Chemical Communication across the Zinc Tetrathiolate Cluster in *Escherichia coli* Ada, a Metalloactivated DNA Repair Protein†

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Received May 16, 2001; Revised Manuscript Received July 20, 2001

ABSTRACT: The *Escherichia coli* Ada protein repairs methylphosphotriesters in DNA through direct, irreversible transfer to a cysteine residue on the protein, Cys 69. Methylation of Cys 69 increases the sequence-specific DNA-binding activity of Ada by 10^3-fold, enabling the methylated protein to activate transcription of a methylation-resistance regulon. The thiolate sulfur atom of Cys 69 is coordinated to a tightly bound zinc ion in the Ada N-terminal domain, and this metal−ligand interaction plays a direct role in promoting the DNA repair chemistry. Ada is thus the founding member of a mechanistic class of proteins that employ metalloactivated thiolates as nucleophiles, other examples of which include protein prenyltransferases and cobalamin-independent methionine synthase. Here we have probed the role of the three other Cys residues in Ada that together with Cys 69 coordinate the zinc through mutation to the alternative ligand residues Asp and His. All of the mutant proteins folded properly and bound zinc, but none of them exhibited measurable levels of DNA repair activity. Significantly, the Cys-to-His mutant proteins retained nearly wild-type sequence-specific DNA-binding activity in the unmethylated state. These findings demonstrate that the three "spectator" Cys ligands communicate chemically with Cys 69 through the bound metal ion.

DNA is subject to spontaneous attack by alkylating agents, leading to the formation of toxic, mutagenic, and carcinogenic adducts (*1*, *2*). Nearly all known organisms express proteins that confer resistance against alkylating agents by repairing the DNA damage such agents cause (*2*). In *Escherichia coli*, the Ada protein is a critical component of the so-called adaptive response, an inducible resistance system that counters the deleterious effects of methylation damage in DNA (*3*).

Ada is composed of two distinct domains: a 20 kDa N-terminal domain (N-Ada)[1] and a 19 kDa C-terminal domain (C-Ada) (Figure 1) (*4*, *5*). C-Ada repairs the mutagenic lesion O^6-methylguanine via direct transfer of the aberrant methyl group to a nucleophilic cysteine residue at position 321 (Cys 321) (*5*, *6*). N-Ada repairs the S_p diastereomer of DNA methylphosphotriesters (MeP) by direct transfer of the methyl group to a second nucleophilic cysteine residue, in this case located at position 69 (Cys 69) (*7*). Both of these methyl transfers to Ada are irreversible, as no known mechanism exists for regeneration of the original Cys residue at either position. Ada is therefore not an enzyme in the true sense but rather is a sacrificial reagent for DNA repair.

Methylation of Cys 69 confers on Ada the ability to bind certain sequences in DNA with high affinity and specificity, thus transforming the protein into an inducer that coordinately activates transcription of a methylation-resistance regulon (*8*−*10*). The unmethylated form of Ada does contain a weak DNA-binding activity, but this is insufficient to furnish significant levels of promoter activation, unless the protein is ectopically overexpressed. Methylation of Cys 69 increases the binding affinity of Ada for its own promoter by a thousandfold, thereby driving promoter occupancy and the ensuing activation of transcription (*11*).

The elements responsible for sequence-specific DNA binding by Ada are contained entirely within the N-terminal domain of the protein (*12*). Furthermore, N-Ada has been found to contain a single, tightly bound zinc ion that is essential for folding of the protein in vitro and in vivo. The solution structure of an amino-terminal fragment of the Ada protein competent for phosphotriester repair but not sequence-specific DNA binding (N-Ada10, Figure 1B) revealed that the zinc atom is tightly bound by four conserved cysteine ligands: Cys 38, Cys 42, Cys 72, and, remarkably, the active site nucleophile Cys 69 (*13*, *14*). These findings suggested that Ada uses zinc to activate the nucleophilicity of Cys 69, a proposal further strengthened by the results of metal substitution studies (*11*). Ada thus became the first example of what is now recognized as an important mechanistic class of proteins that use a metalloactivated cysteine nucleophile to carry out displacement reactions at carbon (*15*). Apart from Ada, zinc-mediated sulfur alkylation has been reported in

† This research is supported by a grant from the National Institutes of Health (GM 47467).

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[1] Abbreviations: N-Ada, the 20 kDa N-terminal domain of Ada (residues 1−178); N-Ada17, the 17 kDa N-terminal domain of Ada (residues 1−153); C-Ada, the 19 kDa C-terminal domain of Ada; MetH, cobalamin-dependent methionine synthase; MetE, cobalamin-independent methionine sythase; ICP-MS, inductively coupled plasma resonance mass spectrometry; HSQC, two-dimensional heteronuclear single-quantum coherence spectroscopy; MeP, methylphosphotriester; T-p(OMe)-T, dinucleoside methylphosphotriester dimer; K_d, dissociation constant; hCys, homocysteine; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol; PAC, phenoxyacetyl.

