Metal-coordination sphere in the methylated Ada protein–DNA co-complex

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Background: The Ada protein of *Escherichia coli* repairs methyl phosphotriesters in DNA by direct, irreversible methyl transfer to one of its own cysteine residues. This residue, Cys69, is ligated to a tightly bound zinc ion in the protein. After methyl transfer, Ada can bind DNA sequence-specifically, inducing the transcription of genes that confer resistance to the toxic effects of methylating agents. Coordination of zinc via a thioether-S is exceedingly rare. We therefore investigated whether methylation causes ligand exchange of Cys69, replacing the thioether with a new zinc ligand with higher affinity for the metal.

Results: We added a ^{13}C-labeled methyl group to Cys69 of Ada and used isotope-edited NMR to observe the behavior of its protons. Comparison of the spectra for the Zn- and ^{112}Cd- bound forms of the methylated protein with that of the ^{113}Cd-bound form provided clear evidence that S-Me-Cys69 is coordinated to the metal in Ada when Ada is bound specifically to DNA.

Conclusions: The transcriptionally competent form of Ada, in which Cys69 is methylated and the protein is bound to DNA, maintains the coordination of S-Me-Cys69 to the metal ion. Thus, ligand exchange is not responsible for switching Ada from a DNA-repair protein to a transcriptional activator. We propose that the lability of the thioether–zinc coordinate bond may provide a mechanism for down-regulation of the adaptive response by inactivation of the Ada DNA-binding domain.

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Introduction

Escherichia coli responds to sub-lethal dosages of methylating agents by inducing the synthesis of proteins that confer protection against the toxic effects of DNA methylation. The Ada protein is essential for this so-called adaptive response (for review, see [1]). Ada repairs one of the most mutagenic DNA adducts, O^6-methylguanine (O^6-meG), by direct, irreversible transfer of the offending methyl group to a cysteine residue in the carboxy-terminal domain (C-Ada19; Fig. 1) [2,3]. Ada also repairs the S_p diastereomer of DNA methylphosphotriesters (MePs) by direct transfer to another cysteine residue, Cys69, located in the amino-terminal domain [4]. Once Cys69 has been methylated, Ada becomes able to activate methylation-resistance genes *in vitro* [5]. Ada is thus essential for the regulation of the induction of the adaptive response, as well as participating directly in DNA repair. The ability of the Ada protein to activate transcription has been traced to an enhancement in its sequence-specific DNA-binding activity upon methylation of Cys69; the methylated protein (S-Me-Cys69 Ada) binds to several sites in the *E. coli* genome, activating expression of a methylation-resistance regulon [5–7]. As suggested by the observation that overexpression of Ada leads to gratuitous induction of the *ada* regulon, the unmethylated protein does have some sequence-specific DNA-binding activity [8]. It has recently been determined that methylation of Cys69 increases the binding affinity of Ada for the *ada* promoter by a factor of 10^3, which translates to a difference in binding free energy (25 °C) of ≈ 4.2 kcal mol^{-1} [8]. Uncovering the molecular basis of this effect will lead to a deeper understanding of *ada* regulon function, and should also provide insight into the general problem of how the post-translational modification of DNA-binding proteins governs their transcriptional activity [9].

The structures responsible for sequence-specific DNA-binding by Ada are contained entirely within the amino-terminal domain of the protein [8,10]. This domain has been found to contain a tightly bound zinc ion that is essential for folding of the protein both *in vivo* and *in vitro* [11]. NMR studies on a ^{113}Cd-labeled version of the 20 kDa amino-terminal domain of Ada (N-Ada20), coupled with similar studies on a truncated version that contains the intact zinc-binding module (N-Ada10; Fig. 1b) and several mutant Ada fragments led to the unexpected discovery that the metal ion is directly coordinated to the active site nucleophile, Cys69 [12]. This finding, confirmed by an NMR solution structure of N-Ada10 [13], suggested that the metal ion of Ada not only serves to stabilize the tertiary structure of the protein, but also participates directly in the methyl transfer chemistry by increasing the nucleophilicity of Cys69. The zinc and cadmium forms of N-Ada10 are nearly isostructural as judged by their NMR spectra [12],

