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First observation of metal ion-induced structural fluctuations of α-helical peptides by using diffracted X-ray tracking

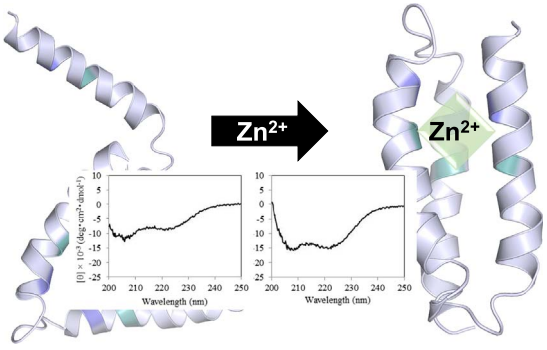
Daiki Usui[a], Satomi Inaba[a,b], Hiroshi Sekiguchi[b], Yuji C. Sasaki[c], Toshiki Tanaka[d], Masayuki Oda[a,*]

[a] Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, 1-5 Hangi-cho, Shimogamo, Sakyo-ku, Kyoto, Kyoto 606-8522, Japan
[b] Research & Utilization Division, Japan Synchrotron Radiation Research Institute, 1-1-1 Kouto, Sayo, Hyogo 679–5198, Japan
[c] Graduate School of Frontier Sciences, The University of Tokyo, Kashiwanoha, Kashiwa, Chiba 277–8561, Japan
[d] Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-chou, Showa-ku, Nagoya, Aichi 466-8555, Japan

HIGHLIGHTS

- We generated peptides whose structures are changed to helix-bundle by metal binding.
- The conformational change depends on the residues in the hydrophobic core.
- The difference in fluctuation was observed by diffracted X-ray tracking methods.

GRAPHICAL ABSTRACT



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ABSTRACT

In order to analyze protein structural dynamics, we designed simple model peptides whose structures changed from random-coil to helix-bundle structures by forming stable hydrophobic core in the presence of metal ions. The strategy involved destabilizing a *de novo* designed three helix-bundle protein by substituting the residues present in its hydrophobic core with histidine and small amino acids. The conformational changes of peptides induced upon binding of Zn^{2+} to histidine were analyzed using circular dichroism spectroscopy, which revealed peptides, HA and HG, to be good candidates for further analyses. The diffracted X-ray tracking experiments showed that the structural fluctuations of both HA and HG were suppressed upon binding of Zn^{2+}. We succeeded in observing the differences in fluctuations of HA and HG in solution between random-coil like and helix-bundle structures. The metal-binding energies determined using the angular diffusion coefficients were in good agreement with those determined using isothermal titration calorimetry.

1. Introduction

To reveal structure - activity relationships of proteins, the elucidation of protein structural dynamics is one of most important subjects. The structure of protein fluctuates in solution and proteins function as a result of conformational changes induced upon ligand binding [1–4]. Several methods have been developed to analyze protein structural dynamics. NMR is one of the experimental methods that has been

* Corresponding author.
E-mail address: oda@kpu.ac.jp (M. Oda).

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widely used to analyze site-specific structural dynamics at an atomic resolution, in addition to protein structural determination. Thermodynamic analysis using differential scanning calorimetry and isothermal titration calorimetry (ITC) could also help in determining the contribution of protein structural dynamics to the "entropy" in solution [5,6]. While both NMR and calorimetry can be used for characterizing the conformational ensembles of protein structures in solution, the recently developed single-molecule analysis enables us to understand protein structural dynamics [7–9]. The diffracted X-ray tracking (DXT) is one of the methods for single-molecule analysis, and it can evaluate protein structural fluctuations by detecting the time-dependent movement of a gold-nanocrystal attached to the target protein [10,11].

A variety of intrinsically disordered proteins are known to play important roles in the living cells [12,13]. They exist as random structures under physiological conditions, but they convert their structures into active rigid forms. For monitoring changes in the structural dynamics of a protein by DXT, we used a peptide, whose structure could be changed in response to external stimuli. In this study, we analyzed the structural dynamics of this simple model peptide whose structure could be changed from a random-coil like conformation to a helix-bundle conformation upon binding of a metal ion to His residues present in the hydrophobic core of the peptide. The different structural conformations adopted by the protein during the folding process would be analyzed at the single-molecule level, and the sum of the structural dynamic changes in the single molecule would correlate well with the dynamic structural ensemble determined using NMR and calorimetry. In this study, in order to generate the model peptide, we chose a *de novo* designed three helix-bundle protein as the template, whose 3D-structure was determined by NMR [14]. The protein, α_3D, is a 73-residue peptide, containing 18 residues facing the hydrophobic core at positions *a* and *d*. The peptide is characterized by the presence of an α-helical coiled-coil structure (Fig. 1), which was used as a framework with additional functions [15,16]. We focused on the hydrophobic residues present at positions *d* and *a*, and changed these residues to His and other residues, Ala and Gly, in order to destabilize the protein structure and refold it into the helix-bundle structure by allowing a metal ion to bind to His, as reported previously [17–19]. The conformational changes induced by metal binding were analyzed using circular dichroism (CD). The CD analysis showed that the destabilization effects largely depended on the substituted amino acids. Of all the peptides designed, two peptides, HA and HG, showed distinct conformational changes induced by the binding of metal ion. Both peptides were further analyzed for understanding the binding thermodynamics using ITC and the structural dynamics using DXT. DXT could detect the motion of peptide in the folding process, and the obtained results were well correlated with the ligand binding energy including the folding energy of helix-bundle conformation determined by using ITC.

