Electrospray ionization mass spectrometry analysis of the apo- and metal-substituted forms of the Fur protein

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Abstract Fur has been purified and reconstituted with Co^{2+} and Mn^{2+}. The ESI-MS spectra of the apoprotein as well as Mn-Fur and Co-Fur under acidic denaturating conditions showed the existence of two species of molecular mass 16660 ± 3 and 16792 ± 3 Da, which correspond, respectively, to the N-terminal methionine 'excised' or 'non-excised' forms of the monomer. This result proves the absence of any other post-translational modification or modification due to metal incorporation. On the other hand, under soft conditions, ESI spectra provided for the first time direct evidence for dimeric metal-containing forms in solution.

Key words: Ferric uptake regulation (Fur) protein; Iron uptake regulation; Electrospray ionization mass spectrometry; *Escherichia coli*

1. Introduction

Iron is necessary to the growth of almost all living cells, but the iron concentration must be highly regulated since Fe^{II} is potentially toxic [1]. The control of the intracellular iron concentration in Gram-negative bacteria such as *Escherichia coli* is effected by altering the rate of iron uptake inside the cell [2]. Fur is a metallo-regulatory protein involved in the control of the expression of various bacterial genes related to iron uptake in these Gram-negative bacteria [3]. It has been proposed that Fur binds iron as a corepressor and then acts as a negative regulator via recognition of DNA sequences at the promoter regions of Fur-regulated genes [3–6]. However, for the specific binding of Fur to DNA in vitro, the presence of any of the divalent metals Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+} and partially Zn^{2+}, is essential [3,7].

Some spectroscopic studies have been previously described using NMR, UV-visible and EPR spectroscopies [8,9]. However, the environment of the metals ions is still unknown. In order to better characterize the metal binding site(s) and to understand the mechanism of action of Fur at a molecular level, manganese- and cobalt-substituted Fur proteins have been prepared. We present here an analysis by electrospray ionization mass spectrometry of the apo- and metal-substituted forms of Fur, showing for the first time direct evidence for dimeric, metal-containing forms in solution.

2. Materials and methods

2.1. Overproduction and purification of the Fur

The T7 RNA polymerase/promoter system was used to overproduce Fur from *Escherichia coli* as previously described [10]. Fur was purified as previously described, but with some modification [11]. The first modification concerns the addition of trypsin-chymotrypsin inhibitors (from Sigma) at 10 mg L^{-1} together with EDTA at 20 mM, PMSF at 1.38 mM, and pepstatin at 5.8 μM in the extraction buffer (0.1 M MOPS buffer at pH = 8 containing 10% w/v sucrose and 10% v/v glycerol) in order to avoid proteolysis. The second modification was the addition of a second step of purification after the chelating Zn-imino acetate column using a gel filtration on Superdex 75 (Pharmacia) in 0.1 M Tris/HCl at pH = 8 (5 C°) containing 0.1 M KCl.

2.2. Protein concentration measurement

Protein concentrations were determined spectrophotometrically using an absorption coefficient at 275 nm of 0.4 mg^{-1} mL cm^{-1} [11] for one monomer of pure apo-Fur.

2.3. Amino acid sequencing

The N-terminal amino acid sequence of the apoprotein was obtained using an Applied Biosystems sequencer (477A) coupled to an on-line PTH-amino acid analyzer (120A). All sequencing reagents were obtained from Applied Biosystems.

2.4. Metal incorporation

The incorporation of manganese was followed by EPR measurement at room temperature as described in the literature [12]. Cobalt incorporation was followed by the appearance of a band at ca. 550 nm in the UV-visible spectrum. Metal quantifications were obtained by analysis using inductive coupling plasma-atomic emission spectroscopy (ICP-AES) on a Fisons 'maxim type' analyzer.

2.5. Mass spectrometry analysis

Poly(propylene glycol) was purchased from Aldrich. Acetonitrile was purchased from Carlo Erba.

2.6. ESI-MS

ESI-MS was performed using a SCIEX API III^+ triple quadrupole mass spectrometer (Perkin-Elmer Sciex) equipped with a nebulizer-assisted electrospray (ionspray) source. Calibration was performed in positive mode using poly(propylene glycol) ions. Mass spectra were analyzed using a Quadra 950 data system (Apple Computer Inc.). MacBioSpec software (Perkin-Elmer Sciex) was used to calculate the mass of the apoproteins from their sequence.