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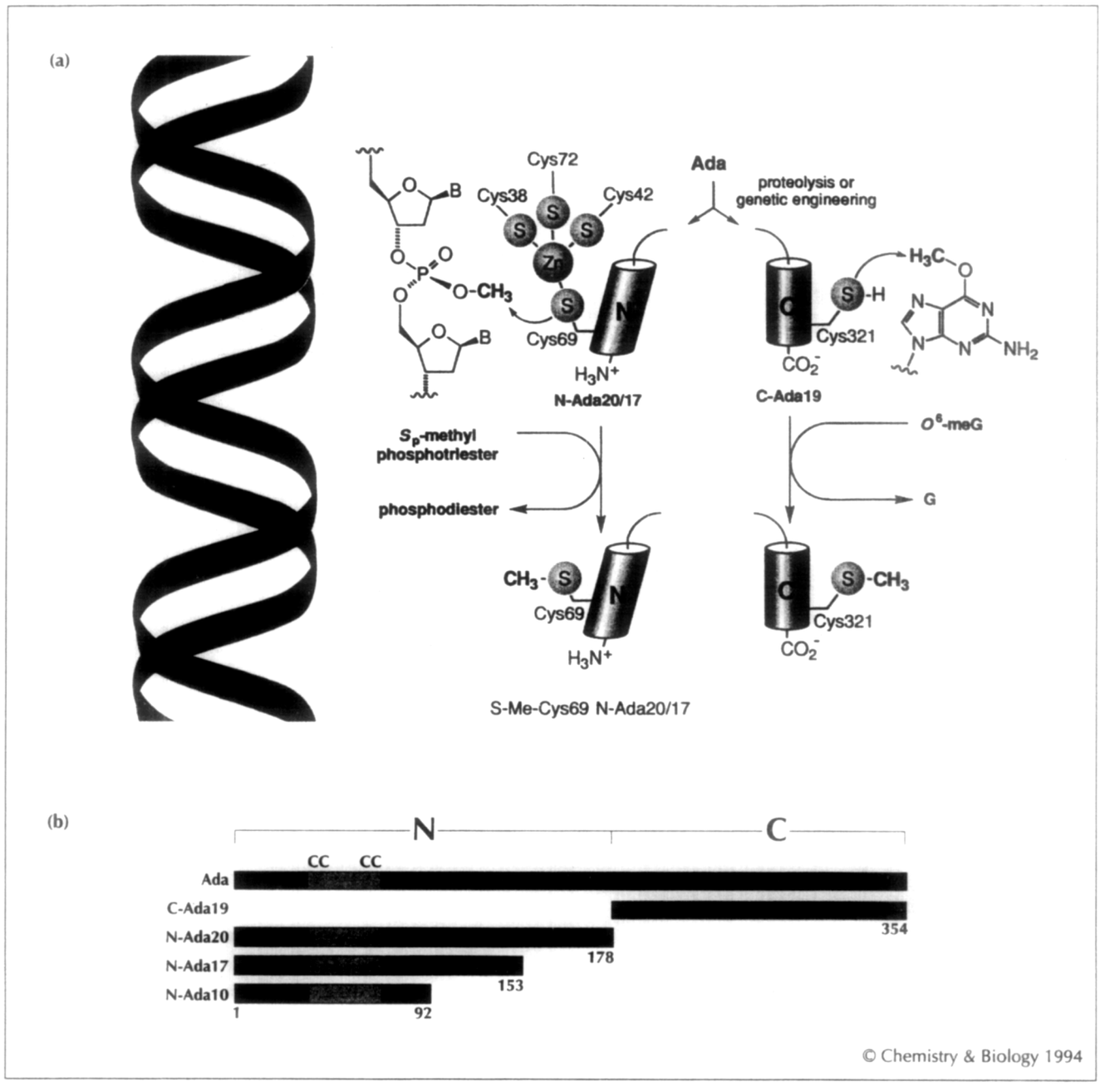

Fig. 1. Schematics of the Ada protein, its constituent domains, and the autocatalytic DNA-repair reaction of the amino-terminal domain. **(a)** Ada consists of two domains. The 20-kDa amino-terminal domain (N-Ada20), or a further truncated fragment (N-Ada17), repairs the S_p diastereomer of methylphosphotriesters and, in its methylated form, binds DNA in a sequence-specific manner; the 19-kDa COOH-terminal domain (C-Ada19) repairs the mutagenic lesion O^6-meG by direct methyl transfer to Cys321. Each domain retains its activity when separated from the other. **(b)** Schematic representation of Ada and its fragments. The metal-binding domain is shaded yellow, with the ligand residues denoted above. N-Ada17 comprises residues 1 to 153. This 17-kDa fragment, generated by gene truncation, fully retains all activities contained in the full amino-terminus (N-Ada20), including methylation-enhanced sequence-specific DNA binding. N-Ada10, which comprises residues 1–92, retains MeP repair and metal binding activity, but is not capable of methylation-enhanced sequence-specific DNA binding.

and have similar affinities for DNA. These two forms of the protein show a four-fold difference in the rate of methyl transfer [8], however, consistent with the notion that the bound metal participates directly in the DNA repair reaction.

The finding that the bound metal interacts directly with the active site Cys suggested that the metal may also form part of the structural switch that converts Ada from a DNA-repair protein to a transcriptional activator. The fact that the thiolate-S of Cys69 is a much stronger zinc ligand than the thioether-S produced by methylation suggested the possibility that methylation might trigger ligand exchange or stereochemical rearrangement about the metal [12]. Ligand exchange seemed a particularly attractive option, as coordination of the thioether-S of

Met residues to zinc occurs rarely, if at all, in proteins. Furthermore, the matrix metalloproteases use a similar type of switch mechanism wherein chemical modification of a zinc-bound Cys results in dissociation of that ligand and subsequent unveiling of the enzyme's masked zinc protease activity [14]. The NMR solution structure of N-Ada10 revealed no obvious candidate ligand for the putative exchange reaction [13]; however, this fragment lacks residues that are essential for sequence-specific DNA binding, even though it is fully competent to demethylate MePs [11].

