Metal Switches

DOI: 10.1002/ange.200902888

# Cobalt(II)-Responsive DNA Binding of a GCN4-bZIP Protein Containing Cysteine Residues Functionalized with Iminodiacetic Acid**

*Yusuke Azuma, Miki Imanishi, Tomoyuki Yoshimura, Takeo Kawabata, and Shiroh Futaki**

The design of proteins with functions that can be controlled by external stimuli is a challenge in peptide/protein engineering.[1] Many natural proteins utilize metal ions to stabilize their structure and regulate their bioactivity. The structural engineering of proteins with effective metal coordination to enable switching between two different structures would thus increase the feasibility of developing novel protein machineries.[2,3] Our previous studies showed that metal coordination of iminodiacetic acid (Ida) moieties yielded a significant decrease in helicity when the Ida moieties were placed at positions $i$ and $i+2$ in helical peptides.[3] This approach is particularly useful for the functional regulation of proteins with stable helical structures, as exemplified through recognition switching between leucine zipper peptides derived from Jun and Fos oncoproteins and through extramembrane gating control of artificial ion channels.[3] However, due to the lack of methodology for the effective introduction of Ida moieties at specific positions in proteins, the application of this concept has been limited to synthetic peptides.[4]

We now present a new method for introducing Ida moieties into proteins. The method involves the specific modification of cysteine residues by treatment with a new functionalization agent, *N*-(2-tosylthioethyl)iminodiacetic acid (Ts-S-IDA, **5**). We demonstrated the practicability of this approach by modifying a protein derived from the yeast transcription factor GCN4 with Ida moieties. Successful switching of the DNA binding in response to a metal was observed for one the resulting proteins.

The GCN4-bZIP protein (bZIP = basic leucine zipper) consists of two helical segments. Dimerization through the leucine zipper segment is critical for binding to the target DNA segment (AP-1 site) by the basic segment.[5] The well-studied structure of the protein has been employed as a model for the design of reversible control of DNA binding through switching of the dimer formation.[6] In the present study, bZIP proteins containing a pair of Ida residues at positions $i$ and $i+2$ in the leucine zipper segment (bZIP-1a, bZIP-1b) were designed to destabilize the helical structure of the bZIP protein by the interaction of Ida with metals to switch DNA binding (Figure 1). The positions of the Ida modification were selected as shown in Figure 1c so as to have a minimum effect on dimer formation in the absence of metals.[7]

