysical studies. Fur–DNA complexes of different molecular masses were observed under the conditions tested. This is in accordance with the sequence analysis of the promoter region of the *fur* gene, which showed the presence of at least two putative binding sites. Moreover, the tendency of this protein to form oligomers, depending on its concentration, suggests that Fur could interact with DNA to establish secondary contacts outside the consensus sequence, causing polymer elongation, as occurs in *E. coli* [31,39]. Therefore the binding of Fur to the target promoters may involve protein–protein interactions in addition to DNA–protein interactions. This is a common feature in gene response regulators of enteric bacteria, where the formation of large oligomers at the enhancer binding site is required for activation of gene transcription [35,36]. As expected, chemically cross-linked Fur did not show DNA-binding activity (results not shown).

Fur is a constitutive protein in *Anabaena* cells

The recombinant Fur protein showed the same mobility in SDS/PAGE as the native one, as can be observed in the Western blot presented in Figure 9. SDS/PAGE of the purified recombinant Fur protein revealed the presence of cross-reactive high-molecular-mass bands, possibly oligomeric forms. This phenomenon has been previously reported for other oligomeric proteins, and Watnick et al. [40], using monoclonal Fur antibodies, found immunoreactive bands in pure preparations, which they interpreted as Fur aggregates. In the cyanobacterial extracts, it is also possible to see the presence of the presumed oligomers, as well as a low-molecular-mass band, which is possibly a Fur degradation product (sometimes also detected by MALDI-TOF MS of the purified protein) or the product of a *fur* homologue present in *Anabaena* PCC 7120 (J. A. Hernández, M. T. Bes, S. López-Gomollón, M. L. Peleato and M. F. Fillat, unpublished work).

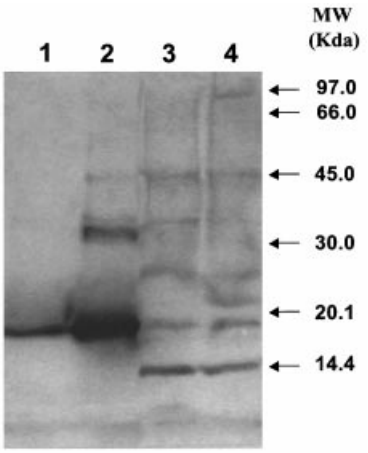

Figure 9 Western blot analysis of Fur in *Anabaena* PCC 7119 extracts

Samples were electrophoresed in an SDS/15%-PAGE gel at 40 V and electroblotted to an Immobilon^{TM}-P (filter type PVDF; pore size 0.45 μm) membrane from Millipore, at 400 mA for 25 min. Lane 1, 0.1 μg of pure protein; lane 2, 1 μg of pure protein; lane 3, 30 μg of crude extract from an iron-deficient *Anabaena* PCC 7119 culture; lane 4, 30 μg of crude extract from an iron-sufficient *Anabaena* PCC 7119 culture. Molecular mass markers are indicated with arrows.

The Western blot in Figure 9 shows that Fur is a constitutive protein in *Anabaena*, with no significant differences in expression depending on the amount of iron available to the *Anabaena* cells. Similar results were described previously for *Vibrio cholerae*, where the difference in Fur abundance when cells were grown in iron-deficient or -sufficient conditions was practically negligible [40]. The polyclonal antibodies raised against Fur from *Anabaena* PCC 7119 did not show cross-reactivity with the Fur protein isolated from *E. coli*, probably due to the fact that Fur from *E. coli* and Fur from *Anabaena* exhibit low sequence identity (33%). The cyanobacterial Fur gene cluster is not in the same location as that encoding Gram-negative bacterial Fur proteins (http://wwwbioq.unizar.es/departamento/investigacion/Fur.htm), and it is not surprising that the lack of cross-reactivity suggests significant structural differences between the regions where the proteins exhibit their antigenic determinants. As expected, the antibodies against cyanobacterial *Anabaena* PCC 7119 Fur cross-reacted with the protein from *Anabaena* PCC 7120, since the sequences of the proteins are identical. Also, cross-reactivity was found with the protein from *Synechococcus* PCC 7942, since the sequence identity is very high (70%). Western blots of *Plectomena*, *Anabaena variabilis* and *Synechocystis* crude extracts showed cross-reactive bands of putative Fur proteins (results not shown).

Concluding remarks

We have reported the purification and an extensive study of the properties of Fur from *Anabaena*, the first member of the Fur family to be purified and characterized from cyanobacteria. The protein exhibits specific features that may give some clues to understanding the structure and function of this family of proteins. From our results, we propose that the presence of structural zinc in the Fur family may be not a general characteristic, although further studies must be performed concerning other members of the family. In the same way, special attention should be paid to the role of cysteine residues in Fur. In *E. coli* Fur there are two cysteines in the C-terminal domain that have been proposed to be necessary for metal-binding ability and DNA recognition. However, in *Anabaena* Fur, four cysteines out of five are not reactive. This result differs from the cysteine redox state described for other Fur proteins, and could be in accordance with the absence of zinc from our protein. The determination of free thiols seems to indicate that, in Fur from *Anabaena*, only one thiol is accessible to solvent. Dimer formation seems to be dependent on the oxidation state of the cysteines, while the cross-linking experiments suggest that, in solution, Fur exists in several discrete oligomeric species, namely monomers, dimers, trimers, tetramers and even higher oligomeric forms, with hydrophobic interactions involved. The quaternary structure of Fur may be of physiological importance, and its study will help to clarify the role of this protein *in vivo*, with possible different promoter targets or differential affinity for the iron boxes of the different oligomers. Metals that promote oligomerization could have some physiological significance in relation to the role of iron as a co-repressor.

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