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The *Salmonella enterica* ZinT structure, zinc affinity and interaction with the high-affinity uptake protein ZnuA provide insight into the management of periplasmic zinc

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ABSTRACT

Background: In Gram-negative bacteria the ZnuABC transporter ensures adequate zinc import in Zn(II)-poor environments, like those encountered by pathogens within the infected host. Recently, the metal-binding protein ZinT was suggested to operate as an accessory component of ZnuABC in periplasmic zinc recruitment. Since ZinT is known to form a ZinT–ZnuA complex in the presence of Zn(II) it was proposed to transfer Zn(II) to ZnuA. The present work was undertaken to test this claim.
Methods: ZinT and its structural relationship with ZnuA have been characterized by multiple biophysical techniques (X-ray crystallography, SAXS, analytical ultracentrifugation, fluorescence spectroscopy).
Results: The metal-free and metal-bound crystal structures of *Salmonella enterica* ZinT show one Zn(II) binding site and limited structural changes upon metal removal. Spectroscopic titrations with Zn(II) yield a K_D value of 22 ± 2 nM for ZinT, while those with ZnuA point to one high affinity ($K_D < 20$ nM) and one low affinity Zn(II) binding site (K_D in the micromolar range). Sedimentation velocity experiments established that Zn(II)-bound ZinT interacts with ZnuA, whereas apo-ZinT does not. The model of the ZinT–ZnuA complex derived from small angle X-ray scattering experiments points to a disposition that favors metal transfer as the metal binding cavities of the two proteins face each other.
Conclusions: ZinT acts as a Zn(II)-buffering protein that delivers Zn(II) to ZnuA.
General significance: Knowledge of the ZinT–ZnuA relationship is crucial for understanding bacterial Zn(II) uptake.

1. Introduction

Zinc (Zn) is the second most abundant transition metal after iron in living organisms where it plays vital roles. In bacteria, Zn has a catalytic function in a large number of housekeeping enzymes or virulence-related ones (*e.g.* β-lactamases or extracellular metalloproteinases) and a structural role in other enzymes (*e.g.* periplasmic copper, zinc superoxide dismutase). On the other hand, Zn at high concentrations is highly toxic for all cell types since it can interact non-specifically with polypeptide chains [1] and in that way inhibit essential enzymes [2]. It follows that the cellular concentration of Zn, as well as that of other metals like iron, has to be controlled very strictly. Bacteria achieve the delicate balance between the requirement for Zn and its toxicity by the coordinated action of high- and low-affinity uptake systems and of export systems that rid the cells of excess Zn. Thus, different transporters acquire the metal from the growth medium to reach a total concentration in the submillimolar range, while transcriptionally controlled zinc uptake and efflux systems maintain the readily exchangeable "free" zinc at very low concentrations [3]. Whereas initial *in vitro* studies suggested that cellular "free" zinc levels are in the femtomolar range [3], more recent studies involving ratiometric zinc biosensors have shown that the *in vivo* "free" zinc is around 20 pM [4].

Investigations carried out on *Escherichia coli*, and confirmed for other microorganisms like *Salmonella enterica*, have established that the activity of the Zn import and export systems is controlled by Zur and ZntR, two metalloproteins that regulate gene transcription depending on their metallation state. Zur comes into play when the Zn concentration in the medium is low [5]; it is known to control the expression of the high affinity Zn uptake system ZnuABC, which is used by Gram-

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negative bacteria to transport Zn from the periplasmic space to the cytosol. ZnuABC is a high affinity ATP-binding cassette-type transporter and like all such systems is composed of three proteins: a soluble periplasmic component (ZnuA) that captures Zn(II) and delivers it to the membrane permease (ZnuB), whereas the ATPase component (ZnuC) provides the energy necessary for ion transport through the inner membrane.