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Abbreviations: Fur protein, Ferric Uptake Regulation protein; Co-Fur and Mn-Fur, Fur protein substituted with cobalt and manganese ions, respectively; Fur-Met, Fur protein without the N-terminal methionine; ESI-MS, electrospray ionization-mass spectrometry; MALDI-TOF, matrix-assisted-laser desorption/ionization-time of flight; HPLC, high pressure liquid chromatography; LC, liquid chromatography; ICP-AES, inductive coupling plasma-atomic emission spectroscopy; PMSF, phenylmethylsulfonyl fluoride

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2.7. Standard conditions

Experiments were performed with an absolute value of ion-spray voltage of about 5000 V and an orifice voltage of 80 V. The interface temperature was set at 55°C. The samples, solubilized in a 10 mM aqueous ammonium acetate buffer at pH 8, were analyzed using as vector solvent a solution of 25% methanol, 1% acetic acid in water at pH=2.6, and infused using a syringe pump (Harvard 22, South Natic, USA) at a flow rate of 5 μL min^{-1}. The mass spectrometer scanned from *m/z* 400 to 1400 in 0.9-Da steps at 2 ms per step.

2.8. Soft non-denaturating conditions

Experiments were performed with an absolute value of ion-spray voltage of about 5000 V and an orifice voltage of 40 V in order to decrease the post-acceleration (declusterization) which might lead to dimer dissociation. The interface temperature was set at 40°C in order to avoid denaturation. The samples, solubilized in a 10 mM aqueous ammonium acetate buffer at pH 8, were analyzed using the same buffer as vector solvent (no organic solvent which could cause denaturation but an aqueous solvent with a pH close from the pHi of the protein) and infused using a syringe pump (Harvard 22, South Natic, USA) at a flow rate of 3 μL min^{-1} (using a lower flow rate is better with non-volatile solvent). The mass spectrometer scanned from *m/z* 1200 to 2200 in 0.9-Da steps at 2 ms per step.

2.9. LC/ESI-MS of the Holoforms

LC was directly coupled to ESI-MS using a 140B syringe pump system (Applied Biosystems). One nmole of protein solution was injected on a Brownlee reverse phase column (C_{18}, 5 μm, 1 mm × 150 mm, Applied Biosystems) using a flow rate of 50 μL min^{-1}. Using a Valco T, a split of 1/3 was applied such that approximately 15 μL min^{-1} was directed to the mass spectrometer. The remaining eluant was diverted to an Applied Biosystems 785 UV detector, monitoring at 214 nm. Peptide separation was achieved using a linear gradient of 0–100% acetonitrile (0.1% TFA) for 5 min. Mass spectra (*m/z* 400–1400) were acquired with a 2 ms dwell time per step of 0.9 Da.

3. Results and discussion

3.1. ESI-MS of apo-Fur under acidic denaturating conditions

The amino acid sequence of the apo-Fur derived from the nucleotide sequence comprises 148 amino acids that together constitute a polypeptide of 16795 Da [13]. The thiol content was previously determined to be 3.4 molecule per Fur molecule, suggesting that all 4 cysteines predicted by the gene sequence are present in the reduced state [5,14]. Furthermore, Fur appears to exist in solution mainly as a dimer with a molecular mass of 34000 Da as determined by HPLC on a Hitachi gel Pack column W-530 [15].

It is also interesting to note that the amino acid analysis described by Wee et al. [11] suggests the presence of only one methionine residue per molecule and the replacement of one Ala by one Thr residue compared to the sequence deduced from the cDNA.

The ESI spectrum under acidic denaturating conditions shows the existence of two species with molecular masses of 16660 ± 3 and 16792 ± 3 Da. These species correspond to the N-terminal methionine excised form (Fur-Met) or non-excised form (Fur) of the apomonomer of Fur, respectively, present in a ratio of 1:2 (as shown on Fig. 1). This result was confirmed by N-terminal sequence analysis. Under denaturating conditions, at no time was a species with a molecular mass corresponding to a dimeric form observed. Therefore, the presence of an intersubunit disulfide bridge in the native protein can be excluded. This result also proves the absence of any other post-translational modification. These molecular masses are in perfect accordance with that deduced from the known se-

