The structures of the CutA1 proteins from *Thermus thermophilus* and *Pyrococcus horikoshii*: characterization of metal-binding sites and metal-induced assembly

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Received 27 December 2013
Accepted 15 February 2014**PDB references:** Na⁺–*Tt*CutA1, 1nza; apo
*Tt*CutA1, 1v6h; Na⁺–*Ph*CutA1, 4nyo

CutA1 (copper tolerance A1) is a widespread cytoplasmic protein found in archaea, bacteria, plants and animals, including humans. In *Escherichia coli* it is implicated in divalent metal tolerance, while the mammalian CutA1 homologue has been proposed to mediate brain enzyme acetylcholinesterase activity and copper homeostasis. The X-ray structures of CutA1 from the thermophilic bacterium *Thermus thermophilus* (*Tt*CutA1) with and without bound Na⁺ at 1.7 and 1.9 Å resolution, respectively, and from the hyperthermophilic archaeon *Pyrococcus horikoshii* (*Ph*CutA1) in complex with Na⁺ at 1.8 Å resolution have been determined. Both are short and rigid proteins of about 12 kDa that form intertwined compact trimers in the crystal and solution. The main difference in the structures is a wide-type β -bulge on top of the *Tt*CutA1 trimer. It affords a mechanism for lodging a single-residue insertion in the middle of β_2 while preserving the interprotomer main-chain hydrogen-bonding network. The liganded forms of the proteins provide new structural information about the metal-binding sites and CutA1 assembly. The Na⁺–*Tt*CutA1 structure unveils a dodecameric assembly with metal ions in the trimer–trimer interfaces and the lateral clefts of the trimer. For Na⁺–*Ph*CutA1, the metal ion associated with six waters in an octahedral geometry. The structures suggest that CutA1 may contribute to regulating intracellular metal homeostasis through various binding modes.

1. Introduction

Heavy-metal ions are important in biological metabolic processes, especially as enzymatic cofactors, in both eukaryotic and prokaryotic cells. The ions also help to stabilize protein structures and macromolecular interactions and maintain cellular osmotic balance. However, excessive levels of heavy metals are harmful, and therefore intracellular metal concentrations must be carefully regulated (Nelson, 1999; Ercal *et al.*, 2001; Adaikkalam & Swarup, 2002; Franke *et al.*, 2003). Copper is an essential trace element required for survival by all organisms; for example, some enzymes require it for electron transfer from substrates to oxygen. However, like other heavy elements, in excess copper is highly toxic to cells because of its chemical redox potential and its ability to participate in free-radical reactions (Gupta *et al.*, 1995; Rensing & Grass, 2003). Therefore, all organisms have developed tightly controlled copper homeostasis mechanisms during evolution. Microorganisms use uptake, chelation and extrusion of the extra copper or enzymatic detoxification to less toxic metal-ion species to uphold the correct cellular balance and thus avoid toxicity (Silver, 1996; DiDonato & Sarkar, 1997; Rosenzweig, 2001; Banci & Rosato, 2003; Arnesano *et al.*, 2003). Deficiency in copper-regulating proteins, or disruption of their activities, may cause many diseases, including Wilson (copper overload) and Menkes (copper deficiency) diseases and possibly even Alzheimer's pathology (Gupta *et al.*, 1995; Bush, 2000; Zhao *et al.*, 2012). Early detection and treatment with metal supplements or chelating agents, or through the activation of enzymes possessing the appropriate metabolic activity

using activators or coenzymes, may prevent the damage caused by imbalances in metal homeostasis.

In microorganisms, the *cop* (*copA*, *copB*, *copY*, *copZ*) and *cut* (*cutA*, *cutB*, *cutC*, *cutD*, *cutE* and *cutF*) genes are assumed to be associated with cellular copper regulation. The *cop* gene family is a well understood system of active transport efflux pumps (Odermatt & Solioz, 1995; Silver & Phung, 1996; Magnani & Solioz, 2005). In contrast, the *cut* gene family is not clearly defined (Gupta *et al.*, 1995; Fong *et al.*, 1995). The *cutA* locus consists of two operons, one containing a single gene encoding a relatively small cytoplasmic protein of ~12 kDa (CutA1) and the second composed of two genes encoding a 50 kDa (CutA2) and a 24 kDa (CutA3) inner membrane protein. Molecular genetics studies showed that in *Escherichia coli* CutA1 (*EcCutA1*), in cooperation with CutA2, is implicated in tolerance to divalent cations (copper, zinc, nickel, cobalt and cadmium; Fong *et al.*, 1995). The CutA1 function may occur by direct ion binding and/or by affecting ion import/export through interaction with membrane transporters. The mammalian CutA1 homologue was found in the brain and supposedly has roles in the processing and trafficking of membrane-bound acetylcholine esterase (AChE) tetramers as well as in lactation, copper homeostasis and ligand transport to membranes (Perrier *et al.*, 2000, 2002; Navaratnam *et al.*, 2000; Naylor *et al.*, 2005; Yang *et al.*, 2009; Liang *et al.*, 2009; Zhao *et al.*, 2012). Although direct structural data for the distinct functions of CutA1 are not available at present, in all known structures a trimeric quaternary framework of a structurally ordered ~100-amino-acid region is found to be conserved (Arnesano *et al.*, 2003; Tanaka *et al.*, 2004; Savchenko *et al.*, 2004; Lin *et al.*, 2006; Bagautdinov *et al.*, 2008; Sato *et al.*, 2011). This implies a similar mechanism of the protein multi-tasking activity that may depend on its cellular location and surrounding conditions. Additionally, the CutA1 trimeric packing with functionally active lateral clefts resembles the arrangement of the well studied signal transducer PII proteins (Xu *et al.*, 2001; Arnesano *et al.*, 2003; Llcer *et al.*, 2007). In order to accomplish multiple functions, the trimeric architecture of CutA1 should be stable across a range of environmental conditions responding to changes of the intracellular metal concentrations. Indeed, recent experiments showed that the protein is characterized by extremely high stability; the heat denaturation temperatures (T_d) of *Pyrococcus horikoshii* CutA1 (*PhCutA1*) and *Thermus thermophilus* CutA1 (*TtCutA1*) are quite high at ~150 and ~113°C, respectively (Tanaka *et al.*, 2006; Sawano *et al.*, 2008; Matsuura *et al.*, 2010, 2012; Sato *et al.*, 2011).