10.1021/bi011001m CCC: $20.00
Published on Web 08/28/2001

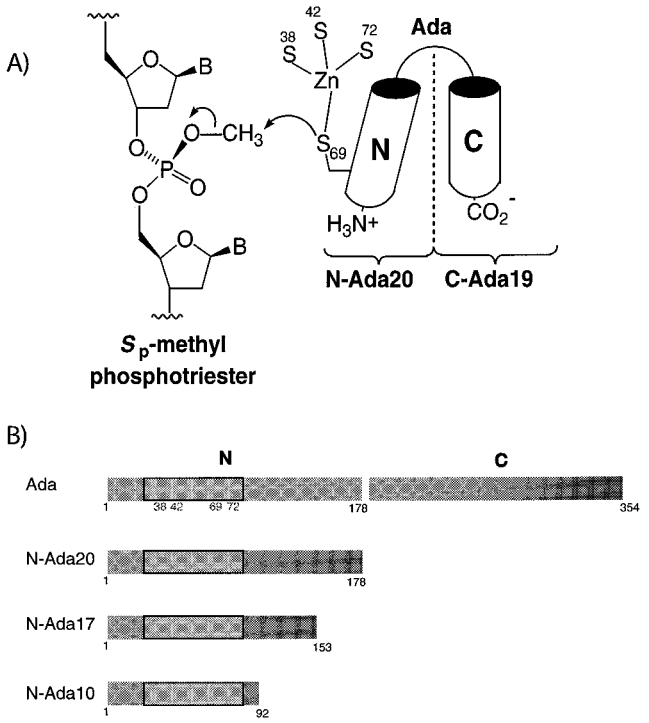

FIGURE 1: The Ada protein, its constituent domains, and the autocatalytic DNA-repair reaction of the amino-terminal domain. (A) Ada consists of two domains connected by a flexible linker. Proteolytic cleavage in the linker generates separate, active proteins comprising the 20 kDa N-terminal domain (N-Ada20) and the 19 kDa C-terminal domain (C-Ada19). The 20 kDa N-terminal domain (N-Ada20) repairs the S_p diastereomer of methylphosphotriesters in DNA through direct, irreversible transfer to Cys 69, which is coordinated to a tightly bound zinc ion in the protein. Methylation of Cys 69 increases the sequence-specific DNA-binding affinity of N-Ada20 10^3-fold, enabling the methylated protein to activate a methylation-resistance regulon. (B) Schematic representation of Ada and its fragments. The metal-binding domain is shaded in gray, with ligand residues numbered below. N-Ada20 and N-Ada17 possess identical DNA-repair and DNA-binding properties. N-Ada10, which comprises residues 1−92, retains MeP repair and metal-binding activity but lacks the ability to bind DNA sequence-specifically.

the protein-prenylation enzymes farnesyltransferase and geranylgeranyltransferanse (*16*, *17*), cobalamin-dependent (MetH) and colbalamin-independent methionine synthase (MetE) (*15*, *18*, *19*), and certain epoxide thiol-conjugating enzymes (*20*). A major point of general interest in such systems concerns the chemical mechanism by which zinc activates the thiol without rendering it susceptible to attack by certain ubiquitous intracellular electrophiles such as oxidized glutathione.

Just as with DNA methylphosphotriesters, the simple chemical methylating agent methyl iodide (CH_3I) exhibits a high degree of selectivity to methylate Cys 69 in Ada, even in the absence of DNA (*21*), thus explaining the ability of CH_3I to induce the *ada* regulon in *E. coli* under conditions in which the extent of DNA alkylation is negligible (*22*). These results indicate that, of the four Cys residues in Ada, Cys 69 is the most chemically reactive. NMR structural analysis of N-Ada10 has revealed that Cys 69 and Cys 72 are roughly equal in accessibility, suggesting that the greater reactivity of Cys 69 is the result of electronic rather than steric factors. Unexpectedly, the sulfur atom of Cys 69 remains coordinated to zinc even after it has been methylated, perhaps the only known example of zinc coordination by a thioether ligand in a native protein (*23*).

A model study on Ada thiol alkylation systems revealed that tetrathiolate coordination to zinc is important for enhancing the reactivity of the ligands toward alkylation (*24*, *25*). Namely, each serial replacement of a thiolate ligand with the nitrogen-based ligand methylimidazole, a histidine analogue, was found to decrease the reactivity of the thiolates by several orders of magnitude.

