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ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · APRIL 2003

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Inert Site in a Protein Zinc Cluster: Isotope Exchange by High Resolution Mass Spectrometry

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It is well known that proteins control the local environment of bound metal ions,[1] and hence their thermodynamic and kinetic properties, for example, redox potentials[2,3] and transfer rates.[4] Metallothioneins (MTs) appear to play an important role in Zn homeostasis and the zinc buffer/distribution system.[5] Mammalian MTs contain Zn_3Cys_9 and Zn_4Cys_{11} clusters,[6] and metal exchange reactions for MTs are usually fast.[7] Bacterial MTs possess only a single zinc cluster,[8] $Zn_4Cys_9His_2$ in the case of the cyanobacterial MT SmtA (Figure 1).[9,10] We have investigated Zn exchange reactions of Zn_4-SmtA by a new method using stable isotope labeling combined with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). We show that the Zn_4 cluster of SmtA, in contrast to the structurally analogous cluster of mammalian MT, contains a kinetically inert Zn site, a feature which can be related to its secondary and tertiary structure, and which is of potential importance to its biological function.

Gentle ionization by electrospray (ESI) has previously been exploited for MTs,[11] and in combination with FT-ICR-MS[12] it is a powerful tool for the analysis of metalloproteins.[13] Deconvoluted ESI-FT-ICR spectra of Zn_4-SmtA containing Zn isotopes in natural abundance[14] and with 93% enrichment[15] with ^{67}Zn are compared in Figure 2. The observed experimental masses of the most intense isotopic peaks in Figure 2A and B (5862.95 and 5868.81 Da, respectively) are in good agreement with calculated values (5863.00 and 5869.00 Da: deviations of 8.5 and 32 ppm, respectively).

The effects of isotope enrichment are pronounced. Exchange of all four Zn atoms causes an increase in mass of the most abundant peak by 6 Da, and the isotopic envelope becomes much narrower (Figure 2B).

To investigate Zn exchange behavior, we incubated natural abundance Zn_4-SmtA with $^{67}ZnCl_2$ for various time intervals at 310 K, removed unbound Zn^{2+} by rapid gel filtration (ca. 3 min), and analyzed the product by FT-ICR-MS.[16] The amount of exchanged Zn at each time point was determined by comparing the experimental data to modeled isotope envelopes for $Zn_{x-4}{}^{67}Zn_x$SmtA ($x = 1-4$, in 0.25 Zn intervals), taking into account the isotopic compositions of both natural abundance Zn and the ^{67}Zn-enriched $^{67}ZnCl_2$ used[14,15] (see Figure S2).

Crucially, the FT-ICR-MS measurements allow direct determination of the metal:protein ratio, without the need for separate measurements of metal and protein concentrations, as is necessary in radioisotope studies, while simultaneously confirming the identity of the intact metal−protein complex.

We find that initial Zn exchange is fast (ca. 1.4 Zn exchanged after 1 h, see Figure S3) as expected for metallothioneins,[7a] but most interesting is the extent of ^{67}Zn incorporation at equilibrium. If exchange occurred at all four Zn sites, the maximum achievable incorporation of ^{67}Zn with a 10-fold excess of 93% enriched ^{67}Zn would be 3.6 ^{67}Zn per mol SmtA. We observed a maximum incorporation of 2.75 ^{67}Zn (Figure 2C) after 99 h of incubation, close to the value of 2.7 ^{67}Zn calculated for exchange at only three sites. This implies that one of the four sites in the Zn_4 cluster (Figure 1B) is inert to exchange, a result consistent with our previous ^{111}Cd

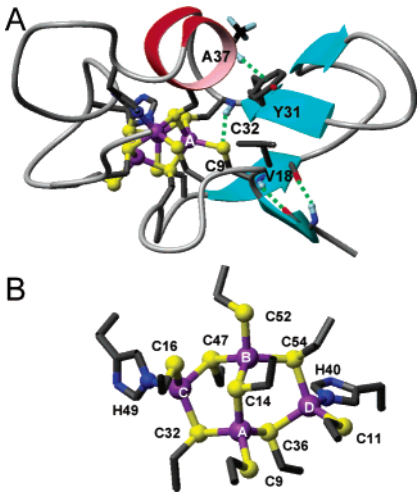

Figure 1. (A) 3D structure of Zn_4-SmtA (PDB 1JJD) showing elements of secondary structure around site A. The amide proton of Cys32 forms an H-bond to the sulfur of Cys9, which accounts for the extraordinary low-field shift of its ^{1}H resonance (10.01 ppm). The tertiary arrangement of the helix and sheet is further stabilized by the CH$-\pi$ interaction between Ala37 and Tyr31. (B) The $Zn_4Cys_9His_2$ cluster of Zn_4-SmtA.

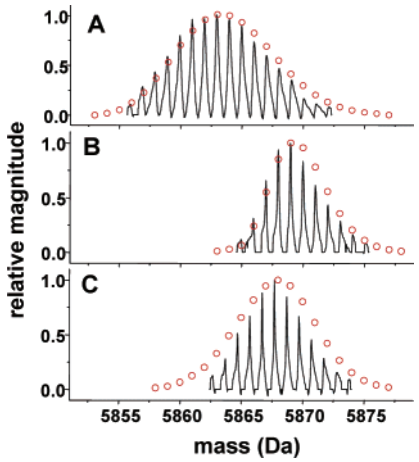

Figure 2. Deconvoluted ESI-FT-ICR mass spectra and modeled mass envelopes (red circles) of (A) natural abundance Zn_4-SmtA (first model circle is 1 Da below monoisotopic peak), (B) 93%-enriched $^{67}Zn_4$-SmtA, and (C) Zn_4-SmtA reacted with a 10-fold molar excess (with respect to Zn) of $^{67}ZnCl_2$ for 99 h at 310 K, and model for exchange of 2.75 Zn.

