Metal induced selectivity in phosphate ion binding in E9 DNase†

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Mass spectrometric and calorimetric data reveal that phosphate ion binding to the active site of colicin E9 DNase is delicately regulated by concomitant binding of specific transition metal ions.

In nature the functioning of proteins depends on subtle, multiple, specific non-covalent interactions with other molecules, such as other proteins, metals, DNA/RNA, co-factors *etc.* Therefore, means to monitor and validate such interactions are important. In this study we use electrospray ionization mass spectrometry (ESI-MS) for the measurement of the ligation of a single phosphate ion to the enzyme colicin E9. In contrast to what was expected from X-ray structural data this single phosphate ion ligation turned out to be specifically regulated by preceding Zn^{2+} ligation to the enzyme. Our ESI-MS data were validated by isothermal titration calorimetry (ITC). As DNA is the substrate of the enzyme we hypothesize that the findings reported here have impact on the detailed functional mechanism of the enzyme and the role of the metal ion co-factors therein.

Colicin E9 is a member of a family of bacterial endonuclease colicins that are protein antibiotics released by *Escherichia coli* into the extracellular medium during times of nutrient or environmental stress. On entrance into target cells the 15 kDa C-terminal DNase domain is translocated into the cytoplasm. In the target cell the lethal effect is accomplished by the hydrolysis of chromosomal DNA. Four highly homologous DNase colicins that use this mechanism have been identified E2, E7, E8 and E9. The intact ~60 kDa proteins consists of three domains: a translocation domain, a receptor binding domain and the aforementioned DNase domain. The DNase domain can be isolated as an active enzyme. A large number of biochemical and structural studies have been carried out previously on these homologous DNase colicins.[1,2] For instance, X-ray structures of the DNases E7 and E9 confirmed that the DNase proteins are able to coordinate a single metal ion within their active site. Zn^{2+} was observed in the E7 DNase structure,[3] whilst Ni^{2+} was observed ligated to E9 DNase.[4] The affinity of E9 DNase metal complexes have been probed by ITC and revealed that both Ni^{2+} and Zn^{2+} bind strongly, with dissociation constants of 0.7 μM and 1–4 nM, respectively.[5] Additionally, a single phosphate molecule has been observed in most of the reported X-ray structures, in proximity to the active site of the DNase (see Fig. 1). These phosphate ions likely originate from the phosphate buffer solutions that are used in the preparation of the samples. This single phosphate ion was observed in the same location in X-ray structures of metal free E9 DNase,[6] Ni^{2+} ligated E9 DNase,[6] Zn^{2+} ligated E9 DNase (1FSJ PDB), and Zn^{2+} ligated E7 DNase[3] and seems thus to be independent of the metal ion. As shown in Fig. 1 the phosphate ion is bridged between the Ni^{2+} and a histidine and arginine residue of the DNase active site. This phosphate ion binding likely denotes the position either of the scissile bond or even the product 5′-phosphate.

Since its introduction, ESI-MS[7] has evolved into a method that may be used to investigate noncovalent interactions.[8,9] Recently, we have shown that specific immunity protein binding and metal ion binding can be monitored by ESI-MS. More specifically, we revealed that the conformation of E9 DNase in solution dramatically alters upon metal ion binding.[10,11] In these ESI-MS experiments we did not use phosphate containing buffers, but a for electrospray more amendable ammonium acetate buffer. Therefore, we were initially unable to observe phosphate ion binding. In this work we set out to investigate whether we could use ESI-MS to observe any specific phosphate ion binding. In Fig. 2 ESI mass spectra are shown for 10 μM colicin E9 DNase in aqueous 50 mM ammonium acetate at pH 7.4, in the presence of low concentrations of metal and phosphate ions. We observed primarily the charge states from 7+ to 9+. The spectra were recorded from solutions containing less than stoichiometric amounts of metal and ammonium phosphate ions, so that metal free E9 DNase was always detectable. The measured mass of the metal free E9 DNase protein is 15088.3 ± 0.3 Da (calculated mass 15088.7 Da). A listing of all detected ions, their masses and assignments is given in the supplementary Fig. S1†. The bottom