To establish the active-site residues and to understand the detailed structure–function relationships, three-dimensional structures of metal ion–CutA1 complexes are desirable. Previous X-ray studies have shown that in the crystalline state the CutA1 proteins bind metal ions at different lattice-dependent positions (Arnesano *et al.*, 2003; Savchenko *et al.*, 2004; Tanaka *et al.*, 2004). Moreover, structural evidence for metal-mediated multimerization of the protein has been detected (Tanaka *et al.*, 2004). Here, in order to reveal the molecular determinants of metal-binding sites in *TtCutA1* and *PhCutA1*, we report the crystal structures of metal-free *TtCutA1* in the hexagonal space group $P3_221$ and sodium-ion-liganded *TtCutA1* in the cubic space group $I23$ at 1.9 and 1.7 Å resolution, respectively, and of *PhCutA1* in the orthorhombic space group $P2_12_12_1$ with Na⁺ at 1.8 Å resolution. The structures provide clues about how CutA1 binds and/or segregates metal ions, which may help to maintain the proper amount of available ions in the cell. The study is of general interest, as the coordination chemistry used by a protein for metal management should be distinctive from that used to carry out catalytic and/or stability functions.

2. Materials and methods

2.1. Purification and crystallization

The protocols used for the expression and purification of *TtCutA1* and *PhCutA1* have been described previously (Tanaka *et al.*, 2006). The homogeneity and identity of the purified samples were estimated by SDS–PAGE (Laemmli, 1970) and their trimeric oligomerization state in solution was confirmed by analytical ultracentrifugation experiments in a Beckman XL-A analytical ultracentrifuge (Beckman-Coulter) using an An-60 Ti rotor.

Prior to crystallization, the purified *TtCutA1* and *PhCutA1* were concentrated by ultrafiltration to 18.9 and 21.0 mg ml^{−1}, respectively, in buffer consisting of 20 mM Tris–HCl, 0.2 M NaCl pH 8.0. Crystallization trials of *TtCutA1* were carried out using the oil-microbatch method (Chayen *et al.*, 1990) in Nunc HLA plates at 22°C using a TERA crystallization robot (Sugahara & Miyano, 2002). Equal volumes of protein solution (0.5 µl) and precipitant solution (0.5 µl) were mixed. The crystallization drop was overlaid with a 1:1 mixture of silicone and paraffin oils (13 µl), allowing slow evaporation of water in the drop. For *TtCutA1*, a precipitant solution consisting of 1.65 M sodium acetate, 0.1 M MES–NaOH pH 6.6 provided the most well defined crystals. Small crystals appeared after 4 d and continued to grow for about two weeks. A thin plate-shaped crystal of dimensions ~0.25 × 0.13 × 0.08 mm was chosen for the X-ray experiment. The condition produced diffraction-quality crystals of the sodium-ion-bound protein. The monovalent Na⁺ is a nonphysiological metal for CutA1, but attempts to crystallize *TtCutA1* in the presence of divalent copper, cobalt and zinc salts were unsuccessful. Other crystallization conditions that yielded metal-free *TtCutA1* crystals were obtained using the sitting-drop vapour-diffusion method (Chayen, 1998). Equal volumes of the protein solution (1.0 µl) and reservoir solution (1.0 µl) consisting of 16.5% (w/w) PEG 20K, 0.1 M sodium HEPES pH 6.1 were equilibrated against 200 µl reservoir solution. The best diffracting crystal grew to maximum dimensions of 0.28 × 0.14 × 0.11 mm after 5 d incubation at 22°C.

Attempts to obtain CutA1 with divalent ions by crystallization of *PhCutA1* in reservoir solution with added copper(II) chloride or cobalt acetate tetrahydrate were unsuccessful. However, an Na⁺–*PhCutA1* crystal could be grown using the sitting-drop method at 22°C by mixing equal volumes (1.5 µl) of the protein solution with a reservoir solution consisting of 17.5% (w/w) PEG 4000, 0.1 M MES–NaOH, 5 mM CuCl₂ pH 6.3. Drops composed of the protein solution mixed with the precipitant solution were equilibrated against 200 µl precipitant solution. Bar-shaped crystals appeared after a few days and grew to a full size of approximately 0.50 × 0.13 × 0.11 mm in 10 d.

2.2. X-ray data collection and processing

Prior to data collection, the crystals were mounted in a nylon-fibre loop, quickly soaked in cryoprotectant [reservoir solution supplemented with 15% (v/v) glycerol] for a few seconds and then flash-cooled in a −173°C dry nitrogen stream. A complete X-ray diffraction data set for Na⁺–*TtCutA1* was collected using synchrotron radiation on the bending-magnet beamline BL44B2 at SPring-8 (Adachi *et al.*, 2001). Data were collected with a MAR CCD165 area detector (MAR Research, Germany) using monochromatic X-rays of wavelength 0.90 Å. A full data set to 1.70 Å resolution was collected as 180 images with 1° oscillation at a crystal-to-detector distance of 100 mm with 1 min exposure times. For other crystals, X-ray diffraction data were collected with an in-house Rigaku FR-D (Cu Kα radiation, λ = 1.54178 Å) rotating-anode generator operating