To address the coordination states of Cys69 after methylation, we have developed a procedure for specific labeling of the methyl group with ^{13}C, to permit selective observation of the attached protons using isotope-edited NMR. Ohkubo *et al.* [15] have pursued the reverse approach, in which all carbons of the protein except the Cys69 methyl group are labeled with ^{13}C. Their results, reported while this manuscript was in preparation, support the notion that in the non-DNA-bound form of the methylated Ada amino-terminus, S-Me-Cys69 is coordinated to zinc. These studies, however, did not address the question of whether S-Me-Cys69 is coordinated to zinc in the transcriptionally competent form of Ada, the state in which the protein is bound specifically to DNA. Here we report that in the transcriptionally competent form of Ada, the active site S-Me-Cys is indeed coordinated to the metal ion. Ada thus represents an exceedingly rare example of zinc ligation by a thioether-S in a protein. These findings have important implications for understanding transcriptional switching in Ada.

Results

Transfer of a ^{13}C-labeled methyl group to Cys69

An Ada fragment containing residues 1-153 (N-Ada17; Fig. 1b) has been shown to have virtually identical metal-binding and methylation-dependent DNA-binding properties to those exhibited by full-length Ada [8]. Sakashita *et al.* [10] have reported a similar observation in a fragment containing residues 1–146 . We have thus focused the present studies on N-Ada17, as this form of the protein represents a suitable candidate for future high-resolution structural studies. As a strategy to determine the ligation state of Cys69 following methylation, we chose to take advantage of the scalar coupling that is often observed between ^{113}Cd and protons that are separated by three contiguous bonds from the metal. In a typical case, the β-protons of a Cys thiolate ligand are coupled to the ^{113}Cd (Fig. 2a); the same three-bond relationship exists, however, for the protons on the methyl group of a metal-bound S-Me-Cys (δ protons; Fig. 2b). We reasoned that the δ-Me-Cys69 could be uniquely identified among all the other atoms of the DNA and protein by specific labeling of the δ-carbon with ^{13}C. This required the development of a method for transfer of a ^{13}C-labeled methyl group specifically to Cys69 of N-Ada17. We have recently shown that a single-stranded thymidine homopolymer containing a single, centrally located MeP, $T_{11}OMe$, alkylates Ada and various amino-terminal fragments efficiently and with 1:1 stoichiometry. This result, taken together with the observed increase in DNA-binding affinity of the Ada proteins methylated by $T_{11}OMe$, establishes that the homopolymer substrate methylates Ada selectively on Cys69 [8].

To label $T_{11}OMe$ with ^{13}C on its single phosphotriester moiety, a dT (^{13}C)-methyl phosphoramidite was synthesized by a standard route [16,17], using ^{13}C-labeled methanol as the source of the isotopically labeled methyl group. This phosphoramidite was used together with the standard dT in the synthesis of (methyl-^{13}C)$T_{11}OMe$ by direct analogy to the known procedure [8].

The specificity of labeling of N-Ada17 with (methyl-^{13}C)$T_{11}OMe$ was examined by comparison of the one-dimensional (1D) ^{13}C-edited ^{1}H spectra of Zn-bound N-Ada17 methylated with either the labeled or unlabeled oligonucleotide. At the concentrations necessary for NMR measurements, we found that methylation of