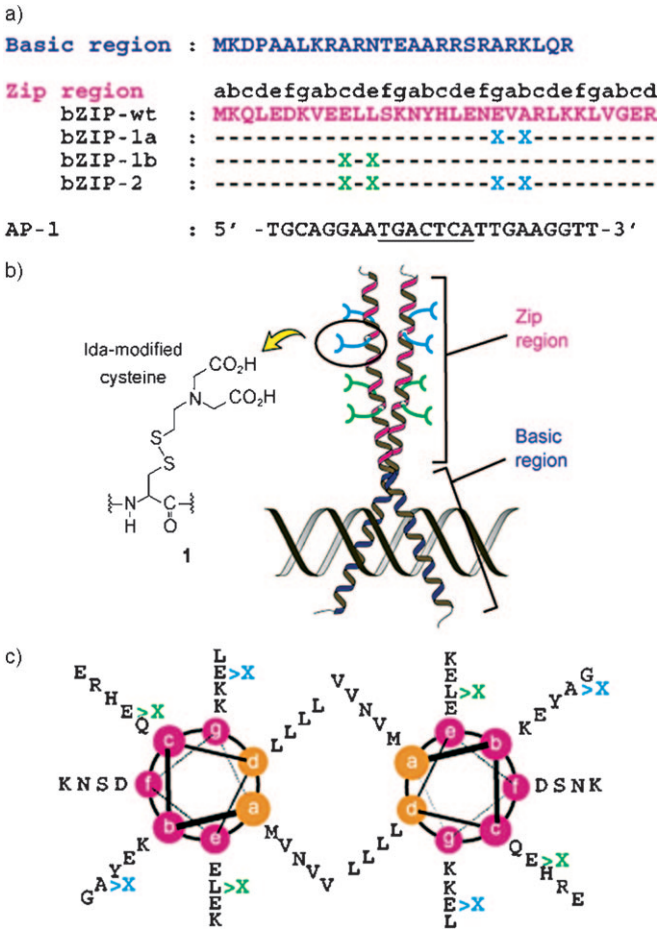


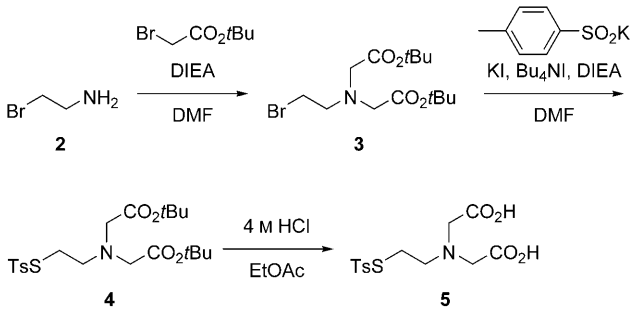

***Figure 1.*** Design of metal-responsive bZIP proteins. a) Sequences of the bZIP proteins studied and the target DNA (AP-1). b) Schematic representation of the bZIP-2/AP-1 complex. c) Helical-wheel projection of bZIP-2. X is an Ida-modified cysteine residue; wt = wild type.

The agent for modification with Ida, Ts-S-IDA (**5**), was synthesized readily from 2-bromoethylamine (**2**) in three steps (Scheme 1). The treatment of the recombinant proteins with Ts-S-IDA (1.5 equiv for each cysteine residue) in 10 mM

[*] Y. Azuma, Dr. M. Imanishi, Dr. T. Yoshimura, Prof. Dr. T. Kawabata, Prof. Dr. S. Futaki
Institute for Chemical Research, Kyoto University
Uji, Kyoto 611-0011 (Japan)
Fax: (+81) 774-32-3038
E-mail: futaki@scl.kyoto-u.ac.jp

[**] This research was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Y.A. is grateful for a JSPS Research Fellowship for Young Scientists.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200902888.




***Scheme 1.*** Preparation of Ts-S-IDA (**5**), an agent for the modification of proteins with Ida. DIEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, Ts = *p*-toluenesulfonyl.

Tris-HCl (pH 7.5; Tris = tris(hydroxymethyl)aminomethane) at 4 °C for 1 h yielded the desired bZIP proteins without difficulty.[8] Monitored by HPLC, the reaction reached completion almost immediately upon the addition of Ts-S-IDA (**5**); no side reactions were detected.

Unexpectedly, CD spectroscopy showed that the addition of metals in the presence of DNA had only a slight effect on the structure of bZIP-1a and bZIP-1b (Figure 2a; see also Figure S1 in the Supporting Information). Similarly, no significant decrease in the affinity of these proteins for DNA was observed in an electrophoretic mobility shift assay (EMSA), even upon the addition of excess amounts of the metals (Table 1; see also Figure S2 in the Supporting Information).[9] Therefore, we designed an alternative protein, bZIP-2, with two pairs of Ida moieties in the leucine zipper segment to cause greater destabilization upon the addition of a metal (Figure 1).[8]

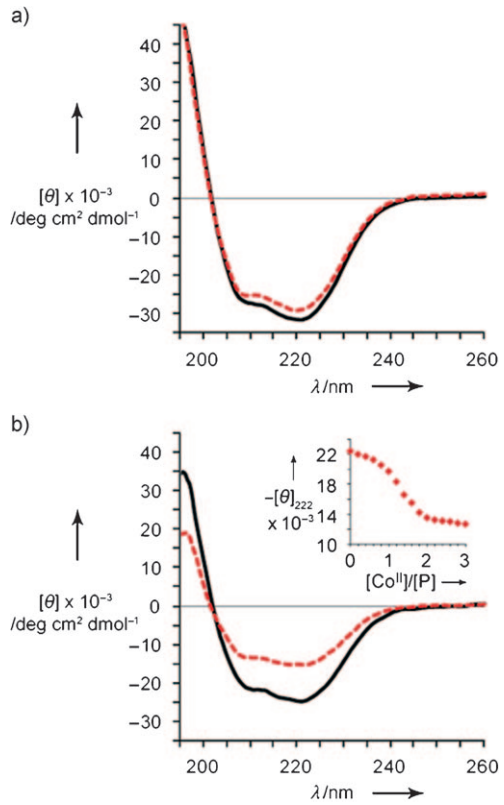



***Figure 2.*** Difference CD spectra of a) bZIP-1a and b) bZIP-2 (10 μM) in the absence (black) or presence (red) of $Co^{II}$ (30 μM). The spectra shown were obtained by subtracting the spectra of AP-1 (5 μM). The inset shows the molar ellipticity at 222 nm, $[\theta]_{222}$, of bZIP-2 as a function of the $Co^{II}$ concentration. [P] is the protein concentration.