at 50 kV and 100 mA and equipped with an R-AXIS IV image-plate detector and an X-stream 2000 low-temperature system (Rigaku, Japan). The crystal-to-detector distance was set to 170 mm and images of 0.5° oscillation were collected for 5 min exposure times. All data were processed using the *HKL-2000* program package (Otwinowski & Minor, 1997). The Na⁺–*TiCutA1* crystal was found to belong to space group *I*23, with unit-cell parameters *a* = *b* = *c* = 83.00 Å. A Matthews coefficient *V*_M of 1.85 Å³ Da^{−1} was obtained for one molecule per asymmetric unit, with a corresponding solvent content of 33% (Matthews, 1968). For the *TiCutA1* apo form, the crystal was found to belong to space group *P*3₂21, with unit-cell parameters *a* = *b* = 71.75, *c* = 100.40 Å, *γ* = 120°. A Matthews coefficient *V*_M of 2.16 Å³ Da^{−1} was obtained for three molecules per asymmetric unit, with a corresponding solvent content of 43%. For the Na⁺–*PhCutA1* form, the crystal was found to belong to space group *P*2₁2₁2₁, with unit-cell parameters *a* = 93.09, *b* = 124.25, *c* = 50.46 Å. Two *PhCutA1* trimers aggregate face-to-face to form a hexamer in the asymmetric unit, yielding a Matthews coefficient *V*_M of 1.73 Å³ Da^{−1}, with a corresponding solvent content of 29%.

2.3. Structure determination and refinement

The crystal structures were solved by molecular replacement. The Na⁺–*TiCutA1* structure was determined using a model of a monomer of *CutA1* from *Thermotoga maritima* (PDB entry 1kr4; Savchenko *et al.*, 2004). Rotation- and translation-function searches and initial rigid-body refinement were carried out using *CNS* (Brünger *et al.*, 1998) and were checked by displaying the transformed coordinates in *TURBO-FRODO* (Roussel & Cambillau, 1992). Further rounds of refinement using standard *CNS* protocols were interspersed with manual rebuilding including insertion of ions, ligands and water molecules using *TURBO-FRODO*. Two sites of significantly different electron density (*2F*_{obs} − *F*_{calc}) were identified

Figure 1
Overall structure of *CutA1*. Ribbon diagram of the trimeric structure viewed down the crystallographic threefold axis. Protomers *A*, *B* and *C* are coloured green, red and yellow, respectively, and protomer *C* is presented with secondary structures. The N- and C-termini are identified in the models of each protomer.

Table 1
Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

	Na ⁺ – <i>TiCutA1</i>	<i>TiCutA1</i>	Na ⁺ – <i>PhCutA1</i>
Space group	<i>I</i> 23	<i>P</i> 3 ₂ 21	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å)			
<i>a</i>	83.00	71.75	93.09
<i>b</i>	83.00	71.75	124.25
<i>c</i>	83.00	100.40	50.46
<i>α</i> = <i>β</i> (°)	90	90	90
<i>γ</i> (°)	90	120	90
<i>V</i> _M (Å ³ Da ^{−1})	1.85	2.16	1.73
Solvent content (%)	32.99	42.61	28.48
Subunits per asymmetric unit	1	3	6
Data collection			
Temperature (K)	100	100	100
X-ray source	BL44B2, SPring-8	Rigaku FR-D	Rigaku FR-D
Detector	MAR CCD 165	R-AXIS IV	R-AXIS IV
Wavelength (Å)	0.9	1.54178	1.54178
Resolution range (Å)	40–1.70 (1.76–1.70)	40–1.90 (1.99–1.90)	40–1.80 (1.88–1.80)
Reflections (total/unique)	324012/10452	801788/23582	324593/53038
<i>R</i> _{merge} [†] (%)	7.3 (47.9)	8.2 (44.6)	6.0 (32.4)
<i>I</i> / <i>σ</i> (<i>I</i>)	51.3 (7.9)	17.5 (6.7)	20.7 (4.1)
Completeness (%)	99.8 (100)	95.4 (90.4)	96.3 (86.2)
Refinement statistics			
No. of protein atoms	819	2462	5250
No. of water atoms	84	386	688
Bound metal atoms	Na1, Na2	—	Na1
Reflections (working set/ test set)	10452/544	23009/1116	53038/2691
<i>R</i> _{work} / <i>R</i> _{free} [‡]	0.23/0.25	0.19/0.23	0.19/0.23
R.m.s. deviations from ideal values			
Bond lengths (Å)	0.005	0.006	0.005
Bond angles (°)	1.3	1.4	1.2
<i>B</i> factors (Å ²)			
Wilson plot	19.80	20.10	23.70
Average	27.70	28.65	26.67
Protein atoms	24.6	26.2	26.1
Water atoms	42.0	43.8	42.5
Ramachandran plot (%)			
Most favoured	93.5	93.8	93.5
Additionally allowed	6.5	5.8	6.5
Generously allowed	0	0.4	0.0
PDB code	1nza	1v6h	4nyo

[†] *R*_{merge} = $\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where *I*_i(*hkl*) and $\langle I(hkl) \rangle$ are the observed intensity of measurement *i* and the mean intensity of the reflection with indices *hkl*, respectively. [‡] *R*_{work} = $\sum_{hkl} ||F_{obs}| - |F_{calc}|| / \sum_{hkl} |F_{obs}|$, where *F*_{obs} and *F*_{calc} are the observed and calculated structure factors, respectively. § *R*_{free} is the *R*_{work} calculated for a subset of 5% of the reflections that were omitted from refinement.

