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In HspA from *Helicobacter pylori* vicinal disulfide bridges are a key determinant of domain B structure

Salvatore Loguercio[a,b], Cyril Dian[b], Angela Flagiello[c], Alessandra Scannella[a], Piero Pucci[c,d], Laurent Terradot[b,*], Adriana Zagari[a,c,*]

[a] *Department of Biological Sciences and CNISM, University of Naples "Federico II", Via Mezzocannone 16, I-80134 Naples, Italy*
[b] *European Synchrotron Radiation Facility, BP 220 F-38043, Grenoble Cedex 9, France*
[c] *CEINGE – Biotecnologie Avanzate Scarl, Naples, Italy*
[d] *Department of Organic Chemistry and Biochemistry, University of Naples "Federico II", Naples, Italy*

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Abstract ***Helicobacter pylori*** **produces a heat shock protein A (HspA) that is unique to this bacteria. While the first 91 residues (domain A) of the protein are similar to GroES, the last 26 (domain B) are unique to HspA. Domain B contains eight histidines and four cysteines and was suggested to bind nickel. We have produced HspA and two mutants: Cys94Ala and Cys94Ala/Cys111Ala and identified the disulfide bridge pattern of the protein. We found that the cysteines are engaged in three disulfide bonds: Cys51/Cys53, Cys94/Cys111 and Cys95/Cys112 that result in a unique closed loop structure for the domain B.**

Keywords: Small heat shock protein; Chaperonin GroES; Nickel binding protein; Mass spectrometry; *Helicobacter pylori*

1. Introduction

Helicobacter pylori is a gram negative bacteria that colonise the human stomach. It is present in more than half of the world's population and causes major diseases such as gastritis, peptic ulcers and stomach cancer [1]. To colonise this highly acidic niche, this neutrophile bacterium has developed peculiar strategies. Among them the production of the urease is the most important one [2,3]. Urease is a nickel-dependent enzyme that hydrolyses urea to produce ammonia, which allows the bacteria to survive at low pH [4,5]. The activity of urease strongly depends on the availability of nickel ions [6]. Other proteins such as Hpn, Hpn-like, NixA, Frpb4 and a heat shock protein, namely HspA, have been demonstrated to bind the metal playing the role of importing, providing or sequestering nickel ions [7–9].

Heat shock proteins (Hsp) synthesis is stimulated in response to abnormal environmental conditions like such as heat shock, oxidative stress, and also exposure to heavy metals. The proteins are found in all domains of life where they actually act as molecular chaperons [10]. A subgroup, referred as to small Hsp, shares common features such as small molecular mass (10–30 kDa).

The small heat shock protein A (HspA) from *H. pylori* is an essential protein with a double localization. Unexpectedly this cytoplasmic protein, has been localised also on the bacterium surface. Whether this protein is released through a specific secretion mechanism or a programmed bacterium autolysis is an interesting matter of debate [11,12].

Owing to its external localization, HspA presents strong antigenic properties [13–15]. A protective immunity from the infection was induced in mice after immunization with HspA. The protein has been then considered appealing as a vaccine component [13].

HspA consists of 118 amino-acids divided in two domains; the A domain (1–91) and the B domain (92–118). The domain A of the protein shares sequence similarity to the GroES sequence [13]. The domain B of HspA is unique to *H. pylori* and *Helicobacter acinonuchis* and contains eight histidines and four cysteines.

This unusual sequence plays a role in Ni uptake and release [9,13,15,16] to assist nickel homeostasis in vivo. The B domain does exhibit high affinity for nickel ions with dissociation constants of 1.8 μM [15] and 1.1 μM [16]. In vitro, the binding to nickel is pH dependent, being the nickel released at low pH ($pH_{1/2} = 3.8$) and was demonstrated by several complementary methods such as dialysis equilibrium, UV–Vis spectroscopy, ICP-MS mass [15,16].

Cys is the less represented amino-acid in the GroES protein family, because it does not serve for the co-chaperonin function. Vice versa, no substitution affects the His/Cys residues of HspA sequence even though several substitutions occur among clinical isolates, suggesting that these amino-acids are specifically involved in nickel homeostasis [9,15]. Therefore, the role played by the B domain should be mediated by a high number of cysteines with capacity of different redox state.