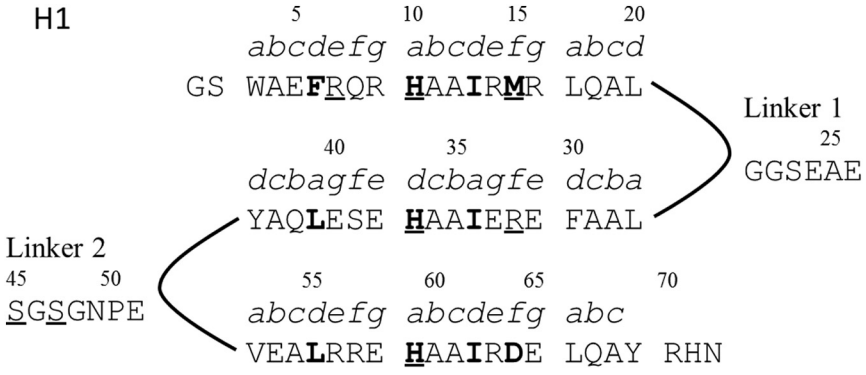
2. Materials and methods

2.1. Expression and purification

The DNA fragments, with *Bam*HI and *Eco*RI restriction enzyme sites at each end, encoding the designed peptides were inserted into pET-32HT and pET-28n plasmid vectors, which were slightly modified from pET-32a(+) and pET28a(+) (Novagen), respectively. *Escherichia coli* BL21(DE3) cells, transformed with the plasmids, were cultured at 37 °C for 4 h in 500 mL of LB medium, followed by further culture at 22 °C for 18 h in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested, resuspended in 20 mL of 50 mM potassium phosphate buffer (pH 8.0) containing 500 mM NaCl, and sonicated. The suspension was centrifuged, and the supernatant fraction was collected. The supernatant was applied to a His-accept resin column (COSMOGEL, 3 mL). The target peptide was eluted with the same buffer containing 500 mM imidazole (10 mL), and each fraction was analyzed by sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (SDS-PAGE). The eluted fraction was subjected to thrombin digestion, to remove the thioredoxin-tag protein from the peptide. Finally, the mixtures were subjected to reverse-phase HPLC and the peptides were eluted with a linear gradient of CH_3CN/H_2O containing 0.1% trifluoroacetic acid.

2.2. CD measurements

CD measurements were carried out on a Jasco J-820 spectrometer,



	helix 1				helix 2				helix 3			
	6	10	13	15	34	37	41		55	59	62	64
	d	*a*	*d*	*f*	*a*	*d*	*a*		*d*	*a*	*d*	*f*
H1	**F**	**H**	**I**	**M**	**I**	**H**	**L**		**L**	**H**	**I**	**D**
H4	**F**	**H**	**A**	**M**	**A**	**H**	**L**		**L**	**H**	**A**	**D**
H7	**A**	**H**	**A**	**M**	**A**	**H**	**A**		**A**	**H**	**A**	**D**
H7G	**G**	**H**	**G**	**M**	**G**	**H**	**G**		**G**	**H**	**G**	**D**
HA	**H**	**H**	**A**	**T**	**A**	**H**	**H**		**H**	**H**	**A**	**M**
HG	**H**	**H**	**G**	**T**	**G**	**H**	**H**		**H**	**H**	**G**	**M**

Fig. 1. Amino acid sequences of H1, H4, H7, H7G, HA, and HG peptides. (upper) The residues of H1 peptide changed from α_3D are underlined and those changed in other peptides are indicated in bold. The positions, *a*–*g*, are also indicated above the amino acids. (lower) The residues at 6, 10, 13, 15, 34, 37, 41, 55, 59, 62, and 64 are indicated, and others are same as those of H1 peptide.