as metal-ion-binding positions. Based on their coordination with ion-binding residues, temperature factors, the strong signals the bound ions are most likely to be Na⁺ from the crystallization mixture. The structure model contains all residues, and in addition there were features in the electron-density maps that appeared to be two Na⁺ ions, four Cl[−] ions, five sulfate ions, six glycerol molecules and 84 water molecules. The structures of the apo *TiCutA1* and Na⁺–*PhCutA1* proteins were determined using a homotrimer of Na⁺–*TiCutA1* around a noncrystallographic triad. The structures of the proteins were solved by molecular replacement with *MOLREP* (Vagin & Teplyakov, 2010) incorporated in the *CCP4* suite (Winn *et al.*, 2011) and refinement procedures were carried out in *CNS* (Brünger *et al.*, 1998) with manual rebuilding using the program *QUANTA* (Accelrys, San Diego). In Na⁺–*PhCutA1*, the ions were assigned to the high peaks in the (*2F*_{obs} − *F*_{calc}) difference electron-density maps and each peak identified as an Na⁺ ion was inspected to determine whether the arrangement of surrounding electron-density features in experimentally phased and electron-density difference maps was octahedral. Here, a fully hydrated Na⁺ ion was found by searching electron-density maps visually for spherical features containing six identifiable water molecules arranged octahedrally.

Figure 2

Sequence alignment of the *TtCutA1* and *PhCutA1* proteins. Secondary α and β structures are highlighted by orange and cyan shading, respectively. Residues highlighted in red are identical. Residues binding the metal ions are indicated by green triangles. Magenta and cyan clamps underlining the *PhCutA1* sequence mark the inner and outer SC clusters, respectively.

Refined density for all of the water molecules was clear. The binding modes for the hexa-aquated sodium, $[\text{Na}(\text{H}_2\text{O})_6]^+$, to the trimers of hexameric *PhCutA1* were found to be similar. Also, there was an additional electron density associated with the S atom of Cys29 from full oxidation of the residue to cysteine-S-dioxide ($\text{C}_3\text{H}_7\text{NO}_4\text{S}$).

The data-collection, processing and refinement statistics are summarized in Table 1. The quality of the electron density was good for all Met1–Gly103 and Met1–Lys102 residues in *TtCutA1* and *PhCutA1*, respectively. No residues fell into the disallowed regions; the final models exhibited good stereochemistry when checked by *PROCHECK* (Laskowski *et al.*, 1993). Protein interactions were analyzed using *PIC* (Tina *et al.*, 2007), the cleft volume was calculated with the program *CASTp* using a 1.4 Å probe (Dundas *et al.*, 2006) and the stabilization centres were detected using *SCide* (Dosztányi *et al.*, 2003). The sequences were aligned using *ClustalW* (Larkin *et al.*, 2007) and the figures were prepared with *PyMOL* (DeLano, 2002; DeLano & Lam, 2005).

The atomic coordinates and structural factors have been deposited in the Protein Data Bank (PDB; Berman *et al.*, 2000) as entries 1nza (Na^+ –*TtCutA1*), 1v6h (apo *TtCutA1*) and 4nyo (Na^+ –*PhCutA1*).

3. Results and discussion

3.1. The overall architectures of the *TtCutA1* and *PhCutA1* proteins

The *TtCutA1* and *PhCutA1* proteins present the same overall structural topology as other known CutA1 proteins (Arnesano *et al.*, 2003; Savchenko *et al.*, 2004; Tanaka *et al.*, 2004; Bagautdinov *et al.*, 2008; Sato *et al.*, 2011). The root-mean-square deviation (r.m.s.d.) of C^α atoms between Na^+ –*PhCutA1* and apo *TtCutA1* and Na^+ –*TtCutA1* as trimers is 1.15 and 1.20 Å, respectively, which confirms a high degree of homology between them. The following analysis concerns both proteins. The ferredoxin-like fold of the protein molecule forms two domain-like parts: a main part composed of an interlocking double sandwich ($\beta_1\alpha_1\beta_2-\beta_3\alpha_2\beta_4$) with an additional strand β_5 and helix α_3 at the C-terminal end and a $\beta_2\beta_3$ lid organized by residues of the C-terminus of β_2 and the N-terminus of β_3 (Fig. 1 and Supplementary Fig. S1¹). The main part forms the compact core of the monomer, bringing residues remote in the primary sequence into close spatial proximity (Fig. 2 and Supplementary Fig. S1a). In the functional biological unit, three protomers assemble into a trimer resembling a flattened barrel. It comprises a large inter-protomer β -sheet: the curved ($\beta_2\beta_3\beta_1\beta_4$) sheet of each monomer interacts with β_2 and β_3 in another chain and β_5 in the third chain to form a seven-stranded sheet (Fig. 1 and Supplementary Fig. S1b). The hydrogen-bonding interactions within extended seven-stranded β -sheet joining

segments of all three protomers should be important for holding the CutA1 trimer together against destabilization from thermal motion and forces arising during function (Supplementary Fig. S1b and Supplementary Tables S1 and S2). Interprotomer interfaces are mainly formed by hydrogen bonds among the equivalent β_2 strands and between β_4 and β_5 . Notably, in the trimer, β_5 flanked by β_4 and α_3 of a paired protomer is an outcome of the packing-influenced interactions. The orthogonal packing of the β -sheets around the crystallographic threefold axis creates an inner hollow arrangement which forms a central cavity that is open to the outside *via* three solvent-accessible lateral channels. The α -helices lie on the outside of the β -barrel in the concavities resulting from the curvature of the sheets. Each elliptical lateral pore is formed by two polypeptides intertwined in head-to-tail mode; the $\beta_2\beta_3$ lid of each protomer covers the entrance of the cleft formed by the main part of the second protomer, while its main part forms a cleft for the $\beta_2\beta_3$ lid of the third protomer. Since the lateral clefts are considered to be active sites, the oligomerization process is essential for the function of CutA1 (Arnesano *et al.*, 2003; Bagautdinov *et al.*, 2008).