To get insight into Cys role, we have produced the wild-type HspA as well as two mutants: Cys94Ala and Cys94Ala/Cys111Ala. By combining biochemical methods with mass spectrometry, we have determined the Cys oxidation state, assigned the disulfide bridge pattern and provided evidence for a unique lasso-like structure of the B domain in HspA.

*Corresponding authors. Address: Department of Biological Sciences and CNISM, University of Naples "Federico II", Via Mezzocannone 16, I-80134 Naples, Italy. Fax: +39 081 2536 603 (A. Zagari).
E-mail addresses: terradot@esrf.fr (L. Terradot), zagari@unina.it (A. Zagari).

Abbreviations: HspA, heat shock protein A; IAM, iodoacetamide; DTT, dithiothreitol

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2. Materials and methods

2.1. Expression and purification of HspA from H. pylori

The wild-type HspA was expressed and purified using the vector pILL948 described in [15]. Single (C94A) and double (C94A/C111A) HspA mutants were constructed using the QuickChange site directed mutagenesis protocol (Stratagene). *E. coli* BL21(DE3)pLysS, carrying the expression vector, was grown in LB medium at 37 °C, and the expression of the recombinant product was induced by 1 mM isopropyl-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation, resuspended in lysis buffer A (20 mM Na_2HPO_4 pH 7.4, 500 mM NaCl and 1 mM $MgCl_2$) and disrupted by sonication. The soluble fraction was isolated from the cell debris by centrifugation and applied to a HiTrap HP column (Amersham Biosciences) charged with 100 mM $NiSO_4$ and equilibrated with buffer A. The protein was eluted from the column using a gradient of 0–100% buffer B (buffer A and 500 mM imidazole), and further purified by size exclusion chromatography (Superdex 200 (10/30); Amersham Biosciences) in a buffer (20 mM Tris pH 7.4, 200 mM NaCl).The purity and homogeneity was first tested by SDS–PAGE and then by mass spectrometry.

2.2. Mass spectrometry measurements

Reduction with DDT and alkylation with IAM were carried out according to [17]. HPLC-desalted samples of wt-HspA and mutants were directly analyzed by ES/MS using a Quattro Micro triple quadrupole mass spectrometer (Waters). Wt-HspA and both mutants samples were digested with trypsin in 50 mM ammonium acetate, pH 6.0 at 37 °C overnight, and the mixtures of tryptic peptides were directly analyzed by MALDI mass spectrometry using a Voyager DE PRO instrument (Applied Biosystem) operating both in linear and reflectron mode.

3. Results and discussion

3.1. Assessment of the oxidation state of HspA Cys residues

The oxidation state of the cysteines occurring in HspA and in its mutants was assessed by determination of the accurate molecular mass of the proteins by electrospray mass spectrometry (ESMS), following a procedure previously developed [17]. Aliquots of the wt-HspA and both mutants samples were directly analyzed by ESMS producing the spectra shown in Fig. 1. Wt-HspA exhibited a molecular mass of 12984.0 ± 0.8 Da, about 6 Da lower than expected for the fully reduced protein (theoretical mass value 12990.9), thus suggesting that the six Cys residues were involved in three disulfide bridges. Accordingly, the molecular mass of HspA shifted to 12989.9 ± 0.4 Da following reduction with DTT, exhibiting the expected increase of about 6 Da, whereas no difference in the mass value was observed following direct alkylation of