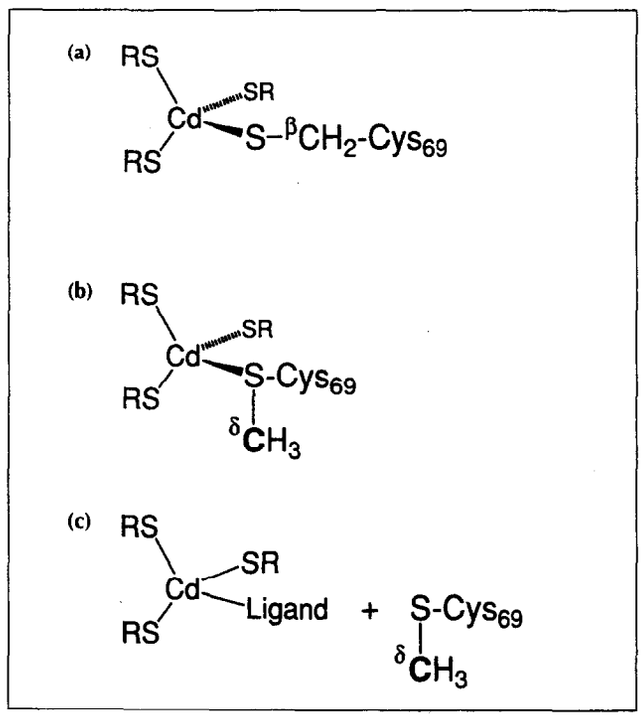

Fig. 2. Ligation schemes for ^{113}Cd in Ada and their detection by NMR. **(a)** In the unmethylated form of ^{113}Cd N-Ada, a three-bond coupling exists between the metal and the Cys β protons. **(b)** If the S-Me-Cys69 remains a ligand in S-Me-Cys69 N-Ada17, then a three-bond coupling should be apparent between the ^{113}Cd and the protons on the ^{13}C-labeled methyl group (signified in bold type). Thus, the NMR signal for the methyl group in the ^{113}Cd form of S-Me-Cys69 Ada bound to DNA would be broadened as compared to the corresponding forms of the protein/DNA complex with a NMR inactive bound metal (Zn or ^{112}Cd). **(c)** If the S-Me-Cys69 does not continue to coordinate the zinc in S-Me-Cys69 N-Ada17, then there should be no three-bond coupling between the ^{113}Cd and the protons on the ^{13}C-labeled methyl group. Thus, the NMR signal for the methyl group in the ^{113}Cd form of S-Me-Cys69 Ada-DNA would not be broadened as compared to the forms of the complex with an NMR-inactive bound metal (Zn or ^{112}Cd).

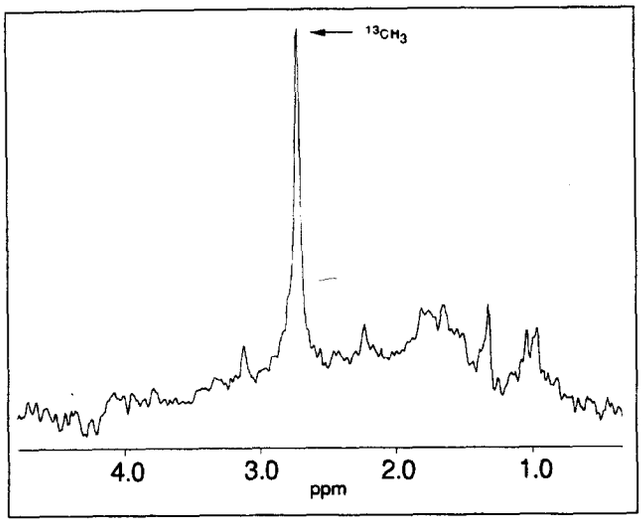

Fig. 3. 1D ^{13}C-edited heteronuclear multiple-quantum coherence NMR spectra of N-Ada17 methylated using (methyl-^{13}C)T_{11}OMe and in a co-complex with a duplex oligonucleotide containing a sequence-specific binding site for the protein.

N-Ada17 results in rapid precipitation of the protein from solution; however, in the presence of a duplex oligonucleotide containing a specific binding site for the protein, it remains in solution and retains the ability to bind DNA specifically [8]. All NMR experiments reported here were therefore carried out in the presence of a specific 21-bp oligonucleotide duplex containing an Ada binding site. Under these conditions, the 1D ^{13}C-edited spectra of N-Ada17 treated with (methyl-^{13}C)T_{11}OMe exhibits a strong, single peak that is not observed in the N-Ada17 treated with unlabeled T_{11}OMe (Fig. 3) and is distinct from the peaks detected for (methyl-^{13}C)T_{11}OMe alone (3.25, 3.47 ppm for the two stereoisomers in $CDCl_3$, data not shown). The chemical shift of the peak (Fig. 3) is consistent with that expected for δ-protons of S-Me-Cys. Taken together with our earlier results, these data establish that (methyl-^{13}C)T_{11}OMe transfers a single ^{13}C-labeled methyl group to Cys69 of N-Ada17.

The coordination status of S-Me-Cys69

It has previously been shown that ^{113}Cd serves as an NMR-active counterpart to Zn in proteins [18]. Indeed, the substitution of Cd for Zn in Ada only weakly affects phosphotriester repair and DNA binding [8]. We therefore incorporated ^{113}Cd into N-Ada17 biosynthetically [12]. The ^{113}Cd-bearing protein was then methylated by treatment with (methyl-^{13}C)T_{11}OMe in the presence of a duplex oligonucleotide containing an Ada-binding site. As the chemical shift of ^{113}Cd in proteins can be diagnostic of the nature of the bound ligands [18], we first attempted to detect the ^{113}Cd directly. No signal was observed, however, presumably due to broadening caused by the large size of the protein–DNA complex (~ 38 kDa), chemical shift anisotropy or dynamic effects [18]. Even if a signal were observed in these experiments, they would not prove conclusive, as appropriate chemical shift standards do not exist for all the possible Cys 3(X) ligation schemes. To address the issue of S-Me-Cys69 coordination unambiguously, we looked for the presence of three-bond coupling between the δ-CH_3 protons and the ^{113}Cd in the protein–DNA complex. Coupling of the δ-CH_3 to ^{113}Cd would not be expected to give rise to a well-defined peak splitting, but rather would be evident as line broadening of the proton peak, as three-bond ^{1}H-^{113}Cd coupling constants are commonly small [18]. It is thus necessary to compare the line width of the potentially scalar coupled system with isomorphous examples that are incapable of metal-ligand scalar coupling. For the latter, we prepared protein-DNA complexes having a ^{13}C label on S-Me-Cys69, but including the NMR-inactive metals Zn or ^{112}Cd. The latter control is especially important, as the structures of the complexes formed with ^{113}Cd and ^{112}Cd are expected to be virtually identical.