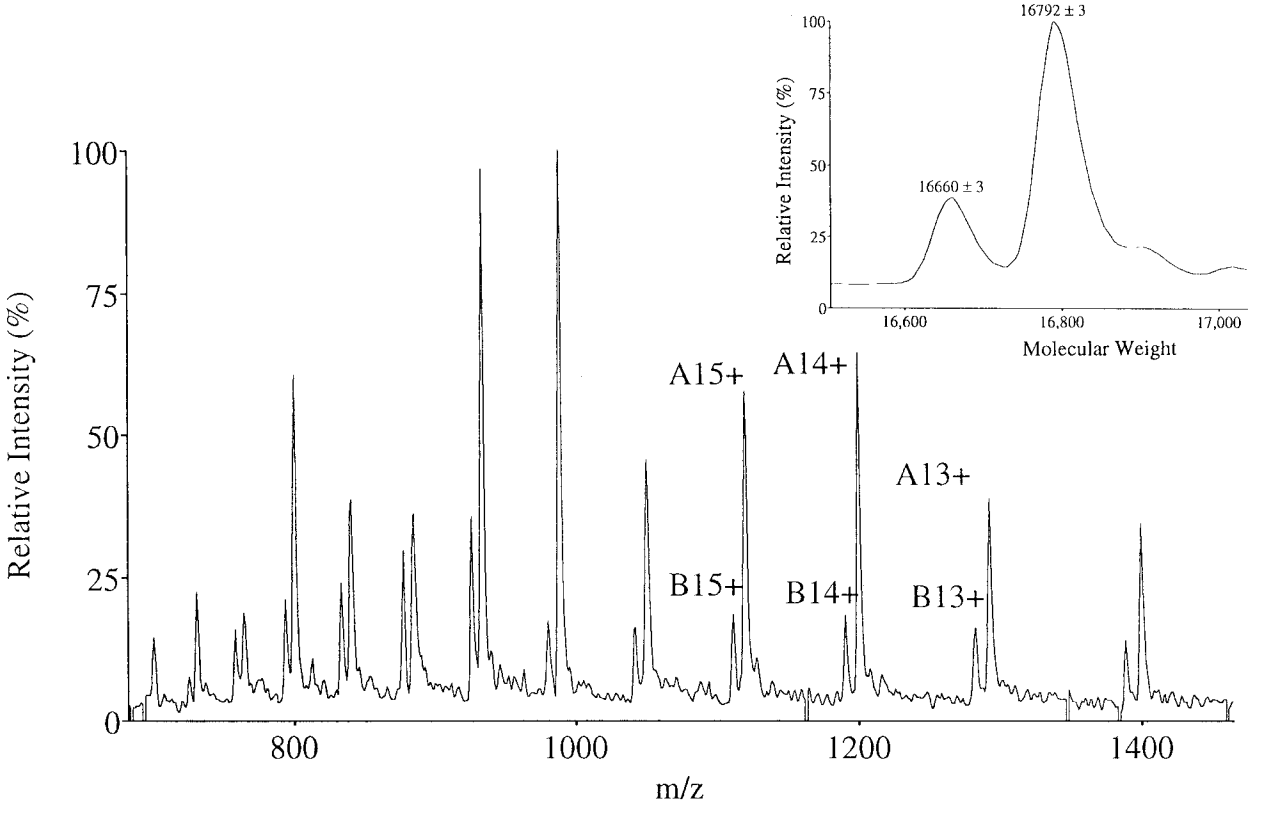

Fig. 1. Electrospray ionization mass spectrum (LC-MS) of Fur under denaturating conditions in positive-ion mode (reconstructed spectrum in inset). A and B labels correspond, respectively, to the name of the signals for the Fur and the Fur-Met forms. The number associated to the letter is the charge *z*.