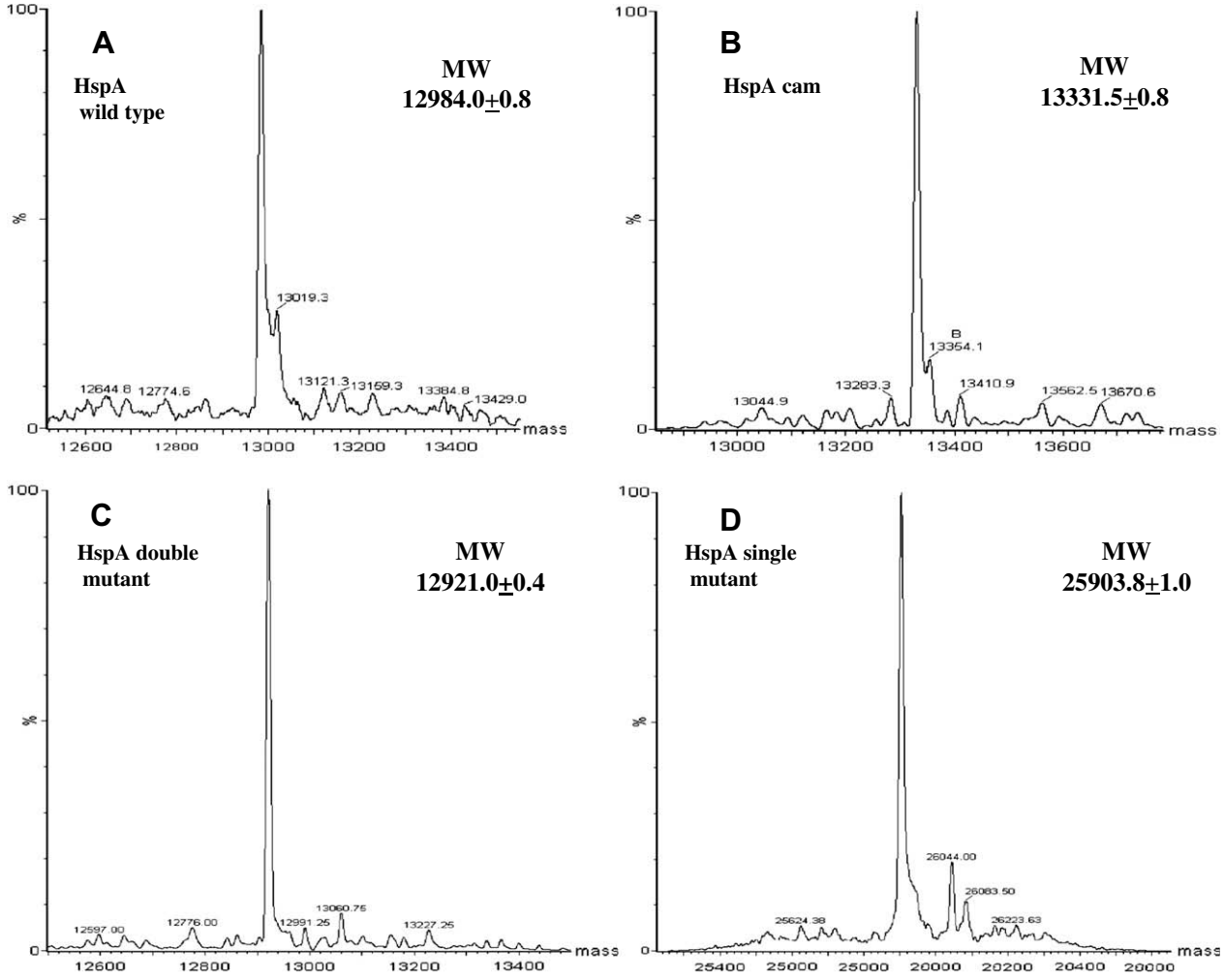

Fig. 1. ESMS spectra. (A) Wt-HspA; (B) HspA carboxymethylated at position 51, 53, 94, 95, 111, 112, after reduction with DDT; (C) C94A/C111A double mutant; (D) dimeric C94A mutant.

the protein with an excess of IAM, confirming the absence of free Cys residues (data not shown). It is worth noting that, after reduction, disulfides quickly reform in air. Finally, when the wt protein was first reduced with DTT and then carboxyamidomethylated with IAM, the corresponding ESMS spectrum exhibited a molecular mass of 13331.5 ± 0.8, as shown in Fig. 1B, with an increase of 340.6 Da, corresponding to the alkylation of six Cys residues.