***Table 1:*** $K_d$ values for the binding of bZIP proteins to AP-1 in the absence or presence of a metal.

| | $K_d$[a] [nM] | | | |
|---|---|---|---|---|
| | no metal | $Co^{II}$ | $Ni^{II}$ | $Mn^{II}$ |
| bZIP-wt | 15 ± 2.6 | 16 ± 1.2 | 17 ± 2.0 | 16 ± 2.5 |
| bZIP-1a | 24 ± 2.7 | 58 ± 15 | 44 ± 4.7 | 38 ± 7.3 |
| bZIP-1b | 22 ± 3.7 | 26 ± 8.1 | 26 ± 3.9 | 27 ± 4.6 |
| bZIP-2 | 22 ± 3.0 | > 1000[b] | 50 ± 15 | 64 ± 10 |
| bZIP-2 + EDTA | 21 ± 1.0 | 22 ± 1.1 | 23 ± 3.1 | 21 ± 2.6 |

[a] The $K_d$ value was determined by an electrophoretic mobility shift assay, as described in the Supporting Information. All values reported are the mean of at least three measurements (± the standard deviation). [b] Protein concentration at which the bound fraction of the DNA is about half the maximum amount ($\theta = 0.5$).

The CD spectrum of bZIP-2 suggested that this protein has a helical structure, although its helical content was somewhat lower than that of the wild-type bZIP protein in the presence of DNA (the $[\theta]_{222}$ values for bZIP-2 and bZIP-wt were $-2.5 \times 10^{-4}$ and $-3.1 \times 10^{-4}$ deg cm$^2$ dmol$^{-1}$, respectively), possibly as a result of electrostatic repulsion between the various Ida moieties (Figure 2b; see also Figure S1d in the Supporting Information). Analysis by EMSA showed that bZIP-2 had a similar affinity for the AP-1 site of DNA to that of the wild type in the absence of metals (the $K_d$ values for bZIP-2 and bZIP-wt were 22 ± 3.0 and 15 ± 2.6 nM, respectively; Figure 3; see also Figure S2a in the Supporting Information).

A significant decrease in the helical content and affinity for DNA of bZIP-2 was observed upon the addition of $Co^{II}$ (Figures 2b and 3). The addition of $Co^{II}$ (30 μM; 1.5 equiv for each Ida pair) induced a 33% decrease in the helical content. Metal titration of the protein showed a saturation in the decrease of the helical content at 2 equivalents of $Co^{II}$ to the protein (Figure 2b, inset). These results suggested that metal coordination to each Ida moiety effectively leads to the structural alteration of bZIP-2. Furthermore, a dramatic decrease in the affinity of bZIP-2 for the target DNA was observed in the presence of 30 μM $Co^{II}$ ($K_d > 1000$ nM; Figure 3, Table 1): the $K_d$ value of the protein was more than 45 times higher than in the absence of $Co^{II}$ ($K_d = 22 \pm 3.0$ nM). The addition of ethylenediaminetetraacetic acid (EDTA; 300 μM) resulted in complete recovery of the DNA-binding affinity ($K_d = 22 \pm 1.1$ nM).[10,11] Thus, the incorporation of two pairs of Ida moieties in the protein structure led to a bZIP protein that interacts reversibly with the target DNA as a result of $Co^{II}$ binding.