In the CutA1 trimer, each $\beta_2\beta_3$ hairpin is very strongly twisted (almost 180°) and participates in two symmetry-related seven-stranded β -sheets. The twist is important to position acidic residues on β_3 in the side clefts. For stabilization, $\beta_2\beta_3$ uses noncovalent interactions with neighbouring atoms, especially in both of the extended sheets. The edges of each β_2 interact with the equivalent β_2 of the other two symmetrically related protomers in head-to-tail and tail-to-head modes, respectively. In *PhCutA1*, $\beta_2\beta_3$ pairs are globally distorted with a uniform bow, but in *TtCutA1* an extra residue insertion into β_2 results in a bulge in the middle of the strand (Figs. 3a, 3b and Supplementary Fig. S2a). The bulge is of the antiparallel wide (AW) type and involves Pro35 and Gly36 on β_2 and Leu53 on the adjacent β_3 (Richardson *et al.*, 1978; Chan *et al.*, 1993). In spite of the bulged Pro35–Gly36 dipeptide on β_2 , the backbone hydrogen-bonding patterns within the two consecutive antiparallel $\beta_2-\beta_3$ and inter-protomer $\beta_2-\beta_2$ interactions in *TtCutA1* are similar to those in *PhCutA1* (Figs. 3b and 3c). For CutA1 proteins, with the exception of those from hyperthermophiles, insertion in the middle of β_2 is fairly common (Figs. 2 and Supplementary Fig. S3). Apparently, for nonhyperthermophilic CutA1s, which are generally more flexible than their hyperthermophilic homologues, the elasticity of the $\beta_2\beta_3$ hairpin tolerates insertions in the middle of β_2 , where pairs of hydrogen bonds are widely spaced. The bulge is accommodated on the top of the trimer structure and it clearly accentuates the twist of $\beta_2\beta_3$. The known CutA1 structures verify that the organization of the inter-protomer $\beta_2-\beta_2$ and $\beta_2-\beta_3$ hydrogen bond is canonical regardless of the bulge in β_2 that is present in a subset of them. In thermophilic and mesophilic CutA1, the conservation of the hydrogen-bond network topology relies on formation of the AW bulge at the middle of β_2 with its extra inserted residue. In CutA1 from the

¹ Supporting information has been deposited in the IUCr electronic archive (Reference: TB5064).

psychrotrophic bacterium *Shewanella* sp. SIB1 (*SsCutA1*), the accommodation of an extra residue produces looping-out of the Gly38-Gln39-Ala40 triad, leading to the split of the β_2 strand (Sato *et al.*, 2011). However, in both cases an inserted residue acts locally, swelling out β_2 at the top of the trimer (Supplementary Fig. S2*b*). The irregularity prevents elongation of β_2 and thus conserves the lateral cleft conformation. Assuming that the functional mechanism is conserved within the CutA1 family, the presence of the local irregularity at the top of the trimer should not perturb the metal-binding-site architecture. The residue propensity of the AW β -bulge in CutA1 is diverse. Pro and Gly are the predominant residues in the disrupted area of β_2 , but in some proteins that are tolerant to conformation change a Gly residue is replaced by Gln or Ser (Supplementary Table S3). Protruded β_2 bulge residues on the solvent-exposed top of the trimer may have destabilizing roles that diminish the protein stability by a relatively small amount. The fundamental difference between hyperthermophilic enzymes with their normal β_2 and non-hyperthermophilic ones with peculiarities in β_2 supports this suggestion. The CutA1 structures with the AW β -bulge present

compacted central but roomier lateral entrance clefts compared with those of the hyperthermophilic proteins (Supplementary Fig. S4 and Table S4). The difference is likely to be the outcome of a relatively high plasticity of the AW bulged $\beta_2\beta_3$ pairs that relieve twisting stress, encouraging orthogonal packing of β -sheets around the threefold axis. In psychrophilic *SsCutA1* the central hollow and lateral clefts are wide, consistent with a gradual loosening of structure in the psychrophiles involving a weakening of almost all types of interactions. Common for all known CutA1 structures is the existence of an antiparallel classic (AC) β -bulge involving the conserved residues Ala (in α -helical conformation), Cys at the start of β_2 and Lys at the end of β_3 (Fig. 3*a* and Supplementary Table S3).

The compact CutA1 trimer presents a pronounced molecular surface and charge complementarity between protomers (Supplementary Fig. S5). Imposing the importance of desolvated interactions among interlocking β -strands, the central core of CutA1 presents the lowest *B* factors (Supplementary Fig. S6). For several CutA1s, strategies for increasing thermotolerance have included the introduction of charged residues, ion pairs and electrostatic interactions (Tanaka *et al.*, 2006; Matsuura *et al.*, 2012). In *PhCutA1*, the ratio of two pairs of preferred (Glu and Lys) and avoided (Gln and His) amino acids (Farias & Bonato, 2003), [(Glu + Lys)/(Gln + His) = 27/2], is higher than in *TiCutA1* (19/4). The stabilization-centre (SC) analysis shows that the proteins modify the number and size of fluctuating domains and interactions between the domains by adopting specific amino acids (Figs. 2, Supplementary Fig. S7 and Supplementary Table S5; Dosztányi *et al.*, 1997, 2003; Bagautdinov & Yutani, 2011).