Similar experiments were also carried out on the HspA mutants. Fig. 1C shows the ESMS spectrum of the Cys94Ala/Cys111Ala double mutant exhibiting a molecular mass of 12921.0 ± 0.4 Da, about 4 Da lower than expected for the fully reduced protein, suggesting the occurrence of two S–S bridges. This result was confirmed by the reduction and reduction/alkylation experiments analogous to those described above for wt-HspA, indicating that the two mutated cystein residues, Cys94 and Cys111, were joined by a disulfide bond in the wt-HspA molecule. Finally, the ESMS analysis of the Cys94Ala single mutant yielded a mass value of 25903.8 ± 1.0 Da, as shown in Fig. 1D, corresponding to a dimeric species stabilised by an intermolecular disulfide bridge likely involving the free Cys111 residue of each HspA molecule.

3.2. Disulfide bridge pattern

Assignment of the disulfide bridges pattern in both wt-HspA and the double mutant was accomplished according to the well established mass mapping strategy [17–20]. Wt-HspA was digested with trypsin at pH 6.0 to avoid scrambling of S–S bridges and the resulting peptide mixture was directly analyzed by MALDI-MS. Fig. 2A shows the high mass region of the resulting MALDI spectrum recorded in linear mode where a series of mass signals was attributed to S–S bridged peptides. The peaks at *m/z* 4234.8 and 3969.6 were interpreted as arising from the peptide 78–103, originated by aspecific cleavage at Tyr77, joined to the fragments 107–118 and 107–116, respectively by two disulfide bridges involving the four Cys residues at positions 94, 95, 111 and 112. Both mass signals were accompanied by satellite peaks at about 335 Da higher (*m/z* 4571.8 and 4304.5) corresponding to the same peptide 78–103 linked to the fragments 104–118 and 104–116, respectively via the same two S–S bridges. Finally the mass signals at *m/z* 3891.1 and 3626.3 were attributed to the peptide 81–103 bridged to the fragments 107–118 and 107–116 by two disulfides involving the above mentioned four cysteines. All these signals disappeared following incubation with DTT.

The mass spectral investigation of wt-HspA provided the overall scheme of cysteine pairings with Cys94, 95, 111 and 112 originating a network of two disulfide bridges and Cys51 and Cys53 forming the third S–S bridge. This interpretation was supported by the weak mass signal at *m/z* 2404.3 observed in the MALDI reflectron spectrum and was further confirmed by the double mutant analysis (see below). This peak occurred 2 Da lower than the peptide 42–64, suggesting the presence of an intermolecular disulfide bridge joining Cys51 and Cys53.

The correct pairings of the Cys residues in HspA was obtained by the MALDI mass spectral analysis of the tryptic digest of the C94A/C111A double mutant. As shown in Fig. 2B, mutation of the two Cys residues seemed not to affect the C-terminal pattern of disulfide bridges. The mass signals at *m/z* 5344.7, 5053.7 (peptides 65–103 and 67–103 joined to 107–116, respectively) and 3907.7, 3564.6 and 3322.4 (peptides 78–103, 81–103 and 83–103 linked to the fragment 107–116) essentially corresponded to the previously observed S–S bridged peptides. This strongly suggests that Cys94 and