To assess the sensitivity of bZIP-2 to $Co^{II}$ during DNA binding, we carried out a metal-titration experiment with

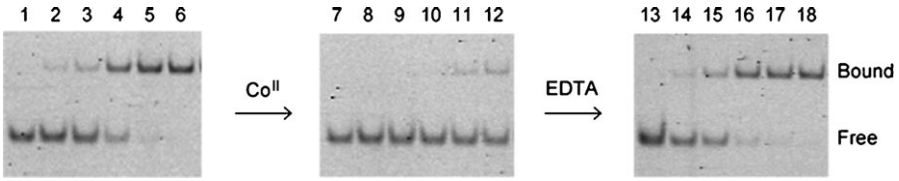



***Figure 3.*** EMSA for bZIP-2 binding to AP-1 in the absence (lanes 1–6) or presence (lanes 7–12) of $Co^{II}$ (30 μM) and after the addition of excess EDTA (300 μM). From left to right in lanes 1–6 as well as in lanes 7–12 and lanes 13–18, the protein concentration was 0, 3.3, 10, 30, 90, and 270 nM.

bZIP-2 (100 nM; Figure 4). Analysis by EMSA showed an almost-quantitative $Co^{II}$-dependent release of bZIP-2 from AP-1 and thus suggested that bZIP-2 was very sensitive to $Co^{II}$.

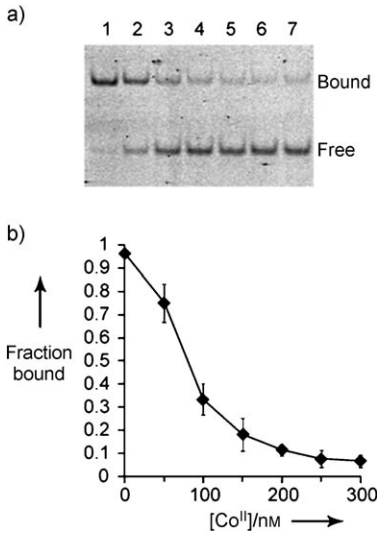



***Figure 4.*** a) EMSA for the binding of bZIP-2 (100 nM) to AP-1 (2 nM) in the absence (lane 1) or presence (lanes 2–7) of $Co^{II}$. In lanes 1–7, the $Co^{II}$ concentration was 0, 50, 100, 150, 200, 250, and 300 nM, respectively. b) Plot of the fraction of fluorescein-end-labeled DNA bound to bZIP-2 as a function of the $Co^{II}$ concentration. The data points represent the average of three experiments (± the standard deviation).

In this study, we established a new method for introducing Ida moieties into proteins by the development of an efficient cysteine-modification agent, Ts-S-IDA (**5**; Scheme 1). We also demonstrated effective and reversible control of the DNA binding of GCN4-bZIP with $Co^{II}$. The difference in the affinity of bZIP-2 for AP-1 in the absence and presence of $Co^{II}$ is significantly greater than that observed with other methods for controlling the DNA binding of GCN4-bZIP.[6] Furthermore, effective chelation of $Co^{II}$ in the submicromolar range with Ida enables structural switching of bZIP-2 to enable bZIP-2 release from AP-1 (Figure 4). The required concentration of $Co^{II}$ is significantly lower than the required metal concentrations for the functional switching of previously reported metal-responsive coiled-coil proteins.[12] The concept of helix destabilization by the $i$ and $i+2$ positioning of Ida would be applicable to the functional switching of other proteins with a stable helical structure. The results obtained in this study should provide an important platform for the design of artificial sensor and regulator proteins.

Received: May 29, 2009
Published online: August 4, 2009

**Keywords:** biosensors · DNA binding · helix destabilization · metal switches · proteins

[1] a) F. Zhang, A. Zarrine-Afsar, M. S. Al-Abdul-Wahid, R. S. Prosser, A. R. Davidson, G. A. Woolley, *J. Am. Chem. Soc.* **2009**, *131*, 2283–2289; b) S. Kneissl, E. J. Loveridge, C. Williams, M. P. Crump, R. K. Allemann, *ChemBioChem* **2008**, *9*, 3046–3054; c) R. S. Signarvic, W. F. DeGrado, *J. Am. Chem. Soc.* **2009**, *131*, 3377–3384; d) J. M. Matthews, F. E. Loughlin, J. P. Mackay, *Curr. Opin. Struct. Biol.* **2008**, *18*, 484–490, and references therein.

[2] M. Albrecht, P. Stortz, *Chem. Soc. Rev.* **2005**, *34*, 496–506.