A series of 2D ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra were measured for the ^{113}Cd, ^{112}Cd and Zn forms of (methyl-^{13}C)S-Me-Cys69N-Ada17. In each spectrum, a single prominent peak was observed that was undetectable, at comparable sensitivity, in the same complex bearing a ^{12}C methyl group (data not shown); this cross-peak can therefore be assigned to the ^{13}C-labeled methyl group. The chemical shift of the cross-peak arising from the ^{13}C-labeled methyl group for the ^{112}Cd and ^{113}Cd forms of the proteins was virtually identical, confirming the isostructural nature of the isotopomers; a corresponding peak in the Zn form was shifted slightly (≈ 0.1 ppm), presumably due to the differing electronic effects of bound Zn and Cd. Cross sections of the proton spectra for the various forms are presented in Fig. 4. To determine the line widths of the cross-peaks arising from the labeled methyl group, the peak widths at half-height were measured in the ^{1}H cross-sections of the HSQC spectra (Fig. 4). The cross-section of the methyl peak of the ^{113}Cd-bound protein is broadened by ≈ 5 Hz relative

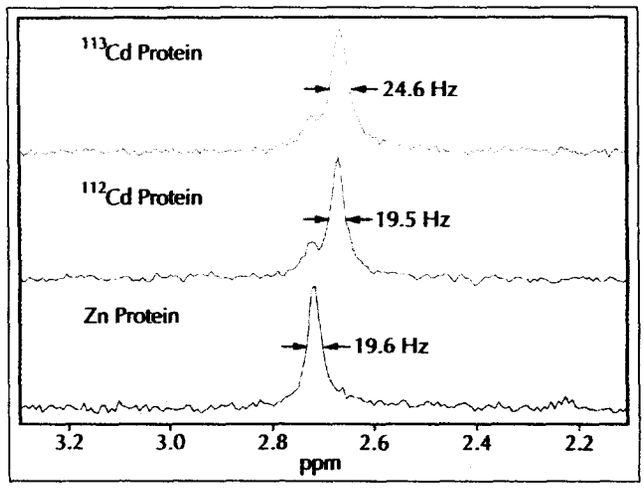

Fig. 4. 1D proton cross-sections of a 2D ^{1}H–^{13}C HSQC spectrum showing the chemical shift and line width measurements for the ^{13}C-labeled methyl group in the (methyl-^{13}C)S-Me-Cys69 Ada-DNA co-complex containing a tightly bound **(a)** ^{113}Cd metal ion, **(b)** ^{112}Cd metal ion and **(c)** Zn metal ion. The small shoulder peak observed in both cadmium samples is likely to originate from minor contaminating amounts of the Zn form of the complex.

to either the ^{112}Cd-bound protein or the Zn-bound protein. This broadening, although small, is highly reproducible and is in a range expected for three-bond couplings of ^{113}Cd to ^{1}H [19]. Furthermore, whereas the line-width of the methyl peak in the ^{113}Cd protein is broader than that in the ^{112}Cd and Zn proteins, the respective natural abundance ^{13}C–^{1}H cross peaks in all three spectra possess the same line widths. The broadening observed in the spectrum of the ^{113}Cd protein is therefore due to scalar coupling of the methyl to the ^{113}Cd and not to variation in sample preparation or spectroscopy conditions. Based on the observation of this methyl–metal coupling, we conclude that the S atom of S-Me-Cys69 in Ada remains ligated to the metal, even when the protein is bound specifically to DNA.

Discussion

Methylation of Cys69 converts Ada from a DNA-repair protein to a potent activator of transcription [5]. This covalent modification of Ada brings about a 10^3-fold change in its specific DNA affinity, which constitutes the molecular basis for Ada's function as a biological switch [8]. The recent finding that Cys69 is activated toward methyl transfer by ligation to a tightly bound zinc ion raised the possibility that the metal may also be directly important in structural changes that may accompany functional conversion to a transcriptional activator. As methylation of Cys69 converts Ada from a strong zinc ligand to a substantially weaker one, it appeared possible that exchange of S-Me-Cys for another ligand, or alternatively stereochemical rearrangement about the metal center, might accompany methylation. Here, we have shown that the ligation status of Cys69 is unaffected by methylation. These results are in general agreement with those of Ohkubo *et al.* [15], who studied an Ada fragment in the absence of specific binding to DNA. There is still no information on whether stereochemical changes accompany methylation, and if so what they are. Preliminary chemical shift data, however, indicate that no major structural rearrangement occurs upon methylation [8,15].