Unexpected electron density was detected in the *PhCutA1* structure close to the side chain of Cys29 that could only be explained by oxidation of the cysteine to a sulfinic form (Cys29-SO₂H; Fig. 4). Apart from the Cys29 modification, no significant structural changes occur, although the Cys29 sulfinic acid forms hydrogen bonds to the neighbouring Asn31, Tyr92 and Lys56 that are likely to affect the

Figure 3

The β -bulges in the *TiCutA1* and *PhCutA1* structures. (a) The AW and AC β -bulges in *TiCutA1*. Those amino acids that are involved in β -bulges are drawn in sticks and outlined by black dotted circles. The structure ribbons are drawn in grey. The AW β -bulge residues Pro35 and Gly36 on β_2 clearly deviate from the regular array. The AC bulges involve Ala, Cys on β_2 and Lys on β_3 and present two narrowly spaced pairs of hydrogen bonds. (b) Comparison of the hydrogen-bonding networks in $\beta_2\beta_3$ pairs. Superimposed $\beta_2\beta_3$ pairs of *TiCutA1* (backbones drawn as sticks in cyan) and *PhCutA1* (backbones drawn as sticks in brown). The AW β -bulge residues of *TiCutA1* are labelled. The main-chain hydrogen bonds are represented by green dashes for *TiCutA1* and magenta dashes for *PhCutA1*. The spatial locations of the β_2 - β_3 main-chain hydrogen bonds of both proteins are similar. The $\beta_2\beta_3$ ribbons of *PhCutA1* are represented in grey. As can be seen the bulge residues of *TiCutA1* deviate from the regular array. (c) Comparison of inter-subunit β_2 - β_3 main-chain hydrogen bonds of the CutA1 proteins. The superposed structures of the *TiCutA1* (yellow) and *PhCutA1* (cyan) and the locations of backbone-backbone hydrogen bonds are shown. Hydrogen bonds are shown as dashed lines. For each position associated with each contact, dashed lines in green and magenta represent hydrogen bonds in *TiCutA1* and *PhCutA1*, respectively, and residue labels are on white and magenta backgrounds for *TiCutA1* and *PhCutA1*, respectively. Pro35 and Gly36 on the bulged part of β_2 in *TiCutA1* are shown as sticks and labelled.

flexibility of the area. Comparison with reported *PhCutA1* structures (PDB entries 1j2v and 1uku; Tanaka *et al.*, 2004) shows that modelling a cysteine sulfinic acid at Cys29 in the present model does not significantly affect the positions of the S atom and/or neighbouring residues. Auto-oxidation of Cys29 to cysteine sulfinic acid was probably promoted by the crystallization procedure, for example, by the presence of peroxide decomposition products in the polyethylene glycol used as a precipitant for crystal growth or by the presence of the copper chloride salt.

3.2. Metal binding and metal-mediated oligomerization in Na^+ -*TtCutA1*

Trimeric apo *TtCutA1* was crystallized in the trigonal space group $P3_221$ with the protomers having very similar structures; all pairwise C^α r.m.s.d.s were less than 0.27 Å. The crystal structure of Na^+ -*TtCutA1* was crystallized in the cubic space group $I23$ and presents one protomer in the asymmetric unit, which is one third of the presumed biological homotrimer. It presents two Na^+ ions (termed Na1 and Na2) close to Glu52 and Asp50 on the twisted β_3 (Figs. 5 and Supplementary Figs. S8, S9 and S10a). The protomer does not constitute a complete metal-binding site, but a closely associated homotrimer forms clefts composed of the necessary metal-binding residues. The *TtCutA1* trimer structures with and without bound Na^+ reveal insignificant conformational changes upon metal binding, with an r.m.s.d. of less than 0.34 Å. The inner surfaces of the lateral clefts are lined with several negatively charged residues and therefore may function as channels to target metal ions and/or other cationic effectors to the interior of the protein. In the Na^+ -*TtCutA1* homotrimer, one Na^+ ion at the Na1 site is held in the deep cleft, while the other Na^+ is at the Na2 site on the homotrimer surface. The Na^+ ion at the Na1 site is bound to Glu52 on β_3 and Thr8 on β_1 and two water molecules, one of which interacts with Cys30 on β_2' of the adjacent protomer through a perfect hydrogen bond (Fig. 5c, Supplementary Figs. S8, S9 and S10a and Supplementary Table S6). Glu52 and Cys30 are conserved residues, implying their importance in the functional metal-binding complex. The conserved Lys58 is in close proximity to the Na1-binding site but does not directly interact with the ion. The

second Na^+ ion at the Na2 site occupies a position of crystallographic twofold symmetry and binds to Asp50 on strand β_3 and two waters and their symmetry mates of the nearest-neighbour homotrimers (Fig. 5d, Supplementary Fig. S9 and Supplementary Table S6). The binding mode offers full octahedral coordination for Na^+ at the junction of the symmetrically related homotrimers. Each Na2 associates two *TtCutA1* homotrimers, and as each homotrimer possesses three equivalent Na2-recognition sites (one site for each protomer), each homotrimer interacts with three neighbouring homotrimers through Na^+ -ion mediation. As a result, a common arrangement of Na^+ -*TtCutA1* involving a tetrahedral distribution of subunit homotrimers creates a new dodecamer assembly composed of 12 identical protomers arranged in a 23 cubic point-group symmetry (Figs. 5a and 5b). The packing appears to have been induced by interactions at the Na2-binding sites between twofold symmetry-related homotrimers. The dodecamer Na^+ -*TtCutA1* nanocage can be viewed as the assembly of four *TtCutA1* homotrimers. The residues Asp50 on β_3 and twofold symmetry Na2 positions play a key role in the inter-trimer interactions. The assembly presents a novel metal-binding motif, and a new mechanism for cell protection based on the sequestration of metal ions. At the same time, the inter-trimer contacts mediated by Na2 can be considered as the mechanism for metal transfer between the homotrimers.

