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The (unusual) aspartic acid in the metal coordination sphere of the prokaryotic zinc finger domain

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ABSTRACT

The possibility of choices of protein ligands and coordination geometries leads to diverse Zn(II) binding sites in zinc-proteins, allowing a range of important biological roles. The prokaryotic Cys_2His_2 zinc finger domain (originally found in the Ros protein from *Agrobacterium tumefaciens*) tetrahedrally coordinates zinc through two cysteine and two histidine residues and it does not adopt a correct fold in the absence of the metal ion. Ros is the first structurally characterized member of a family of bacterial proteins that presents several amino acid changes in the positions occupied in Ros by the zinc coordinating residues. In particular, the second position is very often occupied by an aspartic acid although the coordination of structural zinc by an aspartate in eukaryotic zinc fingers is very unusual. Here, by appropriately mutating the protein Ros, we characterize the aspartate role within the coordination sphere of this family of proteins demonstrating how the presence of this residue only slightly perturbs the functional structure of the prokaryotic zinc finger domain while it greatly influences its thermodynamic properties.

1. Introduction

The comprehension of the factors determining coordination geometry, affinity and specificity of zinc-binding is crucial for the design of new ligands for existing zinc binding sites and of novel Zn-binding proteins. Zinc, in fact, plays indispensable roles in protein structures and functions [1,2]. While in aqueous solution it has a coordination number of six and forms an octahedral structure, tetrahedral coordination is dominant in proteins, although a significant amount of zinc ions display higher coordination numbers [3]. Common ligands for the zinc ion include a variety of combinations of cysteine, histidine, aspartate or glutamate [4]. The possibility of choices of ligands and coordination geometries leads to diverse Zn(II) binding sites in zinc-proteins, allowing a range of important biological roles, such as catalytic, by participating directly in chemical catalysis, co-catalytic and structural role by maintaining protein structure and stability [2,5–10].

In general, the role of the structural zinc site is to maintain the localized structure of a protein which could in turn influence protein folding and/or function. Structural zinc sites have generally 4 protein ligands and no bound water molecule [1,4,7,11]. Cysteine is the preferred ligand for such sites but other ligands are also found: the second most prevalent ligand is histidine which is usually found in combination with cysteine [4]. Non-cysteine structural zinc site are unusual: there are only a few examples where aspartate and glutamate in combination with histidine residues are found [4,12]. The zinc finger represents the typical example of a domain in which metal ion coordination is crucial for folding and maintenance of the native conformation [13–18]. The eukaryotic Cys_2His_2 zinc-finger domain is one of the most common and important structural motifs involved in protein-DNA interactions [19,20] and it is characterized by the tetrahedral coordination of the zinc ion by conserved cysteine and histidine residues [21,22]. We have previously characterized Ros87, the prokaryotic Cys_2His_2 zinc-finger motif included in the DNA binding region of Ros protein from *Agrobacterium tumefaciens* [23]. This protein differs from the eukaryotic counterpart: its globular structure centred around a zinc ion consists of 58 amino acids (from Pro9 to Tyr66) arranged in a ββββαα topology and stabilized by an extensive hydrophobic core (15 amino acid residues) [24]. Ros is the first structurally described member of a large family of bacterial proteins that have interesting and fundamental roles in the bacterial cell life [12,25–32]. Interestingly, Ros homologues present several amino acid substitutions in the positions occupied by zinc-binding residues in Ros and despite these amino acids changes, these proteins maintain the ability to specifically bind the DNA [12,33]. In particular, the three *Mesorhizobium loti* proteins called Ml_1, Ml_2 and Ml_3, which have a cysteine and an aspartic acid respectively in those that

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correspond to the first and the second metal coordination positions in Ros, are able to coordinate zinc and to bind DNA in a zinc-dependent manner [12,33].

We have also previously shown that *M. loti* Ros homologues Ml_4 and Ml_5, lacking of the structural metal ion, retain Ros87 fold and DNA-binding activity [34]. In these zinc lacking homologues a network of hydrogen bonds and hydrophobic interactions, that involve the aspartate in second position and the other residues that occupy the positions of the zinc ligands in Ros87, surrogate the zinc coordination role in the stabilization of the same functional fold [34]. In particular, a key role in this network of hydrogen bonds is played by a basic residue located in the turn between the second and third β-strand (Arg28 in Ml_4 and Lys27 in Ml_5) that contributes to the overall protein stability by interacting with the aspartate [12,34,35]. Interestingly, while the same position in Ros87 is occupied by Gly29, in most of Ros homologues that exploit the $CysAspHis_2$ combination of residues to coordinate the zinc ion, the equivalent position is always occupied by a basic residue suggesting for this residue an important structural role also when the domain natively binds the structural metal ion [12].

Since the coordination of structural zinc by an aspartate in eukaryotic zinc fingers is very unusual, to also elucidate whether and how Ros homologues bind the zinc ion in presence of an aspartate in the second coordination position, here we describe the characterization of a functional point mutant of Ros87 (Ros87_C27D) bearing the mutation of the second coordinating cysteine to aspartate. We have also characterized the double mutant Ros87_C27D_G29K testing its stability together with its metal and DNA-binding capability.

2. Materials and methods

2.1. EMSA

All of the proteins used for the DNA-binding experiments were expressed and purified from *Escherichia coli* BL21 host strain as GST fusions, after cloning the coding sequence in the pGEX-6P-1 expression vector [12]. 15 pmol of the purified proteins were incubated with 2.5 pmol of the oligonucleotide 5′-GATTTTATATTTCAATTTTATTGTAATATAATTTCAATTG-3′, in the presence of 25 mM HEPES (pH 7.9), 50 mM KCl, 6.25 mM $MgCl_2$, 1% NP-40, 5% glycerol. After incubation, the mixture was loaded on a 5% polyacrylamide gel (29:1 acrylamide:bisacrylamide ratio) and run in 0.5xTBE at room temperature (200 V for 75 min). The gel was stained with Syber Green (Invitrogen) and the image acquired with Typhoon Trio^{++} (Amersham).