The finding that a thioether is ligated to zinc in Ada is both unexpected and extraordinary. Although all the Met residues in proteins have thioether-S groups analogous to that in S-Me-Cys, there appears to be no known example in which Met is used to coordinate a zinc ion in a protein that normally binds zinc. The S atom of Met, however, is commonly used as a ligand in copper-containing proteins such a plastocyanin. It has proven possible to replace the bound metal in plastocyanin with zinc or cadmium [20], and NMR evidence suggests that, at least in the case of the Cd-substituted protein, the metal is coordinated to Met [20,21]. The observation of such a rare coordination scheme in Ada raises the question of whether the thioether ligation has a function apart from that of maintaining the folded structure of the protein and, perhaps, positioning the methyl group. Although further experiments will be required to determine whether this is the case, one possibility is that thioether coordination serves as a mechanism for deactivating the adaptive response. We have consistently noted that the S-Me-Cys69 forms of all amino-terminal Ada fragments are substantially more labile toward irreversible unfolding than are the corresponding unmethylated proteins. It is plausible that this is a consequence of the weaker zinc coordination in the methylated protein, which appears to destabilize the zinc cluster and its attendant elements of the protein core [11]. Although sequence-specific binding to DNA dramatically stabilizes the methylated protein, the intracellular concentration of specific genomic binding sites is low. Non-specific binding to DNA by the methylated Ada protein may also stabilize the protein, but the protein readily dissociates from such sites. Therefore, the majority of methylated Ada protein is expected to spend some time free in solution, during which period it is highly prone to thermal inactivation. It thus appears that the adaptive response may be regulated by spontaneous unfolding resulting from ligand labilization, as well as by proteolysis of Ada. This possibility may also help explain the down-regulation of transcription for the *alk*A promoter; from which the methylated N-terminal of Ada alone serves as a sufficient transcriptional activator [7,22].

If ligand reorganization does not contribute to transcriptional switching in Ada, how is it that methylation brings about a 10^3-fold change in binding of the protein to DNA? Comparing the data of Ohkubo *et al.* [15] with those reported here, we note that the methyl protons of S-Me-Cys69 Ada experience ≈ 0.5 ppm downfield shift upon specific binding to DNA. This significant change provides evidence for an alteration in the environment surrounding the methyl group upon interaction of the protein with DNA. Barring stereochemical rearrangement about the metal, which seems unlikely, the simplest explanation for such a change in environment would be that the methyl group is in direct contact with DNA. Although 4.2 kcal mol^{-1} exceeds the greatest free energy change observed for interactions involving a single methyl group (3.2 kcal mol^{-1}; [23]), it may be that the difference reflects a loss of repulsive interactions that are present in the unmethylated protein, in addition to a gain of favorable interactions involving the methyl group. It seems possible that the sort of cooperative interactions that so frequently confer such remarkable strength and specificity on protein–DNA interactions may contribute to an exceedingly sensitive mechanism to detect the presence or absence of a single methyl group.

Significance

Aberrant methylation of DNA by endogenous agents, such as S-adenosyl-L-Methionine and exogenous electrophiles gives rise to a variety of genotoxic adducts which are the target of protein-based repair systems [24]. From the points of view of organismal evolution and human health, the most serious of these lesions is O^6-meG, which causes G•C to A•T transition mutations by base-

pairing with thymine during replication. The prototype for studies of O^6-meG repair has been the *E. coli* Ada protein, which reverses this lesion and also repairs DNA methylphosphotriesters by direct, irreversible transfer of the methyl group to cysteines in its carboxy-terminal domain (Cys321) and amino-terminal domain (Cys69), respectively. The methylation of Cys69 reveals a sequence-specific DNA-binding activity in the amino-terminal domain, enabling Ada to activate transcription of a methylation-resistance regulon. The repair of DNA methylphosphotriesters is auto-catalyzed by coordination of the acceptor residue, Cys69, to a tightly bound zinc ion. This finding raised the question of whether Cys69 methylation affects the coordination sphere of the bound metal, and, if so, how such changes would enable Ada to bind DNA in a sequence-specific manner.

The NMR studies presented here show that when S-Me-Cys69 N-Ada17 binds specifically to DNA, there is a three-bond coupling between the ^{113}Cd ion that is tightly bound to Ada and the protons on the S-Me-Cys69. These experiments were possible because we were able to label the S-Me-Cys69 with a $^{13}CH_3$ group by synthesizing a ^{13}C-labeled methylphosphotriester substrate for Ada. We could thus use isotope-edited NMR to observe the ^{13}C-S-Me-Cys69 three-bond coupling directly. As the S-Me-Cys69 still remains coordinated to the bound metal in the transcription-competent form of the protein, the role of the bound metal in the transition that enhances sequence-specific DNA binding is probably a passive one. The weak S-Me-Cys69 ligand makes Ada more sensitive to thermal denaturation, however, perhaps providing a mechanism for transcriptional down-regulation of the adaptive response by inactivation of the transcription-competent form.

