Methylation Dependent Functional Switch Mechanism Newly Found in the *Escherichia coli* Ada Protein

Tadayasu Ohkubo,*,† Hitoshi Sakashita,† Takahiko Sakuma,‡ Masatsune Kainosho,‡ Mutsuo Sekiguchi,§ and Kosuke Morikawa†

Protein Engineering Research Institute
6-2-3, Furuedai, Suita, Osaka 565, Japan
Tokyo Metropolitan University
1-1, Minami-ohsawa, Hachioji-shi, Tokyo, 192-03 Japan
Medical Institute of Bioregulation
Kyushu University, Fukuoka 812, Japan

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Transcriptional regulatory proteins often alter their binding affinities to DNA by the attachment of small ligand molecules, resulting in the activation or suppression of gene expression like a switch.[1] The genetic switch is modulated by a substantial ligand-induced conformational change in the protein. However, the molecular basis for the genetic switch, which is induced by a conformational change coupled with ligand binding, remains unclear. Using a ^{113}Cd NMR technique, we have definitively demonstrated the mechanism by which a conformational change controls the genetic switch in the *Escherichia coli* Ada protein. This report communicates a new insight into the ligand-induced switching mechanism of the Ada protein, which is coupled with transcriptional regulation.

The Ada protein acquires the ability to bind specific DNA sequences, through a direct and irreversible methyl transfer to its own cysteine residue (Cys69) from a methylated phosphotriester within aberrantly alkylated DNA.[2] This modification of the protein results in the activation of several methylation-resistant genes. The Ada protein has been found to possess a tightly bound Zn(II) ion that is essential for protein folding, both *in vivo* and *in vitro*.[3] NMR analyses of the zinc coordination and the tertiary structure of N-terminal fragments of the Ada protein suggested that methylation of Cys69 may drive ligand reorganization at the metal coordination center in the switch process.[4-7] However, the switch mechanism has not yet been concretely delineated on the basis of experimental evidence, since all of the N-terminal fragments analyzed by NMR were of the nonmethylated form, and they also lacked the peptide region for specific binding to DNA. Therefore, further analyses on the methylated and nonmethylated Ada proteins by the ^{113}Cd NMR technique are required to obtain direct information about the conformational change that controls the genetic switch.

We have previously reported that the N-terminal fragment of the Ada protein, consisting of residues 1–146 (N-ada16k), retains activities both for the DNA methyltransferase and for sequence-specific DNA binding after methylation of Cys69.[4] The methylated N-ada16k (me-C69 N-ada16k) used in this work was prepared by reacting the N-ada16k with a large amount of the alkylated DNA.[8,9] The Zn(II) ion was isostructurally replaced with the $^{113}Cd(II)$ ion by biosynthetic labeling with $^{113}CdCl_2$.[10]

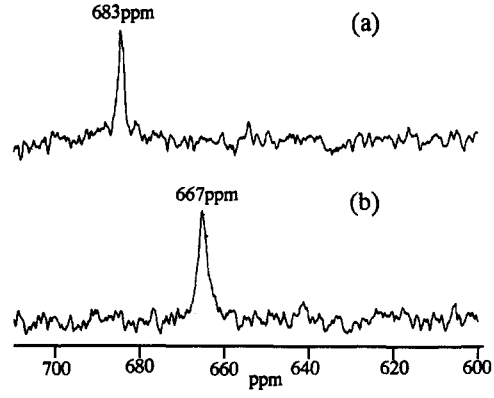

Figure 1. ^{113}Cd NMR spectra obtained with a 2 mM polypeptide concentration of the ^{113}Cd-substituted N-ada16k of the (a) nonmethylated and (b) methylated forms in 90% H_2O/10% D_2O, 50 mM sodium phosphate, pH 6.5, at $T = 30$ °C. The chemical shifts of the ^{113}Cd signals were measured relative to an external standard of a ^{113}Cd–EDTA complex with a signal at 86 ppm.