[3] a) S. Futaki, T. Kiwada, Y. Sugiura, *J. Am. Chem. Soc.* **2004**, *126*, 15762–15769; b) T. Kiwada, K. Sonomura, Y. Sugiura, K. Asami, S. Futaki, *J. Am. Chem. Soc.* **2006**, *128*, 6010–6011.

[4] Iminodiacetic acid (Ida) moieties can be introduced into peptides by the selective removal of ε-amino protecting groups of specific lysine residues on a solid support prior to conversion into Ida.[3] Alternatively, Ida derivatives of lysine may be introduced as building blocks through solid-phase peptide synthesis: a) F. Q. Ruan, Y. Q. Chen, P. B. Hopkins, *J. Am. Chem. Soc.* **1990**, *112*, 9403–9404; b) I. Hamachi, Y. Yamada, T. Matsugi, S. Shinkai, *Chem. Eur. J.* **1999**, *5*, 1503–1511.

[5] a) M. A. Weiss, T. Ellenberger, C. R. Wobbe, J. P. Lee, S. C. Harrison, K. Struhl, *Nature* **1990**, *347*, 575–578; b) T. E. Ellenberger, C. J. Brandl, K. Struhl, S. C. Harrison, *Cell* **1992**, *71*, 1223–1237; c) R. V. Talanian, C. J. McKnight, P. S. Kim, *Science* **1990**, *249*, 769–771; d) B. Cuenoud, A. Schepartz, *Science* **1993**, *259*, 510–513; e) Y. Aizawa, Y. Sugiura, M. Ueno, Y. Mori, K. Imoto, K. Makino, T. Morii, *Biochemistry* **1999**, *38*, 4008–4017, and references therein.

[6] a) G. A. Woolley, A. S. I. Jaikaran, M. Berezovski, J. P. Calarco, S. N. Krylov, O. S. Smart, J. R. Kumita, *Biochemistry* **2006**, *45*, 6075–6084; b) A. M. Caamaño, M. E. Vázquez, J. Martínez-Costas, L. Castedo, J. L. Mascareñas, *Angew. Chem.* **2000**, *112*, 3234–3237; *Angew. Chem. Int. Ed.* **2000**, *39*, 3104–3107.

[7] The inclusion of Ida in sequences at positions a and d was avoided so as not to hamper the hydrophobic interaction between the helices. A combination of the e and g positions (as $i$ and $i+2$) was avoided, as it would result in the positioning of negatively charged Ida moieties in close proximity, at position e in one helix and position g in the other, and would thus destabilize dimer formation: a) J. M. Mason, K. M. Arndt, *ChemBioChem* **2004**, *5*, 170–176; b) W. D. Kohn, C. M. Kay, D. Brian, B. D. Sykes, R. S. Hodges, *J. Am. Chem. Soc.* **1998**, *120*, 1124–1132.

[8] The Ida-modified structures of the proteins were confirmed by ESI-MS. bZIP-1a: $m/z$ calcd for $[M+H]^+$: 7221.43; found: 7221.77; bZIP-1b: $m/z$ calcd for $[M+H]^+$: 7263.50; found: 7263.03; bZIP-2: $m/z$ calcd for $[M+H]^+$: 7609.93; found: 7610.03.

[9] No effect of metal addition was observed for wild-type bZIP (see Figures S1 and S2 in the Supporting Information).

[10] The simple addition of EDTA led to the recovery of DNA binding but not to the complete refolding of bZIP-2. Complete refolding only occurred when the protein was heated to 80 °C

and cooled slowly in the presence of EDTA (300 μM; see Figure S3 in the Supporting Information). This result suggests that bZIP-2 may form a metastable structure in the complex with $Co^{II}$.

[11] The effects of $Ni^{II}$ and $Mn^{II}$ on the helix destabilization and DNA binding of bZIP-2 were less pronounced than the effect of $Co^{II}$ (see Figure S4 in the Supporting Information).

[12] a) K. Suzuki, H. Hiroaki, D. Kohda, H. Nakamura, T. Tanaka, *J. Am. Chem. Soc.* **1998**, *120*, 13008–13015; b) T. Mizuno, K. Murao, Y. Tanabe, M. Oda, T. Tanaka, *J. Am. Chem. Soc.* **2007**, *129*, 11378–11383.