Materials and methods

Oligonucleotide synthesis

Oligonucleotides were produced using an Applied Biosystems 380A DNA synthesizer using a standard phosphoramidite coupling cycle. The ^{13}C labeled homopolymer (methyl-^{13}C)T_{11}OMe was synthesized by coupling five units of 5'-dimethoxytrityl thymidine 3'-β-cyanoethyl phosphoramidite (ABN) to a CPG thymidine resin (Milligen), then one unit of 5'-dimethoxytrityl thymidine 3'-(methyl-^{13}C) methyl phosphoramidite, then five more units of 5'-dimethoxytrityl thymidine 3'-β-cyanoethyl phosphoramidite. The resin-bound oligonucleotide was 5'-detritylated and then deprotected by a modification of the procedure of Kuijpers *et al.* [25] using ^{13}C methanol, by analogy to a procedure described in detail elsewhere [8]. The crude oligonucleotide product d(TpTpTpTpTpTp(O^{13}Me)-TpTpTpTpT) was used directly without further purification. The ^{13}C-labeled phosphoramidite was synthesized by analogy to a published procedure [16,17], using ^{13}C methanol (Cambridge Isotopes, Woburn, MA) as the source of the label.

A duplex 21-mer containing a specific binding site for Ada:

5'-d(GCAAATTAAAGCGCAAGATTG)-3'
3'-d(CGTTTAATTTCGCGTTCTAAC)-3'

was synthesized by standard methods. The individual strands were purified by 20 % denaturing polyacrylamide gel electrophoresis and recovered by a crush-and-soak protocol using 1 M triethylammonium bicarbonate (TEAB) followed by adsorption onto a Sep-Pak cartridge (Waters, Milford, MA) and elution using 30 % CH_3CN/70 % 0.1 M TEAB. Following removal of the solvent by Speed-Vac (Savant), the strands were dissolved in TE Buffer (10 mM Tris, pH 7.5, 1 mM EDTA), quantified by UV, and mixed together in equimolar parts. The combined strands were heated to 95 °C for 3 min, then allowed to cool slowly to room temperature. Other experiments [8] have shown that the duplex 21-mer binds Ada with an affinity equal to that of the intact *ada* promoter.

Protein biosynthesis and purification

N-Ada17 was produced by growth of an overproducing *E. coli* strain [8] in rich media (Zn form) or metal-supplemented minimal media (^{113}Cd and ^{112}Cd) as described [11,12]. The proteins were purified as described previously [12]. ^{113}Cd and ^{112}Cd were purchased in the form of the elemental metal (Icon Services, Summit, NJ) and used as the respective chloride salts. Solutions of the chloride salts were formed by dissolving the elemental metal in 1N HCl with heating followed by neutralization with NaOH.

Formation of the methylated protein–DNA co-complex

The complex was formed by adding equimolar amounts of N-Ada17 and the duplex 21-mer along with a 10-fold molar excess of (methyl-^{13}C)T_{11}(O-Me) and incubating at 37 °C for 2 h in a reaction buffer containing 50 mM Tris/HCl, 1 mM EDTA, 10 mM DTT. Ultrafiltration using a 10-kDa cutoff filter (Amicon, Waltham MA) was performed to separate the homopolymer from the protein–DNA co-complex and to exchange the complex into a buffer suitable for NMR (25 mM $NaPO_3$, pH 6.4, 50 mM NaCl, 2 mM ^2H-DTT from Isotec Inc., Miamisburg, OH).

NMR spectroscopy

Measurements were made using 1 mM ^{13}C S-Me-Cys69 N-Ada17–DNA co-complex samples at pH 6.4 and 37 °C. All samples were lyophilized and resuspended in D_20 (99.99 %) before spectroscopic measurements. The ^1H-^{13}C heteronuclear single-quantum coherence (HSQC) and ^1H-^{13}C heteronuclear multiple-quantum coherence (HMQC) spectra were recorded on a Bruker AMX-600 spectrometer. The 2D ^1H-^{13}C HSQC spectra were taken using a proton spectral width of 8333.33 Hz and 2048 complex points, giving a spectral resolution of 2.1 Hz per point.

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References

1. Lindahl, T., Sedgewick, B., Sekiguchi, M. & Nakabeppu, Y. (1988). Regulation and expression of the adaptive response to alkylating agents. *Annu. Rev. Biochem.* **57**, 133–157.
2. Teo, I., Sedgewick, B., Demple, B., Li, B., & Lindahl, T. (1984). Induction of resistance to alkylating agents in *E. coli*: the *ada*$^+$ gene product serves both as a regulatory protein and an enzyme for repair of mutagenic damage. *EMBO J.* **3**, 2151–2157.