^{113}Cd NMR spectra were obtained to identify the number and type of zinc ligands (Figure 1).[11,12] The ^{113}Cd NMR spectrum of N-ada16k exhibited a single peak at 683 ppm, which is in agreement with those observed in other tetrahedral tetrathiolate cadmium sites (610–750 ppm).[11] Notably, the chemical shift value for the ^{113}Cd signal of me-C69 N-ada16k (667 ppm) obviously lies within a range expected for a tetrahedral tetrathiolate cadmium site.[13] The proton chemical shifts of the corresponding ligands coordinated with the metal were obtained from ^{113}Cd–^1H HMQC experiments (Figure 2). A ^{113}Cd–^1H 1D HMQC NMR spectrum of N-ada16k revealed three-bond coupling between the metal and the $C_\beta H_2$ of Cys38, Cys42, and Cys72.[14] The signal, which arises from coupling between the ^{113}Cd and the $C_\beta H_2$ of Cys69 spins, was not readily observed in any of the ^{113}Cd–^1H HMQC spectra. The previous ^{113}Cd NMR study also failed to reveal any direct evidence for the coordination of Cys69 by NMR measurements.[6] The lack of this signal could result from either a small heteronuclear ^{113}Cd–^1H coupling constants or kinetic lability of the metal–ligand bond.

To demonstrate the direct interaction of me-Cys69 with the metal ion, we assigned the me-Cys69 $C_\delta H3$ signal by measuring ^{13}C-filtered 1D HMQC spectra of uniformly ^{13}C-labeled me-C69 N-ada16k[15] (Figure 3). Protons bound to ^{12}C are exclusively

* Author to whom all correspondence should be addressed: Protein Engineering Research Institute, 6-2-3, Furuedai, Suita, Osaka 565, Japan. Tel.: 81(Japan)-6-872-8218. Fax: 81(Japan)-6-872-8210. E-mail: ohkubo@asp.peri.co.jp.
† Protein Engineering Research Institute.
‡ Tokyo Metropolitan University.
§ Kyushu University.

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(9) Methylated DNA, prepared by treatment with *N*-methyl-*N*-nitrosourea, was added to the purified N-ada16k, and the mixture was incubated at 37 °C for 30 min.[8] Reaction conditions were optimized to produce sufficient amounts of protein for NMR analysis. Under these optimized conditions, nearly 100% of the N-ada16k was methylated, as estimated from peak intensity in the NMR spectra.
(10) Cross-peak patterns and intensities observed in ^{15}N–^1H 2D HMQC and ^{15}N–^1H 3D HMQC-NOESY spectra of Cd and Zn N-ada16k were matched to each other, demonstrating that ^{113}Cd substitution did not cause significant structural perturbation.
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(13) Optical absorption spectra of the cobalt-substituted protein were measured to confirm the ^{113}Cd NMR results. Three intense absorption bands characteristic of the tetrahedral coordination were observed in the visible region.
(14) For the nonmethylated protein, both spectra showed ^{113}Cd and $C_\beta H$ signals of Cys residues with chemical shifts very similar to those previously reported for the N-ada10k.
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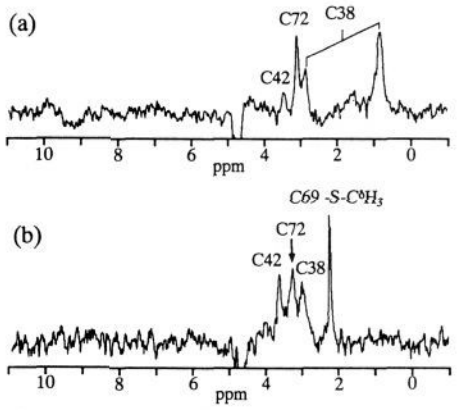

Figure 2. Proton-detected ^{1}H–^{113}Cd 1D HMQC spectra of N-ada16k (a) and me-Cys69 N-ada16k (b). The defocusing and refocusing delay was set to 5 ms. ^{1}H chemical shifts were referred to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

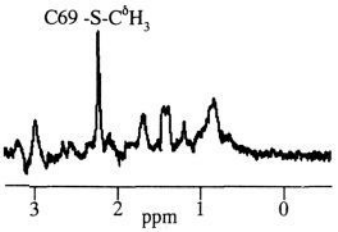

Figure 3. ^{13}C-Filtered 1D HMQC spectrum of uniformly $^{13}C/^{113}Cd$-labeled me-C69 N-ada16k, showing the me-Cys69 $^{12}C_{\delta}H_3$ signal. Protons not bound to ^{13}C are exclusively observed in the spectrum.