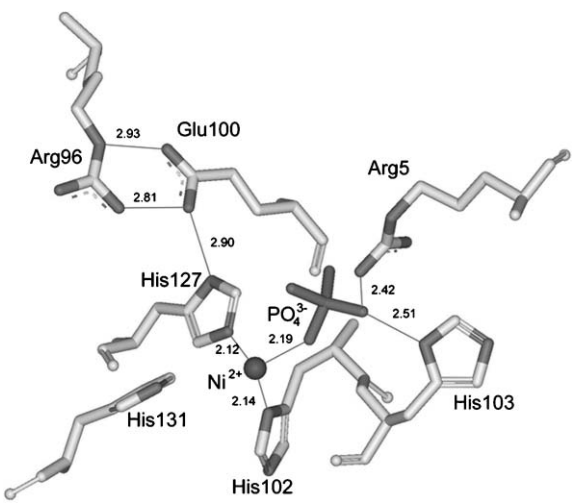

Fig. 1 X-ray structure of the E9 DNase active site,[4] whereby the location of the Ni^{2+} and phosphate ion and their interactions with the enzyme are highlighted. Values indicate distances in angstroms (Å).

† Electronic supplementary information available: Figure S1. See http://www.rsc.org/suppdata/cc/b4/b415709e/

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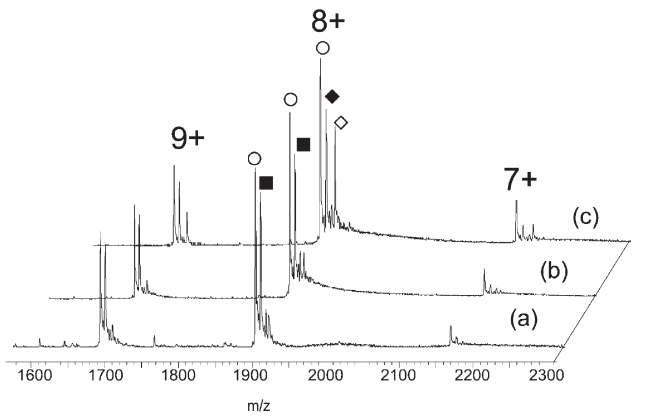

Fig. 2 ESI-MS spectra of E9 DNase (10 μM) in the presence of (a) Ni^{2+}, (b) Ni^{2+} and PO_4^{3-}, and (c) Zn^{2+} and PO_4^{3-}. Ligand concentrations are ~ 6 μM. Signals indicated by (○) represent the metal-free; (■) Ni^{2+}-ligated; (◆) Zn^{2+}- ligated and (◇) Zn^{2+}-PO_4^{3-} ligated DNase.

mass spectrum (Fig. 2a) shows ion signals of both the metal-free and Ni^{2+}-ligated E9 DNase, which are differentiated by a mass increase of approximately 56 Da (58–2H). Upon addition of ammonium phosphate to the solution used in Fig. 2a, the resulting spectrum in Fig. 2b revealed clearly no phosphate ion ligation (+98 Da) to either the metal free E9 DNase and the Ni^{2+}-ligated E9 DNase. Performing similar experiments now with Zn^{2+}, instead of Ni^{2+}, provided a very different picture, as shown in Fig. 2c. The ESI spectrum of the solution containing a less than stoichiometric amount of Zn^{2+} resulted in a spectrum (Fig. S1†) very similar to that shown in Fig. 2a, with ion signals of both the metal-free and Zn^{2+}-ligated E9 DNase, differentiated by a mass increase of approximately 63 Da (65–2H). When ammonium phosphate was added to this solution the Zn^{2+}-ligated E9 DNase unexpectedly ligated additionally a single phosphate ion (as indicated by the mass increase of 98 Da). No ligation is observed for the metal-free E9 DNase. It should be noted that these observations are monitored from a single solution, sprayed under identical conditions, undoubtedly indicating that phosphate ion binding is Zn^{2+} dependent. Increasing the concentrations for both Zn^{2+} and ammonium phosphate resulted finally in a fully saturated 1:1:1 E9–Zn^{2+}–PO_4^{3-} ternary complex (Fig. S1†). In contrast, increasing concentrations further for Ni^{2+} and ammonium phosphate merely resulted in a fully saturated 1:1 E9–Ni^{2+} binary complex (Fig. S1†). The formation of the ternary complex for the Zn^{2+}-bound state was unexpected, since from the reported X-ray structures it was suggested that phosphate binding is not Zn^{2+} specific, and not even metal ion dependent,[3,6] although we reiterate that in the crystallography experiments the phosphate ion concentration was approximately 1000-fold higher, allowing possible non-specific binding.

