# Direct Activation of the Methyl Chemosensor Protein N-Ada by $CH_3I$

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The *Escherichia coli* Ada protein functions as a chemosensor for DNA methylation damage in the cell, activating a defense pathway in response to sublethal dosages of methylating agents. This event is triggered by the direct, autocatalytic transfer of a methyl group from the $S_p$-diastereomer of DNA methylphosphotriesters (MeP) to a cysteine residue ($Cys_{69}$) located in the $NH_2$-terminal domain of the Ada protein (N-Ada)[1] (Figure 1). Methyl transfer to $Cys_{69}$ increases the specific affinity of N-Ada for DNA by $10^3$-fold,[2] thereby conferring on Ada the ability to activate transcription of methylation-resistant genes that together protect the integrity of the *E. coli* genome. N-Ada possesses a tightly bound zinc ion that not only stabilizes the folded structure of the protein[3,4] but also participates directly in the methyl transfer chemistry by coordinating $Cys_{69}$ and potentiating its nucleophilicity.[2,5] Evidence from small-molecule coordination complexes suggests that transition metal-coordinated thiolates possess intrinsically high nucleophilicity,[6−9] and yet DNA methylphosphotriesters alkylate only one of the four cysteines coordinated to the metal in Ada; furthermore, most other proteins that contain structural $Cys_4Zn$ centers are refractory toward electrophilic attack, suggesting they have evolved mechanisms to suppress the nucleophilicity of their thiolate ligands. These considerations prompted us to examine in detail the structure and chemistry of the $Cys_4Zn$ center in Ada, with the aim of uncovering the mechanisms that control the nucleophilicity of zinc-coordinated thiolates in proteins. Here we have used a structurally simple electrophile, methyl iodide (MeI), to probe the relative contributions of intrinsic thiolate nucleophilicity and substrate docking interactions in determining the regiochemical specificity of the DNA repair reaction involving N-Ada.

MeI is a synthetically important methylating agent, which despite its limited capacity to produce methylphosphotriesters in DNA,[10] can activate Ada-dependent gene transcription *in vivo* and *in vitro*[11−13] and enhance DNA binding by N-Ada *in vitro*.[2]

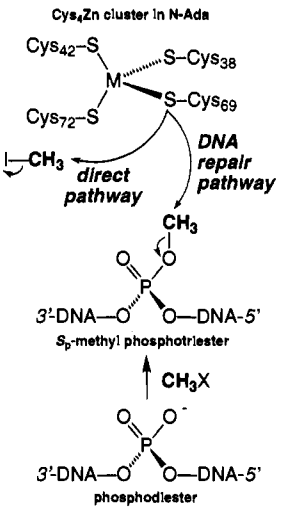


**Figure 1.** Pathway for activating the DNA-binding capacity of N-Ada by metalloactivated transfer of a methyl group to $Cys_{69}$. Residue $Cys_{69}$, present either as a metal-bound thiolate or as a transiently free thiolate, attacks the methyl carbon of a DNA MeP (DNA repair pathway) or directly attacks the methylating agent itself (direct pathway).

To account for these findings, it has been suggested that $CH_3I$ is capable of alkylating $Cys_{69}$ directly, resulting in the conversion of Ada to its transcriptionally active form.[14] This hypothesis has gone untested, and even if correct, it raises the issue of the extent to which MeI is selective for $Cys_{69}$ over the numerous other nucleophilic sites in N-Ada. To determine the preferred site(s) of MeI attack on N-Ada, we treated a 17 kDa fragment of the protein (N-Ada 17) with $^{13}CH_3I$ in the presence of a specific duplex oligonucleotide[15] and followed the fate of the adduct methyl groups using isotope-edited NMR. For comparison, an authentic standard of N-Ada17 bearing a single [$^{13}$C]-methyl group on $Cys_{69}$ was prepared by treatment of N-Ada17 with a single-stranded 11-mer polynucleotide containing a single [*methyl*-$^{13}$C]phosphotriester ([*methyl*-$^{13}$C]$T_{11}$OMe).[16]

The two dimensional (2D) $^1H-^{13}C$ heteronuclear single quantum coherence (HSQC) spectrum[17,18] of N-Ada17 protein methylated with [*methyl*-$^{13}$C]$T_{11}$OMe (Figure 2A) shows a prominent cross-peak at 2.72 ppm ($^1$H)/21.2 ppm ($^{13}$C), which has previously been assigned to the $Cys_{69}-S^{13}CH_3$ group in the methylated protein−DNA complex;[15,16] all other peaks arise from isotopes present at natural abundance. A substantially similar spectrum (Figure 2B) was obtained for N-Ada17 methylated by treatment with a 20-fold molar excess of [$^{13}$C]-MeI:[19] the single prominent peak originating from a $^{13}$C-labeled methyl group corresponds to $Cys_{69}-S^{13}CH_3$. Since the aim of the present study was to determine the kinetic selectivity of alkylation of N-Ada17 by MeI, no attempt was made to arrive at reaction conditions that would produce stoichiometric methylation of $Cys_{69}$; on the basis of relative peak intensities, we estimate that the sample in Figure 2B is methylated ~50−60%.