Although the complex of *CutA1* with Na^+ obtained in crystallization conditions is not relevant from the physiological point of view, the structural information on the Na^+ -binding sites should be useful for divalent metal-binding modes. It is well known that despite the variety of bonding arrangements displayed by metal ions, many metal sites in proteins share a common feature: they are centred in a shell of hydrophilic ligands surrounded by a shell of carbon-containing groups (Yamashita *et al.*, 1990). Since both the Na1- and Na2-binding modes to the negatively charged clefts are primarily due to the charge of the ion (Supplementary Fig. S10a), replacing the Na^+ ion with the physiological divalent ions may result in a similar effect.

3.3. Metal binding in the Na^+ -*PhCutA1* trimer

In the crystal unit cell, the trimeric units of Na^+ -*PhCutA1* were coupled to form a hexameric assembly in a top-to-top face mode (Fig. 6a). The trimers are similar, with an r.m.s.d. of ~ 0.35 Å over all C^α atoms, and they interact *via* the relatively apolar top faces, with the negatively charged bottom faces exposed to the solvent (Supplementary Fig. S10b). The structure revealed metal ions coordinated by six solvent water molecules arranged in an octahedral site and placed within the bottom entry of the central cavity (Fig. 6 and Supplementary S10b). The entrance is relatively large and has a negatively charged environment (Supplementary Figs. S4b and S10b). The binding modes of hexa-aquated $[\text{Na}(\text{H}_2\text{O})_6]^+$ to the trimers are similar, with the six-coordinated tetragonal bipyramidal metal–water complexes forming crystallographically independent units. Pairs Asp84 and Asp86 from each protomer of trimers hold the metal–water complex through hydrogen bonds (Figs. 6b and 6c; Supplementary Table S7). The binding site is accessible to the central trimeric hole and through it to the three solvent-accessible lateral channels. The ions are bound to the protein indirectly, in an ‘outer-sphere’ coordination mode through water molecules. This implies that small ions might move freely in and out of the trimer without directly binding to any residue around the pore.

Several attempts to grow the physiologically relevant M^{2+} -bound form of *PhCutA1* were unsuccessful. In spite of adding CuCl_2 to the crystallization buffer of the protein, the crystal form appears in complex with Na^+ , most likely acquired from the crystallization

Figure 4
Part of the lateral cleft in Na^+ -*PhCutA1*. The residues (Ile27–Asn31), including the Cys29 sulfinic form (Cys29- SO_2H ; CSW29), are shown as sticks and labelled. The $2F_o - F_c$ electron-density map contoured at 1.0σ and coloured dark green shows unambiguous density for the sulfinic form. The highlighted residues refer to the *B* protomer, but the electron-density peculiarity at the Cys29 position is characteristic for all six protomers in an asymmetric unit.

cocktail. Indeed, the observed metal–water distances are rather too long for Cu^{2+} but are fully consistent with Na^+ (Fig. 6c, Supplementary Table S7). Also, the sodium ions present essentially similar vibrations as the donors. Notably, the detected hexaaqua-metal species possess octahedral geometry, without the tetragonal distortion specific to the $[\text{Cu}(\text{H}_2\text{O})_6]^{2+}$ complex ion owing to the Jahn–Teller effect (Ohtaki & Maeda, 1974; Pasquarello *et al.*, 2001).

3.4. Proposed mechanism for the regulation of ion uptake by CutA1 proteins

The metal–CutA1 structures reveal metal binding and metal-induced protomer association features of the protein. Metal-mediated interactions among Na^+ –*Ti*CutA1 trimers suggested that the protein associates into dodecamers owing to the location of metal ions on the protein surface. Therefore, it may represent a new structural model for metal-responsive assembly of proteins. The structure of dodecameric Na^+ –*Ti*CutA1 crystallized in the cubic space group *I*23 offers new insights into the ion uptake by the protein. The assembly results in extra ion-binding sites at the trimer–trimer contact areas and reveals a plausible route to metal regulation by a simple and effective feedback mechanism. It is known that intracellular divalent copper in the free form may produce damaging

Figure 5

Sodium-ion binding in *Ti*CutA1. (a) The dodecamer assembly and sodium-binding sites of Na^+ –*Ti*CutA1. Ribbon representation of the dodecamer composed of four identical trimers coloured green, magenta, gold and cyan. Binding Na^+ ions are shown as red balls. (b) The outer molecular surface of a complete dodecamer. Surfaces of different trimers are depicted in a different colour. The bound ions inside the putative active site (Na1 position) and on the border of trimer–trimer surfaces (Na2 position) are depicted as purple spheres. (c) Coordination of the Na^+ ion at the Na1 site. Close-up view of the side cleft of the trimer, with Na^+ shown as a brown sphere. The chelated residues are shown as sticks and labelled and waters forming hydrogen bonds to Na^+ are shown as small blue balls. The bonds are represented by red dashes and distances are given in Å. The symmetry-related protomers are coloured differently. (d) Coordination of the Na^+ ion at the Na2 site. Close-up view of the trimer–trimer interface with Na^+ shown as a purple sphere. Symmetrically related Asp50 residues and waters binding to Na^+ are shown as sticks and small spheres, respectively, and are labelled. The symmetry-related protomers and waters are coloured differently in cyan and yellow. The monodentate carboxylate binds to Na^+ and waters stabilize the metal-free carboxylate group of Asp50. The bonds are represented by purple dashes and distances are given in Å.

radicals. Therefore, the metal status of the cell may modulate quaternary transformations of CutA1: at normal ion levels, binding at lateral clefts would prevent uptake on the trimer surface and thus prevent dodecamerization. When the cell accumulates excess metal ions CutA1 can help to store the excess ions by dodecamer association. At the same time, the CutA1 assembly may act as a 'buffer' for the cell against ion deficiency, since at low ion concentrations the dodecamer may dissociate to release ions. Generally, dodecamer formation and division can be considered to be a dynamic process.