2.2. Sample preparation

Unlabeled, ^{15}N and ^{15}N–^{13}C-labeled proteins used for all the experiments were over-expressed and purified as previously reported [23].

2.3. NMR experiments

NMR samples contained 1 mM of single or double-labeled proteins, 20 mM phosphate buffer adjusted at pH 6.8, 0.2 M NaCl and 90% H_2O/10% ^2H_2O. All the NMR experiments were acquired on a Varian Unity INOVA 500 MHz spectrometer and processed by using Varian (VNMR 6.1B) software. ^1H and ^{15}N chemical shifts were calibrated indirectly by using TMS as external references. The program XEASY [36] was used to analyze the spectra. The structures were analyzed and visualized by using the programs MOLMOL [37], PyMOL [38] and Chimera [39].

Triple resonance NMR experiments including 3D HNCA [40–42], 3D CBCANH [42], and 3D CBCA(CO)NH [42] were collected to enable sequence-specific backbone and $^{13}C_\beta$ resonances assignment. The side-chain ^1H and ^{13}C NMR signals were assigned from (H)CCH-TOCSY experiments [43]. NOE were evaluated from 3D ^{15}N- and ^{13}C-edited NOESY spectra and 2D [^1H,^1H]-NOESY. The NOESY spectra have been acquired with mixing times of 100 ms, 80 ms and 100 ms respectively. The C_γ chemical shift of aspartate 27 has been assigned by mean of a 2D ct-H(CA)CO [40].

In order to determine which residues in Ros87_C27D exhibit significant changes in the chemical shift with respect to Ros87, the normalized weighted average chemical shift differences (Δ_{av}/Δ_{max}) for amide protons (H_N) and nitrogen (^{15}N) of each residue were calculated as follows [44]: $\Delta_{av}(HN) = \sqrt[2]{\frac{\Delta H^2 + (\frac{\Delta N}{5})^2}{2}}$ where ΔH and ΔN are the chemical shift differences between the Ros87_C27D and Ros87 [24]. To evaluate the chemical shift variation for C_α and C_β nuclei $\Delta_{av}(HNC_\alpha C_\beta) = \sqrt[2]{\frac{\Delta H^2 + (\frac{\Delta N}{5})^2 + (\frac{\Delta C_\alpha}{2})^2 + (\frac{\Delta C_\beta}{2})^2}{4}}$ was also used [45]. For glycine and proline residues:

$$\Delta_{gly} = \sqrt[2]{\frac{\Delta H^2 + \left(\frac{\Delta N}{5}\right)^2 + \left(\frac{\Delta C_\alpha}{2}\right)^2}{3}} \qquad \Delta_{pro} = \sqrt[2]{\frac{\left(\frac{\Delta C_\alpha}{2}\right)^2 + \left(\frac{\Delta C_\beta}{2}\right)^2}{2}}$$

2.4. Uv–vis spectroscopy

Uv–vis spectra were recorded on a Shimadzu UV-1800 spectrophotometer from 200 to 800 nm at room temperature. The apoRos87, apoRos87_C27D and apoRos87_C27D_G29K concentrations were determined by evaluating the absorbance at 280 nm. Co(II)-apoRos87, Co(II)-apoRos87_ C27D and Co(II)-apoRos87_ C27D_G29K binding constants were determined in 30 mM HEPES and 100 mM NaCl at pH 7.4 by direct titration of the apo-protein solution (30 μM) with $CoCl_2$ solution (1.0 mM) up to 1.5 Co(II)/protein ratio. The absorbance at 340 nm indicative of the $S^- \rightarrow Co(II)$ ligand-to-metal charge-transfer (LMCT) transition [46] was monitored and used to calculate the cobalt binding affinity constant [47–49]. To estimate the zinc binding affinity, the reverse titration experiment was carried out adding $ZnCl_2$ solution (1.0 mM) to Co(II)-Ros87, Co(II)-Ros87_C27D or Co(II)-Ros87_C27D_G29K complexes up to a Zn(II)/protein ratio of 2.5 and the collected data were fitted as previously reported [47].

2.5. CD spectroscopy

The thermal denaturation of the Ros87_C27D and Ros87_ C27D_G29K proteins was evaluated using a JASCO J-815 CD spectropolarimeter equipped with Peltier temperature control. Data were collected using a quartz cuvette with a 1 cm path-length in the 200–260 nm wavelength range with a data pitch of 1 nm, a band width of 1 nm and scanning speed of 50 nm/min. CD spectra were measured at regular intervals in the 278–372 K range and normalized against reference spectra to remove the background contribution of buffer. After the final measurement at 372 K, the samples were cooled back to 298 K and a final set of spectra collected. The data obtained were fitted into two-state folding model [50].

2.6. Molecular dynamics (MD) simulations

Simulations were performed using GROMACS software [51]. The Amber99SB force field [52] with explicit TIP3P water was used. All simulations used a 2 fs inner step and were carried out in NVT ensemble using a Nose-Hoover thermostat after equilibration to constant box volume in the NPT ensemble. All simulations were run at 298 K. The time-averaged NOE distance restraints were imposed with a force constant of 6000 kJ mol^{-1} nm^{-2} and was used a memory relaxation time of 20 ps [53,54]. The 1 ns MD simulation were performed and snapshots were saved every 10 ps in order to have a total of 100 conformers for the ensemble. As starting point for the simulation was used the representative