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10.1021/ja0284409 CCC: $25.00

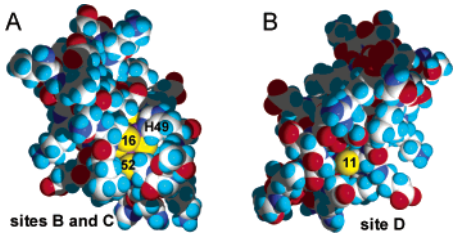

Figure 3. Space-filling models showing accessibility of the terminal Cys and His ligands in sites B, C, and D (Cys S yellow, N blue, O red, C white, H cyan). Site A is completely buried.

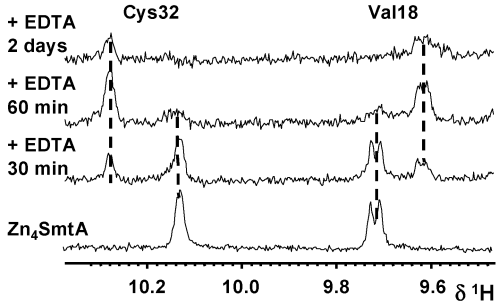

Figure 4. Low-field region (NH peaks) of the ^{1}H NMR spectrum of Zn_4-SmtA, and 30 and 60 min after reaction with ca. 6 mol equiv of EDTA, and 2 days after reaction with a further 10 mol equiv of EDTA. Zn removal was indicated by the decrease in intensity of the ethylene singlet of free EDTA ($\delta = 3.256$) and appearance of the analogous singlet for $[Zn(EDTA)]^{2-}$ ($\delta = 2.873$; Figure S5).

NMR observations[9] of reactions of $^{111}Cd^{2+}$ with Zn_4-SmtA, in which Zn(A) is inert. The formation of Cd_3Zn-SmtA is substantiated by ICP-AES and ESI-MS data (Figure S4). Normally, all-cysteine sites are thermodynamically stronger binding sites for Cd^{2+} as compared to Zn^{2+} (e.g., $10^2\times$ stronger for Cys_4 zinc fingers,[17] and $10^4\times$ stronger for mammalian MTs[18]), and Zn^{2+} replacement by Cd^{2+} in MTs is fast and stoichiometric.[19]

Zn exchange is likely to involve attack of $^{67}Zn^{2+}$ on an accessible ligand atom: S of Cys or N of His. It can be seen in Figure 3A and B that the terminal sulfurs in sites B, C, and D are accessible from the protein surface, but this is not the case for site A, for which all of the ligands (Cys 9, 14, 32, and 36) are buried.

Site A is surrounded by elements of secondary structure, an α-helix and two short antiparallel β-sheets, structural features which are not found in mammalian MTs, and these give rise to an H-bond between the S of Cys9, a ligand in site A, and the backbone NH of Cys32, a ligand in sites A and C (Figure 1A). Such an arrangement probably prevents intramolecular metal exchange into site A. The secondary structure around Zn(A) appears to be maintained during removal of Zn^{2+} from Zn_4SmtA by EDTA,[20] as indicated by the behavior of the low-field shifted NH resonances of Cys32 and Val18 (Figure 4; see also Figure 1A). It seems likely therefore that Zn-(B,C,D) are removed by EDTA more rapidly than Zn(A).

These findings show that the Zn_4 cluster in bacterial metallothionein confers novel properties on the protein. Site A and the surrounding secondary structure constitute a zinc finger fold of the kind found in GATA[21] and LIM[22] proteins which recognize other zinc finger proteins and DNA. Metal exchange and transfer reactions of proteins[4,23] are currently presenting important and challenging questions, and it is clear that FT-ICR-MS can make a major contribution to studies of both their thermodynamics and exchange dynamics.

Acknowledgment. We thank the European Community (Marie Curie Fellowship for C.A.B. under the IHP program), SHEFC, Scottish Enterprise, Bruker Daltonics, the Wellcome Trust (Edinburgh Protein Interaction Centre), BBSRC (for N.J.R. and M.D.H), EPSRC, the Royal Society, and Wolfson Foundation for support.

Supporting Information Available: Experimental details, Figures S1−S5: FT-ICR-MS raw data, examples of fitted MS data, time-dependent zinc exchange, ESI mass spectra of Zn_4-SmtA before and after reaction with $CdCl_2$, ^{1}H NMR spectra of EDTA reaction (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Natural abundances of Zn isotopes: ^{64}Zn 48.6%, ^{66}Zn 27.9%, ^{67}Zn 4.1%, ^{68}Zn 18.8%, ^{70}Zn 0.6%.
(15) $^{67}Zn_4$-SmtA was prepared from apo-SmtA and $^{67}ZnCl_2$ (93.11% ^{67}Zn, remainder ^{64}Zn 1.37%, ^{66}Zn 2.58%, ^{68}Zn 2.89%, ^{70}Zn 0.05% supplied by Oak Ridge National Laboratory, TN, as ZnO and dissolved in HCl).
(16) Zn_4-SmtA (200 μM) in 10 mM ammonium acetate pH 7.4 was incubated with a 10-fold molar excess (with respect to Zn) of ^{67}Zn (8 mM) at 310 K. Unbound Zn was removed from 20 or 40 μL aliquots at various time intervals on a Pharmacia PD10 column using 10 mM NH_4Ac as eluant. The eluate (3.5 mL) was concentrated to ca. 23 μM in protein using an Amicon YM3 filter before FT-ICR-MS analysis. The final sample solutions (10 μM) also contained ubiquitin (5 μM), 30% MeOH, and 0.05% formic acid.
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JA0284409