The rigidity of the CutA1 trimers supports conservation of the boundary amino-acid interactions for communication between modules. At the same time, the bound ions owing to coordinated water molecules are mobile. The Na^+ -*Ti*CutA1 dodecameric state constitutes a new structural model for metal-responsive protein assembly. Previously, it was found that the inter-trimer interface binding of Cu^{2+} in *Ph*CutA1 induces metal-related in-plane multimerization of the protein (Tanaka *et al.*, 2004). In both cases the identical Asp on β_3 (Asp50 in *Ti*CutA1 and Asp48 in *Ph*CutA1) is a key residue in mediating oligomerization. The protein multimerization should be important for the ability of CutA1 to store and release ions in a controlled fashion. Further experiments are required to test this model.

As metal binding and storage prevent unwanted chemical reactions, formation of metal-CutA1 complexes in the cell contributes to metal homeostasis. However, the key to metal homeostasis is transport: the equilibrium between metal uptake and efflux. Apparently, CutA1 uses the rather flexible $\beta_2\beta_3$ lid covering the entrance of the lateral cleft to bind and release ions. The trimer-trimer bridging ion in the dodecamer can be viewed as participating in ion passing between the trimers. It will be beneficial in maintaining the correct concentrations of ions in the different cellular compartments or transport to other *cutA* gene products.

The metal ions tend to bind to CutA1 indirectly *via* water molecules, *i.e.* in an 'outer sphere' coordination mode. In *Ph*CutA1 the Na^+ ions bound at the presumably non-active site utilize six water molecules and in *Ti*CutA1 the Na^+ sites contain two water molecules at lateral clefts or four water molecules at trimer-trimer interface sites. Conformational rigidity of the protein allows small metal ions to anchor to the binding pocket where the metal-water exchange takes place. Water in these sites may be exchanging rapidly between the cleft and the bulk solvent. The presence of waters in the coordination chemistry used by CutA1 for metal binding permits further ion manipulation such as binding/release dynamics associated with

Figure 6

Sodium-ion-binding sites in *Ph*CutA1. (a) Hexameric structure of Na^+ -*Ph*CutA1 (cyan). In the crystalline unit cell, the trimeric units couple to form a hexameric assembly. Locations of the metal ions and waters are marked as spheres. Water molecules interacting with the ions are shown as small red spheres. (b) Close-up view of Na^+ binding with six water ligands in an octahedral coordination. The metal ion is shown as a red ball and metal-bound waters (labelled with the letter W) are shown as small blue balls and are arranged in an octahedron connected by thin lines. Asp84 and Asp86 hydrogen bonding to metal-bound waters are shown as sticks and the bonds are shown as broken red lines with distances in Å. The symmetry-related subunits are coloured differently. (c) Schematic representation of the hexa-aquated sodium ion, $[\text{Na}(\text{H}_2\text{O})_6]^+$. The ion is shown in red and metal-coordinated waters are shown as blue balls arranged in an octahedron connected by thin lines. The waters are arranged at the vertices of an octahedron centred on the metal ion. The red dotted lines indicate the ranges in Å of the metal-bound waters and Na^+ .

intracellular metal regulation. It is likely that the metal ions release their water shell to activate and/or pass through ion pumps in membranes.

The association of Na⁺–TiCutA1 is intriguing since the quaternary-structural arrangement of a protein is frequently responsible for regulating complex cellular functions. In fact, ion binding to the $\beta_2\beta_3$ lid on the trimer surface allows interactions outside the trimer and promotes inter-trimer interactions. The inter-protomer linkages assembling the CutA1 trimer and dodecamer structures form long-range contacts that may mediate signalling and information transfer.

4. Conclusion

The crystal structures of TiCutA1, Na⁺–TiCutA1 and Na⁺–PhCutA1 have been solved and analyzed to clarify the metal-binding properties of CutA1. They present a compact trimer structural motif with tightly intertwined interactions amongst the β -strands. TiCutA1 presents an AW-type β -bulge on the top of the trimer. A possible role of the AW bulge is to incorporate the extra residue in β_2 while preserving the interprotomer main-chain hydrogen-bonding pattern that is vital to the functional trimer folding of CutA1. The superior stability of PhCutA1 compared with TiCutA1 has been supported by allocation of densely interacting clusters over the entire structure that are linked to synchronized fluctuating microstates.

The CutA1 structures present several ion-binding sites with little effect on the local structural conformation. The Na⁺–TiCutA1 structure reveals Na⁺ inside of the lateral clefts of the trimers and at trimer–trimer interfaces. It is likely that ion binding to the surface of the trimer affects the dodecameric assembly of the protein. In Na⁺–PhCutA1, the hexa-aquated ion represents an effective response mechanism for regulating ions as the metal–water cluster in the pore and thus prevents direct contact of essential cellular components with the metal. The isolation of ions by binding inside of the lateral clefts, trimer–trimer interfaces and full hydration by the hexa-aquated arrangement may allow CutA1 to control the damaging effects of metal ions by sequestering them away from other cellular components. Owing to its compact fold, CutA1 has functionally important areas distributed over a large portion of its structure. The presentation of different positions for ion binding may be specific to CutA1 crystals. It appears that CutA1 undergoes dynamic trimeric, dodecameric or other associations depending on the metal status of the cellular compartment in which it is contained and a variety of other factors. Modifications of assembly states may allow CutA1 to operate in different parts of the cell in various environments for a range of challenges.

The author thanks the staff of RIKEN Genomic Sciences Center for providing the plasmid, Dr C. Kuroishi for protein production and the staff of the BL44B2 beamline at SPring-8 for facilities and help. This work was supported by the ‘National Project of Protein Structural and Functional Analysis’ funded by the MEXT of Japan. I thank Professor D. Beckett (University of Maryland) for critical reading of the manuscript.

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