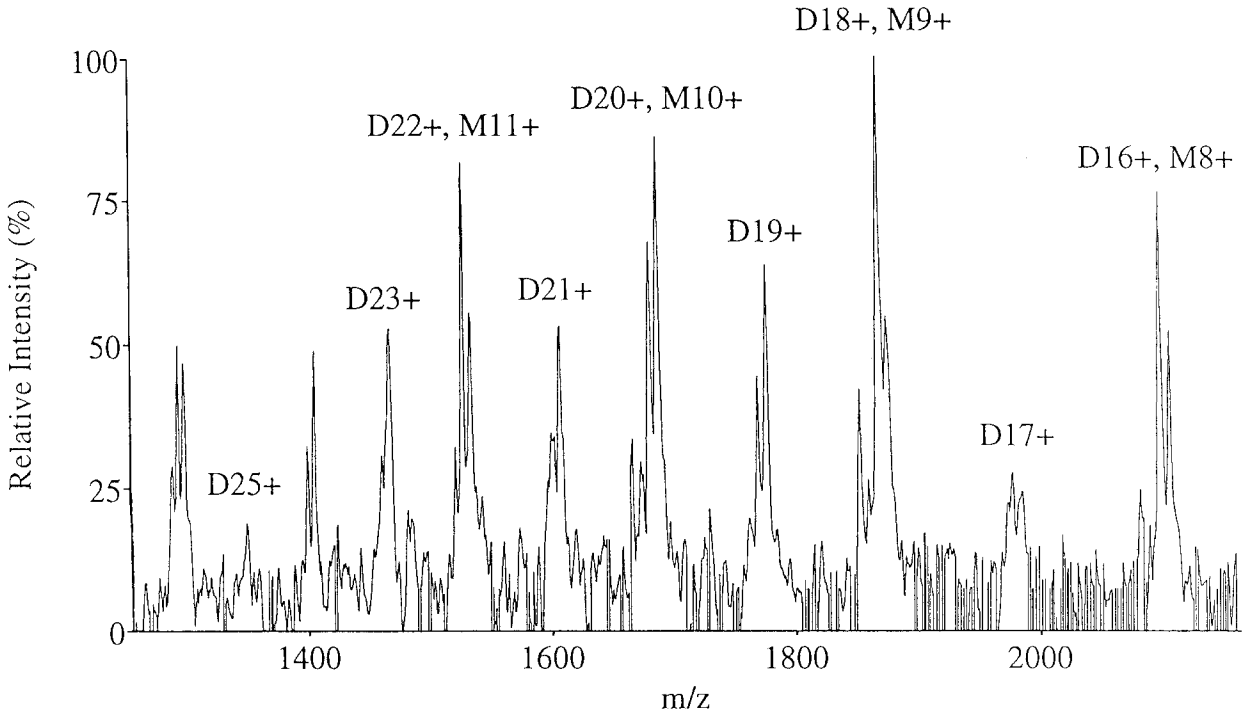

Fig. 2. Electrospray ionization mass spectrum of the Mn-Fur protein under soft conditions in positive-ion mode. D and M correspond to the name of the signals for the dimer and monomer signals, respectively. The number associated to the letter is the charge z. In the conditions of the experiment, monomeric subunits may exist under several charge states z: +10, +11, +12...; but the dimeric forms could be the association of two monomers with identic charges given a resulting charge ($2z$) but also association of two monomers with different charges (z and $z+1$, for example) given a different resulting charge ($2z+1$), for example: (+10; +11) = +21, (+11; +12) = +23.... Then, monomeric species would give m/z ratio but dimeric species would give either $2m/2z$ (equal value to m/z) or $2m/2z+1$. The $2m/2z+1$ type of signals is then the signature of the existence of a dimeric form.

quence, implying neither a modification of the sequence upon cloning (as was the case for Fur overexpressed and purified by Neilands and coworkers [11]) nor a post-translational modification. In order to compare to our results, the ESI spectrum of Neilands' protein (kind gift from J.B. Neilands to D. Touati) under denaturating conditions was recorded. This spectrum confirms the modification of the alanine and threonine contents (Ala^{11} and Thr^{6} instead of Ala^{10} and Thr^{7} from the deduced sequence) and the absence of the N-terminal methionine [11] since we observed a molecular mass of 16 632 ± 4 Da in agreement with the calculated mass of 16 634 Da.

3.2. Proteolyzed species

In the absence of protease inhibitors and without temperature control (heating of the sample if the sonification is done without cooling steps between each sonification step) of the extract during the sonification step (3 times 30 s), two distinct bands were detected on SDS-PAGE corresponding to intact Fur and a shorter protein lacking N-terminal 9 amino acids, as proved by ESI-MS. This proteolytic product had a molecular mass of 15 805 ± 5 Da (15 806.8 Da calculated). Cleavage after the lysine in position 9 is in accordance with a trypsin type proteolysis, and probably corresponds to Fur described by Coy et al. [5] obtained in vitro by using limited proteolysis with trypsin. The proteolysed form also eluted as a dimer during gel filtration shows that the first 9 amino acids are not involved in dimerization. This result is in accordance with the recent data from Stojiljkovic et al. [6] which show that the dimerization site resides in the C-terminal part of the protein.