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(14) The possibility that the methylation of Ada occurs through the intermediary of a methylphosphotriester generated by MeI has previously been ruled out.[12,13]
(15) Methylation of Ada in the presence of a specific oligonucleotide prevents the otherwise rapid precipitation of the free methylated protein from solution.[16]
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(18) Measurements were made using 0.5 mM $Cys_{69}$-$S^{13}CH_3$-N-Ada17/DNA co-complex samples at pH 6.4 and 37 °C. All samples were lyophilized and resuspended in $D_2O$ (99.99%) before spectroscopic measurements. $^1H-^{13}C$ Heteronuclear single-quantum coherence (HSQC) and 2D $^1H-^{13}C$ HSQC spectra were recorded on a Bruker AMX-600 spectrometer as described.[16]

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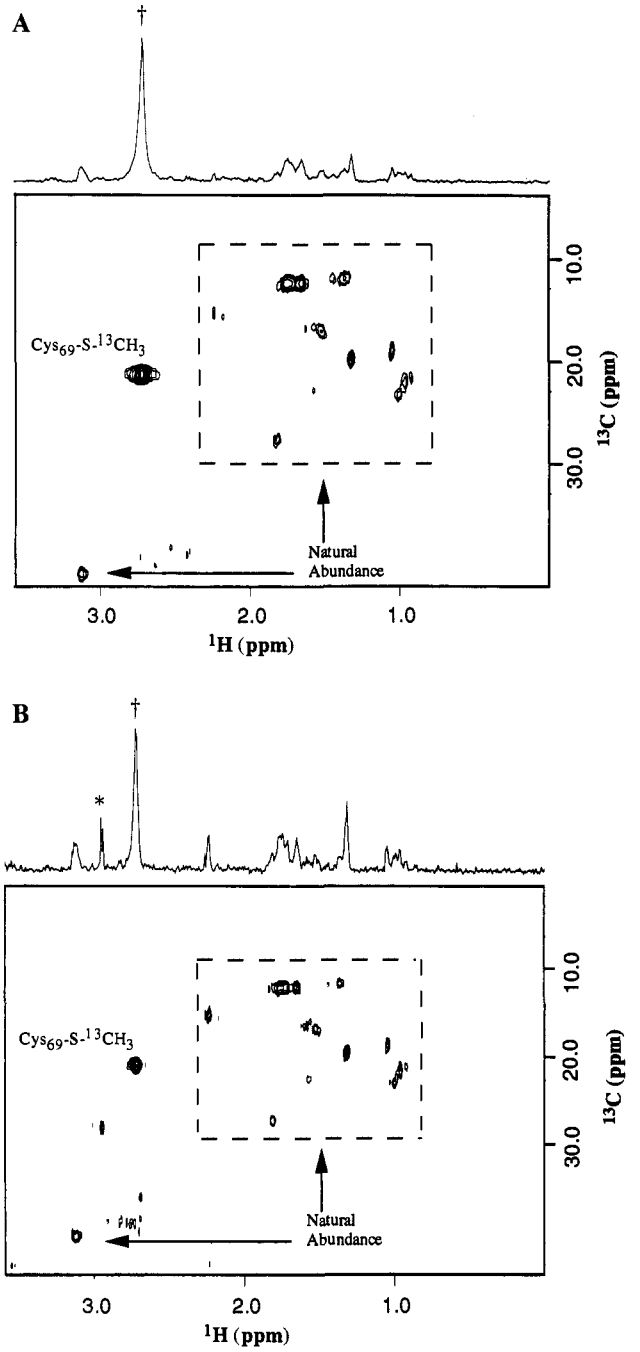


**Figure 2.** NMR spectra from a 2D $^1H-^{13}C$ HSQC experiment on (A) a $Cys_{69}S$-$^{13}CH_3$-N-Ada17/DNA co-complex formed using [*methyl*-$^{13}C$]$T_{11}$OMe as the methylating agent and (B) a $Cys_{69}S$-$^{13}CH_3$-N-Ada17/DNA co-complex formed using [*methyl*-$^{13}C$]MeI as the methylating agent. The major cross-peak arising from the $Cys_{69}$-S-$^{13}CH_3$ is denoted in each spectrum, as well as the cross-peaks arising from natural abundance signals. The signal arising from $Cys_{69}$-S-$^{13}CH_3$ is designated by a † on the one-dimensional $^1H$ projection of each spectra (top). The * denotes a minor impurity, the narrow line width of which indicates that it is unlikely to be part of the protein–DNA complex.