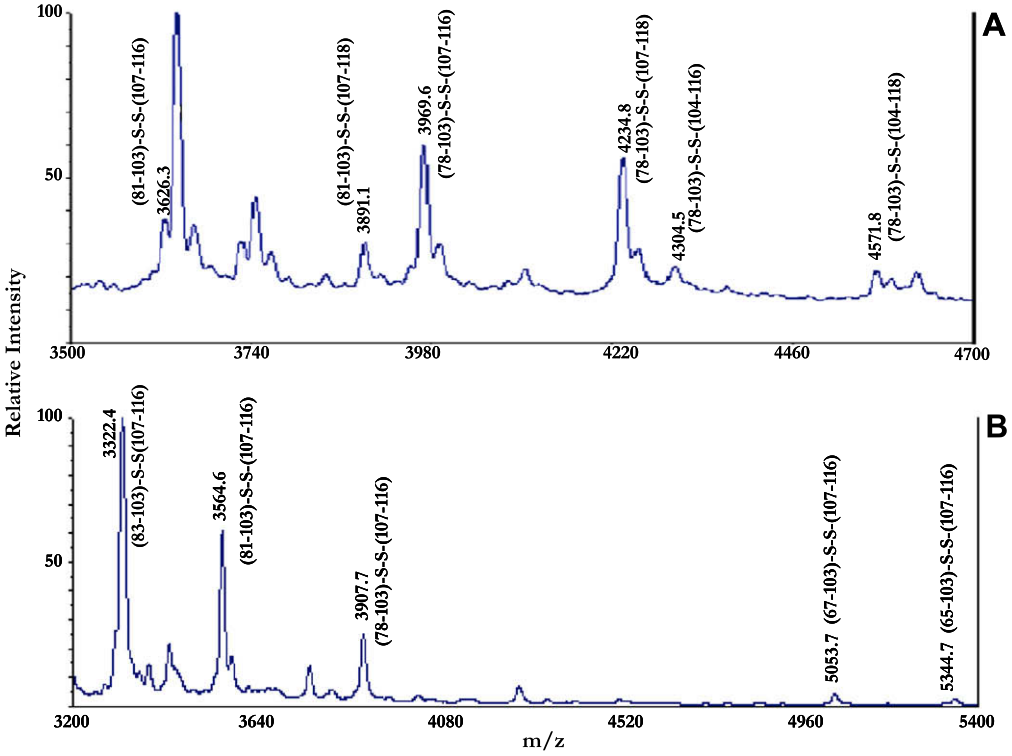

Fig. 2. MALDI-MS spectra after tryptic digestion of: (A) Wt-HspA; (B) C94A/C111A double mutant.

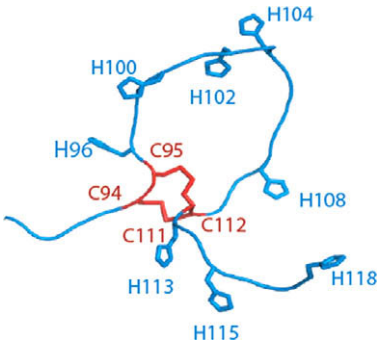

Fig. 3. Model of domain B (92-GSCCHTGNHDHKHAKE-HEACCHDHKKH-118) in HspA. The disulfide bridge pattern generating the lasso-like structure is indicated in red (Cys94-Cys111 and Cys95-Cys112). The side chains of cysteines and histidines are displayed in sticks. Coordinates were obtained by manual building in COOT [22].

Cys111 are held together by an S–S bridge in wt-HspA. The C-terminal fragments are then still joined by the second disulfide bond involving Cys95 and Cys112. The third S–S bridge involving Cys51 and Cys53 was confirmed by the mass signal at *m*/*z* 964.5 (peptide 47–55 containing an intramolecular disulfide bridge) leading to the unambiguous assignment of the S–S bridge pattern in HspA.

4. Conclusion

In this study, we found that HspA contains three intramolecular disulfide bridges. The bonds involve either the GroES-like domain A (Cys51–Cys53) or the nickel-binding domain B (Cys94–Cys111 and Cys95–Cys112). In the latter domain, the small 4-Cys ring we identified constitutes a strong and very rare structural determinant, being observed only in the conotoxin, a short peptide (pdb code 1wct) [21]. Indeed, in HspA the two consecutive S–S bridges form a rigid knot that dramatically affects the topology of the C-terminal domain generating a "lasso-like" structure, here described for the first time (Fig. 3). A large and flexible ring, formed by 18 residues, embodies five His residues leaving out of the lasso the neighboring three C-term His. This lasso-like structure would be ideal to position the ligand side chains in conformation suitable to bind up to two nickel ions [15,16]. The Ni binding role may be played by HspA either through intracellular or extracellular Ni sequestration, as the protein was also found in association with the outer membrane and as a subcellular material [11,12]. Along this line, it is tempting to speculate that HspA might switch from an oxidised to a reduced state depending on the environment. This holds for the cell surface, where the protein is embedded in an oxidizing environment and then possesses the S–S bridges; whereas, in cytoplasm, the disulfide bridges may exhibit a diverse stability, depending on the conditions (pH and Ni depletion or overload).

In conclusion, being the cysteines strongly conserved in different strains they may have the role to assist the Ni binding/release. Then the unique lasso-like structure, built-up from vicinal disulfide bridges is relevant to the Ni dependent functioning of HspA in *H. pylori*.

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