Although a vast amount of data exist nowadays validating the use of ESI-MS for the investigation of non-covalent interactions in solution, we further addressed this unexpected phenomenon by a complementary method in the condensed phase. We therefore performed isothermal titration calorimetry (ITC). As in the ESI-MS experiments we first made sure by thorough dialysis that no spurious phosphate ions were present in the solutions used. We titrated aqueous sodium phosphate solutions into aqueous 50 mM triethanolamine solutions containing respectively metal-free E9 DNase, Ni^{2+} saturated and Zn^{2+} saturated E9 DNase (pH 7.4, 25 °C). The resulting data are shown in Fig. 3. These ITC results show that no phosphate ion ligation could be observed in the solutions containing either metal-free E9 DNase and Ni^{2+} saturated E9 DNase. Performing identical titrations in the presence of Zn^{2+} resulted in a complete single phosphate ion binding isotherm. The dissociation constant for phosphate ion binding to the Zn^{2+} saturated E9 DNase was 15 μM. The ITC measurements therefore confirm the ESI-MS findings.

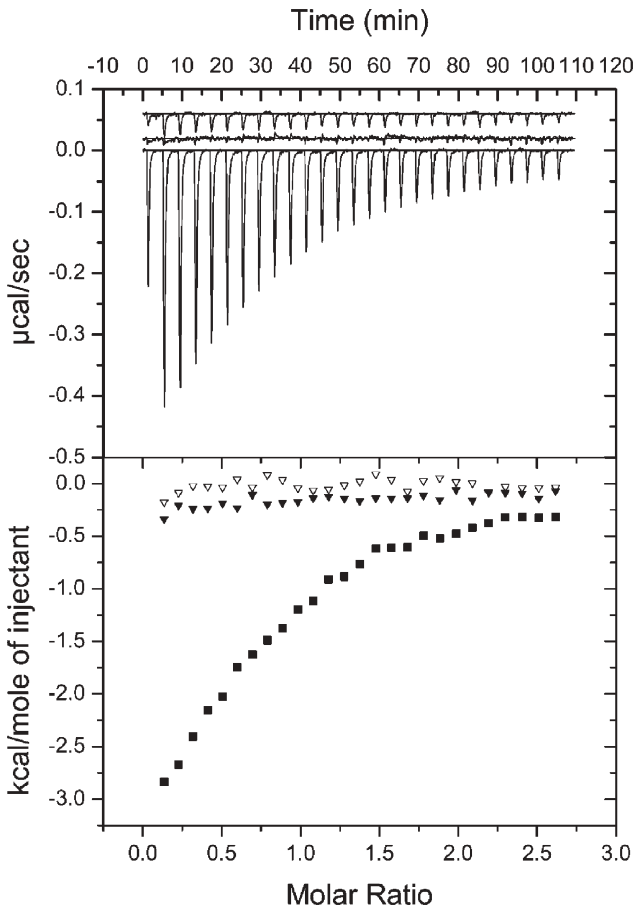

Fig. 3 ITC data for the binding of a single phosphate ion to E9 DNase. Top panel shows the calorimetric response of 25 × 10 μl injections of 510 μM phosphate to 40 μM E9 DNase in 50 mM triethanolamine buffer, pH 7.4 and 25 °C. Bottom panel shows integrated injections heats for the above data. The produced injection heats indicated by (▼), (▽) and (■) are from metal-free, E9-Ni^{2+} and E9-Zn^{2+}, respectively.

Our combined findings indicate that metal ion binding and phosphate ion binding (and possibly DNA substrate binding) are intriguingly regulated in the E9 colicin DNase system. This is remarkable as the X-ray structures available provide no clue for the observed behavior as both the position of the metal ion as well as the interactions with the amino acids in the catalytic site of the E9 DNase seem to be very similar. At present we are pursuing our studies by investigating the effects of specific single amino acid mutants in the catalytic site, which potentially are involved in metal ion binding and/or phosphate ion binding. Additionally, we hope to further elucidate the functional role of the metal ions on the biological activity of the DNase enzymes and intend to study protein–DNA interactions of these DNase's.

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