Here we have shown that MeI reacts preferentially with the same cysteine residue in N-Ada as that which is alkylated by methylphosphotriesters in DNA. The selectivity of MeI attack on $Cys_{69}$ is remarkable, considering the large excess of reagent used and the presence of multiple potentially nucleophilic sites in N-Ada17, especially the free thiols $Cys_6$ and $Cys_{91}$ and metal-coordinated thiolates $Cys_{38}$, $Cys_{42}$, and $Cys_{72}$. These results provide direct experimental evidence in support of an alternative mechanism for triggering of the methylation-resistance response in *E. coli*[12,13] involving direct alkylation of $Cys_{69}$ by the methylating agent rather than indirect transfer through the intermediary of DNA methylphosphotriesters (Figure 1). The existence of this alternate mode may provide a clue to the long-standing puzzle of why Nature chose phosphotriesters as the inducing stimulus in this pathway, when some methylating agents (such as MeI) produce this lesion in minor amounts relative to more genotoxic adducts. On the other hand, the observation of a direct activation mechanism does not obviate phosphotriester repair as an inducing stimulus under at least some conditions: the high rate acceleration for autocatalytic attack of $Cys_{69}$ on DNA methylphosphotriesters,[2] the exquisite stereochemical specificity of the reaction, and the ability of some methylating agents (i.e., *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) to generate substantial amounts of DNA methylphosphotriesters together provide compelling evidence for the physiological relevance of the DNA repair pathway for activation of Ada. In any event, it should be borne in mind that the methylating agents to which *E. coli* is exposed in the wild remain to be identified unambiguously.

The facile and selective reaction of MeI with $Cys_{69}$ reveals unambiguously that this zinc-bound thiolate possesses enhanced nucleophilicity as compared to the three other thiolate ligands in Ada. What are the structural origins of these differences in reactivity toward $CH_3I$? We have noted previously that all four zinc-coordinated sulfur atoms in N-Ada appear to be free of hydrogen bond donation, an interaction that would suppress the intrinsically high reactivity of zinc-coordinated thiolates in other proteins.[20] It could also be envisioned that Ada precludes reaction at all sites other than $Cys_{69}$ through steric shielding; however, this seems unlikely because the structure reveals that $Cys_{72}$ is at least as accessible as $Cys_{69}$.[4] Thus, rather than deactivate either sterically or electronically the other ligand thiolates, Ada appears to maintain $Cys_{69}$ in a high state of electronic activation. Interestingly, $Cys_{69}$ is the only ligand residue in N-Ada that failed to exhibit resolved scalar coupling of its $\beta$-protons to $^{113}Cd$,[5] which suggests *S*-$Cys_{69}$ is not as tightly bonded to the metal. Although any explanation proposed for this kinetic lability of the metal–ligand bond will necessarily be speculative, inspection of the structure offers one suggestion: the main-chain amide N–H of $Gln_{73}$, which is in slow chemical exchange with solvent,[21] approaches to within ~3.2 Å of the thiolate S of $Cys_{69}$. Since this distance is too far to allow for simultaneous coordination of $Cys_{69}$ to zinc and hydrogen bonding to $Gln_{73}$-NH, these two electropositive centers may essentially compete for the interaction with the $Cys_{69}$ thiolate. Hence, the thiolate may shuttle between the two competing centers, and the naked thiolate that exists "in flight" might be highly susceptible to electrophilic attack. Whatever the factors that give rise to the accentuated reactivity of $Cys_{69}$, the observation that DNA phosphotriester repair by Ada is stereospecific implies that docking interactions with a DNA substrate can also play a role in the overall reaction.

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(19) Purified samples of the N-Ada17 protein and a 21-mer oligonucleotide bearing the *ada* promoter binding site were produced as previously described.[16] The complex was formed by adding equimolar amounts of N-Ada17 and the duplex 21-mer along with a 20-fold molar excess of [*methyl*-$^{13}C$]MeI (Cambridge Isotope Laboratories, Andover MA), diluted (20:1, v/v) in dimethyl sulfoxide, to a reaction buffer containing 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, and 10 mM DTT at 0 °C. The concentration of the protein and DNA in the reaction buffer was ~10 $\mu$M. The reaction was kept in the dark at all times and was slowly warmed to 37 °C over the period of 1 h. The reaction was allowed to continue at 37 °C for a period of 8–12 h. Ultrafiltration using a 10-kD 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 sodium phosphate, pH 6.4, 50 mM NaCl, 2 mM $^2H$-DTT (Isotec Inc., Miamisburg, OH)].

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