3.3. Monomeric species

Pure Fur samples were shown to comprise a mixture of monomer and dimer by gel filtration on Superdex 75. These forms can be separated. They are not in equilibrium since loading of the separated species on gel filtration does not give a mixture but still gives pure monomeric or dimeric species. ESI-MS studies under denaturating conditions showed that the two isolated forms have the same molecular mass (16 793.4 ± 4 Da) which indicates that the monomeric form does not result from a covalent modification but rather from an irreversible conformational change which renders formation of the dimer impossible.

3.4. ESI-MS of the Mn- and Co-Fur under acidic denaturating conditions

Metal incorporation in metalloproteins can induce oxidation or covalent modification catalyzed by the metal. For example, in copper amine oxidases, the copper induces the hydroxylation of the ring of one tyrosine which upon oxidation gives dopa quinone [16]. It was important to prove that metal incorporation in Fur which activates Fur does not induce a covalent modification. The manganese and cobalt substituted forms have been prepared. The incorporation of the metal ions was monitored by EPR spectroscopy for Mn-Fur and by UV-visible spectroscopy for Co-Fur. The spectroscopic properties of these metal-containing forms will be described elsewhere (manuscript in preparation). The apo-Fur form is able to bind one divalent metal (Co^{2+} or Mn^{2+}) per monomer as determined during the incorporation and confirmed by ICP analysis. Apo- and metal-substituted forms

also contain 0.5 to 0.8 Zn molecule per monomer as measured by ICP analysis. These values do not allow us to conclude if there is 1 Zn per monomer or 1 Zn per dimer, however an underestimation of the Zn amount in Fur is possible since the protein samples were treated with EDTA after the chelating Zn-imino acetate column. The Mn-Fur and Co-Fur samples injected under acidic denaturating conditions gave the same spectra as the apo-Fur sample, showing the absence of covalent modifications which would be due to the incorporation of the metal.

3.5. *ESI-MS of Mn- and Co-Fur under soft non-denaturating conditions*

Under soft conditions, ESI-MS of Mn-Fur shows peaks at odd *m/z* close to 33 700 Da (Fig. 2). This spectrum constitutes the first direct evidence of the presence of dimeric metal-containing forms. The presence of two protein forms with and without the terminal methionine makes the number of potentially observable species very important (33 328 Da for [Fur-Met]$_2$, 33 459 Da for [Fur-Met; Fur], 33 590 Da for [Fur]$_2$ and the corresponding metal-containing forms). Nevertheless, the major peak at 33 726 ± 4 Da can only correspond to a metal-containing Fur dimer. However the precision of this experiment does not allow us to conclude whether the metal ions were one zinc and one manganese ion, two zinc ions or two manganese ions. Under these soft conditions, the observation of dimeric forms was not always possible, but then a monomeric form containing a zinc ion was clearly detected. We were not able to observe any tetrameric or higher molecular mass form in the conditions used, even on a high *m/z* mass spectrometer analyser (MALDI-TOF). This is in contrast with the observations of E. Le Cam et al. [17,18].

The difficulty to observe a spectrum indicating metal-containing forms of Fur is not surprising because the K_d for the metal-protein complexes are only in the range of 10–100 μM depending on the metal and on the methods [3,5,19]. It must be kept in mind that Fur is, in vivo, a sensor of the iron concentration and must be able to liberate iron in case of a drop of intracellular iron concentration. Therefore its affinity for the metal must be moderate.

In conclusion, ESI-MS has proven to be a very informative technique to study Fur both under acidic denaturating conditions and under soft conditions. It has allowed for a dimeric metal-containing form to be detected for the first time. In addition, it showed that (i) the dimerization is independent of thiol oxidation, (ii) there is no post-translational modification of Fur and (iii) the incorporation of Co(II) or Mn(II) does not modify the primary structure of the protein. Further experiments are currently in progress to better characterize the metal substituted forms of the protein and their interaction with DNA.

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