observed in the ^{13}C-filtered HMQC spectrum. The *S*-methylcysteine-69 $C_{\delta}H3$ signal is easily assigned to the peak at 2.3 ppm, because only the *S*-methyl carbon transferred from the alkylated DNA contains ^{12}C. Thus, it is concluded that four ligands, Cys38, -42, -69, and -71, retain coordinate bonds with the metal after the acceptance of the methyl group at Cys69. In order to investigate more precisely the conformational change induced by the methylation of the Cys69 thiolate, we have compared the ^{15}N–^{1}H 2D HMQC spectra between N-ada16k and me-C69 N-ada16k (data not shown). These spectra were almost identical, except for signals derived from residues lying in the vicinity of the metal binding site.

These results allow us to propose a model which can account for the switching mechanism of the Ada protein. Two possible pathways for the functional switch are outlined as follows. A pathway shown in Figure 4 indicates that the ligand organization at the metal center is retained after methylation. The sequence-specific DNA binding ability can be acquired by a relatively small conformational change, or by direct contact of the attached methyl group to the DNA duplex with the cognate sequence. In contrast, an essential feature of the alternative pathway, proposed previously, is that methylation-induced disruption of the coordination bond at Cys69 drives ligand reorganization at the metal center to form a new coordinate bond to a water molecule.[6] The conformational change resulting from the cleavage of the single coordinate bond could cause the functional conversion of the Ada

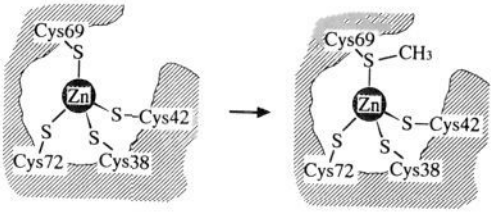

Figure 4. Schematic drawing representing the mechanism for the ligand-induced functional switch in the *E. coli* Ada protein.

protein from a methyltransferase to a sequence-specific DNA binding protein. Similar activation mechanisms have been found in a matrix metalloprotease, and they are currently known as "the cysteine switch".[16] The present results clearly show that the thioether-S ligand of Cys69 remains coordinated to the Zn(II) ion after the acceptance of the methyl group from the methylated phosphotriester backbone and, hence, supports the scheme shown in Figure 4. Thus, the location of the Cys69 thiolate group is not drastically altered. A small conformational change induced by the attachment of the methyl group should become the trigger of the functional switch. On the other hand, these findings are inconsistent with the concept that the methylation at Cys69 induces a conformational change large enough to expose a DNA binding site buried within the protein molecule. For instance, direct contact of the *S*-methyl group to the DNA duplex, which would contribute to the sequence-specific interaction of the Ada protein, seems to be more plausible.[17] The Zn(II) ion appears to act as an anchor that controls the correct positioning of the *S*-methyl group upon DNA binding.[19] To draw a more definite conclusion, structural studies of the protein–DNA complex are in progress.

These findings are generally consistent with the reaction mechanism proposed for methyl transfer, in that the thiolate of Cys69 directly attacks the methyl group of a methylated phosphotriester, and the Zn(II) ion serves to activate the nucleophilicity of Cys69 by stabilizing the thiolate form.[6] However, our present results provide new insight into the methyl transfer reaction of the Ada protein; the thiolate is liganded to the Zn(II) ion, both before and after the reaction. No experimental indication for H_2O coordination to the metal ion was detected. Many other proteins containing Zn(II) ions at the active site are known to utilize H_2O as a catalytic ligand.[20] The Ada protein is the first example in which a cysteine residue acts as a catalytic ligand.

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(17) Recent thermodynamic studies on the stability of specific protein–DNA complexes have revealed that local changes in conformation occur upon binding.[18] We cannot exclude a switch model, where, upon DNA binding, such an induced fit is more likely to take place with the methylated than the unmethylated Ada protein. However, it remains unclear whether an induced fit actually takes place in the Ada protein.

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