3. Nakabeppu, Y., Kondo, H. Kawabata, S.-I., Iwanga, S., & Sekiguchi, M. (1985). Purification and structure of the intact Ada regulatory protein of *Escherichia coli* K12, O^6-methylguanine-DNA methyltransferase. *J. Biol. Chem.* **260**, 7281–7288.
4. Sedgewick, B., Robins, P., Totty, N., & Lindahl, T. (1988). Functional domains and methyl acceptor sites of the *Escherichia coli* Ada protein. *J. Biol. Chem.* **263**, 4430–4433.
5. Teo, I., Sedgewick, B., Kilpatrick, M.W., McCarthy T.V. & Lindahl, T. (1986). The intracellular signal for induction of resistance to alkylating agents in *E. coli*. *Cell* **45**, 315–324.
6. Sakumi, K. & Sekiguchi, M. (1989). Regulation of expression of the *ada* gene controlling the adaptive response. *J. Mol. Biol.* **205**, 373–385.
7. Akimaru, H., Sakumi, K., Yoshikai, T., Anai, M. & Sekiguchi, M. (1990). Positive and negative regulation of transcription by a cleavage product of Ada protein. *J. Mol. Biol.* **216**, 261–273.
8. Myers, L.C., Jackow, F. & Verdine, G.L. Metal dependence of transcriptional switching in *Escherichia coli* Ada. *J. Biol. Chem.* (in press).
9. Hunter, T. & Karin, M. (1992). The regulation of transcription by phosphorylation. *Cell* **70**, 375–387.
10. Sakashita, H., *et al.*, & Morikawa, K. (1993). Folding topology and DNA binding of the N-terminal, 14 kDa fragment of Ada protein. *FEBS Lett.* **323**, 257–260.
11. Myers, L.C., Terranova, M.P., Nash, H.M., Markus, M.A. & Verdine, G.L. (1992). Zinc binding by the methylation signaling domain of the *Escherichia coli* Ada protein. *Biochemistry* **31**, 4541–4547.
12. Myers, L.C., Terranova, M.P., Ferentz, A.E., Wagner, G. & Verdine, G. L. (1993). Repair of DNA methylphosphotriesters through a metalloactivated cysteine nucleophile. *Science* **261**, 1164–1167.
13. Myers, L.C., Verdine, G.L., & Wagner, G. (1993). Solution structure of the DNA methyl phosphotriester repair domain of *Escherichia coli* Ada. *Biochemistry* **32**, 14089–14094.
14. Springman, E.B., Angleton, E.L., Birkedal-Hansen, H., & Van Wart, H.E. (1990). Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a 'cysteine switch' mechanism for activation. *Proc. Natl. Acad. Sci. USA* **87**, 364–368.
15. Ohkubo, T., Sakashita, H., Sakuma, T., Kainosho, M., Sekiguchi, M. & Morikawa, K. (1994). Methylation dependent functional switch mechanism newly found in the *Escherichia coli* Ada Protein. *J. Am. Chem. Soc.* **116**, 6035–6036.
16. Tanaka, T., Tamatsukuri, S. & Ikehara, M. (1986). New approach to the synthesis of deoxynucleoside-phosphoramidite derivatives. *Tetrahedron Lett.* **27**, 199–202.
17. Wada, T., Ishikawa, K. & Hata, T. (1993). Nucleoside 3'-N,N-dialkylphoshonamidites as novel nucleotide units for the solution-phase oligonucleotide synthesis. *Tetrahedron* **49**, 2043–2054.
18. Summers, M.F. (1988). ^{113}Cd NMR spectroscopy of coordination compounds and proteins. *Coordination Chemistry Rev.* **86**, 43–134.
19. Zerbe, O., Pountney, D.L., von Philipsborn, W. & Vasak, M. (1994). Vicinal ^{113}Cd, $^{1}H^{\beta}$-cysteine coupling in Cd-substituted metalloproteins follows a Karplus-type dependence. *J. Am. Chem. Soc.* **116**, 377–378.
20. Engeseth, H.R., McMillin, D.R. & Otvos, J.D. (1984). Comparative Cd-113 nuclear magnetic resonance studies of Cd(II)-substituted blue copper proteins. *J. Biol. Chem.* **259**, 4822–4826.
21. Live, D.H., Kojiro, C.L., Cowburn, D. & Markley, J.L. (1985). Identification of proton NMR signals from the metal ligands in cadmium-substituted plastocyanin via two-dimensional multiple-quantum detection in the absence of explicitly resolved ^{1}H–^{113}Cd coupling. *J. Am. Chem. Soc.* **107**, 3043–3045.
22. Shevell, D.E. & Walker, G.C. (1991). A region of the Ada DNA-repair protein required for the activation of *ada* transcription is not necessary for activation of *alkA*. *Proc. Natl. Acad. Sci. USA* **88**, 9001–9005.
23. Fersht, A.R., Shindler, J.S. & Tsui, W.-C. (1980). Probing the limits of protein-amino acid side-chain recognition with the aminoacyl-tRNA synthetases. Discrimination against phenylalanine by tyrosyl-tRNA synthetases. *Biochemistry* **19**, 5520–5524.
24. Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature* **362**, 709–715.
25. Kuijpers, W.H.A., Huskens, J., Koole, L.H. & van Boeckel, C.A.A. (1990). Synthesis of well-defined phosphate-methylated DNA fragments: the application of potassium carbonate in methanol as deprotecting reagent. *Nucleic Acids Res.* **18**, 5197–5205.

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