Panina et al. [6] identified other Zur-regulated genes interspersed within the bacterial chromosome. These include the gene for a putative metal-binding protein named ZinT, categorized initially as a member of the *E. coli* cadmium stress stimulon [7]. ZinT was proposed to decrease the cadmium concentration in *E. coli* cells during cadmium stress. This putative function was ruled out by later studies which indicated clearly that ZinT is involved in Zn homeostasis [8–11]. In particular, *Salmonella* and *E. coli* strains deleted of the *zinT* gene were shown to be impaired in their ability to grow in media poor of this metal [10,11]; moreover, ZinT accumulation was shown to depend on zinc availability in the medium. As in the case of ZnuA, *zint* expression increases in bacteria growing in Zn-poor media and is repressed in the presence of abundant Zn. Importantly, the expression of *zint* is deregulated in bacteria lacking ZnuA, but not *vice-versa*. As a result, bacteria lacking the *zint* gene are able to grow in a medium with low zinc concentrations, despite the reduced duplication rates, whereas bacteria lacking the *znua* gene are unable to grow [10,11]. These results indicate that the ZinT and ZnuABC activities are strictly related and that ZinT may be considered an accessory member of the ZnuABC transporter. The tight linkage between ZinT and the ZnuA and ZnuB components of the transporter is evidenced also by other experimental data. *Salmonella* mutant strains deleted either of the whole *znuABC* operon or of the single *znuA* gene—and thus potentially able to express ZnuB, but not ZnuA—are equally impaired in the ability to import environmental zinc [10]. It follows that ZnuB cannot mediate Zn(II) import in the absence of ZnuA. Further, ZinT cannot compensate for the lack of ZnuA, indicating that the role of ZinT is likewise dependent on the presence of ZnuA [10].

The data on the structural basis of this functional relationship are limited. It has been established that ZinT and ZnuA do not interact when metal-free, but form a stable complex when metal-bound [10]. However, the dependence of complex formation on the metallation state of the individual proteins and the mode of their interaction are not known. Interestingly, in some Gram-positive bacteria, Zn(II) transport is ensured by AdcA, a lipoprotein constructed by two domains resembling ZnuA and ZinT [6,12].

Even though several bacteria relying on the ZnuABC transporter to import Zn(II) do not possess ZinT, these studies suggest that the contribution of ZinT to metal recruitment within the periplasmic space is considerable, at least under conditions of severe Zn shortage and provided ZnuA is present [10]. It may be hypothesized that the ZinT contribution to Zn(II) recruitment entails binding of the metal with high affinity, followed by formation of a complex with ZnuA that allows metal to be transferred. To prove this contention, we have chosen to work on the proteins from *S. enterica* in view of the wealth of data accumulated in recent years on the ZnuABC transporter from this microorganism [10,13–15].

Firstly, X-ray crystal structure studies on *S. enterica* ZinT (*Se*ZinT) in the metal-free and metal-bound forms were undertaken. The only ZinT structure known pertains to metal-bound *E. coli* ZinT (*Ec*ZinT, indicated originally as YodA) [16]. *Ec*ZinT is composed of two domains: a major one related structurally to the lipocalin/calycin protein family and a smaller helical domain. The metal-binding site, formed by histidine side chains, is buried at the domain interface, along the side of the calycin domain [16]. It was not established whether the binding of Zn(II) gives rise to protein conformational changes.

In contrast to the ZinT proteins, the X-ray structures of several members of the ZnuA family are known, namely those of the proteins from *E. coli* (*Ec*ZnuA, PDB codes: 2OGW; 2OSV; 2PRS) [17–19], *Synechocystis 6803* (PDB code: 1PQ4) [20] and *S. enterica* (*Se*ZnuA, PDB code: 2XQV) [13]. The ZnuA family belongs to the so-called cluster 9 of periplasmic solute-binding proteins (PBPs) and displays their well conserved architecture comprising a pair of $(\alpha/\beta)_4$ sandwich domains and a connecting long, tightly packed α-helix. The distinctive characteristic of all ZnuA proteins consists of a histidine rich (His-rich) loop located at the entrance of the Zn(II) binding site at the interface between the two domains. The *Se*ZnuA structures with the Zn(II) binding site occupied either partially or fully, and the structure of a deletion mutant lacking a large part of the loop (*Se*ZnuA Δ118–141), where the site is empty (PDB code: 2XH8), all indicate that the His-rich loop plays an important role in the Zn(II) management process. In fact, their comparison unveiled for the first time the occurrence of Zn(II)-induced conformational changes that are likely of functional relevance in metal sequestration from the periplasm and/or its delivery to ZnuB. In particular, His140, placed on the C-terminal part of the *Se*ZnuA His-rich loop, appears of importance as it replaces His60 (*Ec*ZnuA numbering) [13], one of the otherwise conserved metal binding histidine residues in Zn- and Mn-specific PBPs. Moreover, structural–dynamical investigations pointed to a high mobility of the His-rich loop and suggested that the fluctuations may be influenced by Zn(II) binding either at the primary site or at the His-rich loop itself [14].

The hypothesis that the contribution of ZinT to the Zn(II) management processes in the *S. enterica* periplasm is based on its interaction with ZnuA and on the subsequent transfer of bound Zn(II) to the latter protein is strengthened by the data presented in this paper. Thus, the X-ray structures of metal-free and Zn(II)-bound *Se*ZinT disclose Zn(II)-induced conformational changes that are confined to the area surrounding the Zn(II) binding site. An additional Zn(II)-bound *Se*ZinT structure was solved that displays a PEG molecule in the inter-domain cleft, in a similar position as that occupied by the *Se*ZnuA His-rich loop in the model of the *Se*ZinT–Zn(II)–*Se*ZnuA complex derived from SAXS experiments. In the modeled complex the Zn(II) binding sites of the two proteins face each other, in an arrangement that allows metal to be transferred. Significantly, *Se*ZnuA has a higher affinity for the metal (K_D < 20 nM) than SeZinT (K_D 22 ± 2 nM) and the interaction of ZinT with ZnuA takes place only when Zn(II) is bound to ZinT.

2. Material and methods

2.1. Protein purification

Cells harboring plasmid pSEzinT [10] were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin. Protein expression was induced overnight with 0.1 mM isopropyl β-D-1-thiogalattopiranoside (IPTG) when the absorbance of the culture at 600 nm reached 0.5. Cells were harvested by centrifugation for 15 min at 5000 rpm and periplasmic proteins were extracted by lysozyme treatment. Spheroplasts were separated from periplasmic proteins by centrifugation and the supernatant was applied to a Ni-NTA column pre-equilibrated with 50 mM Na–phosphate, 250 mM NaCl, pH 7.8 and eluted with a linear gradient of 0–500 mM imidazole. ZinT eluted at 250 mM imidazole, due to the presence of a naturally occurring His-rich N-terminal sequence which confers to the protein the ability to strongly interact with immobilized metal ions. Fractions containing ZinT (>98% pure according to SDS-PAGE analyses) were pooled, dialyzed against 20 mM HEPES, 10 mM NaCl, pH 7.0, concentrated to 20 mg/ml by ultrafiltration, using Amicon Ultrafiltration Discs YM-10, and stored at −20 °C. About 20 mg of purified protein was obtained per liter of bacterial culture. The protein concentration has been evaluated using the extinction coefficient at 280 nm ($\varepsilon = 37{,}485\ M^{-1}\ cm^{-1}$) based on the protein amino acid composition (http://web.expasy.org/tools/protparam/protparam-doc.html). Wt *Se*ZinT contains the signal peptide typical of periplasmic proteins and has 215 amino acids, whereas the recombinant protein comprises 186 residues. In the X-ray structure (see below) the first residues (HGHHAHGA) are not visible.