To address the influence of the nonnucleophilic ligands in Ada on the reactivity of Cys 69, we individually mutated the three structural coordination ligands of the zinc ion, Cys 38, Cys 42, and Cys 72, to either histidine or aspartate, other residues known to coordinate zinc. The methylation repair potential of the mutant proteins was measured and compared to that of the wild-type Ada protein. All of the mutations diminished the repair activity of the protein to below detectable levels. The three histidine mutant proteins retained the ability to bind *ada* promoter DNA in the unmethylated state, while the aspartate mutants exhibited significantly reduced DNA-binding activity. These findings provide evidence for electronic communication of the nonnucleophilic residues in Ada through the metal cluster to Cys 69.

MATERIALS AND METHODS

Plasmid Construction. Ada17 bacterial expression plasmid was constructed by cloning a fragment of the N-terminal Ada gene (1−153) into the *Eco*RI and *Hin*dIII sites of the bacterial expression vector pLM1 (*26*). All mutants of the Ada17 were prepared by megaprimer mutagenesis using *pfu* polymerase (Stratagene). All mutations were confirmed by dideoxynucleotide DNA sequencing.

Expression and Purification of Ada17 Proteins. Recombinant Ada17 proteins were expressed in *E. coli* BL21(DE3) cells (Stratagene). Uniformly ^{15}N-labeled proteins were expressed in M9 minimal media with $^{15}NH_4Cl$ as the sole nitrogen source. Protein expression was induced with 1 mM IPTG, and the cells were harvested after a 4 h induction at 37 °C. The proteins were purified to homogeneity under native conditions as described (*12*). Ada17 protein samples for NMR measurements were prepared by dialyzing the purified proteins into a buffer containing 25 mM sodium phosphate, 50 mM NaCl, and 5 mM deuterated DTT at pH 6.5 (NMR buffer) with a final protein concentration of 0.5 mM.

The methylated Ada17−DNA complex was formed by adding equimolar amounts of Ada17 and a duplex 18mer oligonucleotide (5′-GCAAATTAAAGCGCAAGA-3′, 3′-CGTTTAATTTCGCGTTCT-5′) along with a 20-fold excess of $^{13}CH_3I$ (Cambridge Isotope Laboratories) in a buffer containing 20 mM Tris-HCl, 50 mM NaCl, and 10 mM DTT at pH 7.4. The mixture was incubated first on ice for 5 min before being slowly warmed to room temperature and further incubated at room temperature overnight. The protein−DNA cocomplex was exchanged into NMR buffer using Centricon filters (Amicon).

NMR Spectroscopy. ^{15}N−^{1}H heteronuclear single-quantum coherence (HSQC) spectroscopy and ^{13}C−^{1}H heteronuclear single-quantum coherence (HSQC) spectroscopy experiments were carried out at 25 °C on a Varian INOVA 600 MHz spectrometer equipped with a triple resonance probe.

Phosphotriester Single-Stranded Substrate Repair Assay. Protein (2 nmol) was incubated with an equimolar amount of T_{11}OMe (*11*) in 20 μL of buffer containing 20 mM Tris-HCl, 50 mM NaCl, and 10 mM DTT at pH 7.4. The incubation time is typically around 2 h at 25 °C. Overnight and longer incubation periods were conducted for the mutant proteins. The reactions were terminated by diluting the reaction mixture with the reaction buffer to 200 μL and loaded onto a MonoQ HR 5/5 column (Amersham Pharmacia) preequilibrated with buffer A (20 mM Tris-HCl, 10 mM β-mercaptoethanol, pH 7.5). At a flow rate of 1 mL/min, a linear gradient from 0% to 60% buffer B (20 mM Tris-HCl, 1 M NaCl, 10 mM β-mercaptoethanol, pH 7.5) was used. T_{11}OMe eluted at 210 mM NaCl concentration and T_{11} at 220 mM.

Phosphotriester Double-Stranded Substrate Repair Assay. A duplex 16mer containing one single phosphotriester [T(OMe)]

5'-CCCACCT(OMe)TAACCTCCC-3'

3'-GGGTGGA ATTGGAGGG-5'

was synthesized by standard methods using PAC-protected 5′-dimethoxytrityl-3′-β-cyanoethylphosphoramidite and 5′-dimethyoxytritylthymidine 3′-methylphosphoramidite (Glen Research). The resin-bound oligonucleotides were deprotected with 0.05 M K_2CO_3 in anhydrous methanol at room temperature for 12 h. The individual strands were purified by 20% denaturing polyacrylamide gel electrophoresis. The duplex was formed by heating the combined strands to 90 °C for 3 min, followed by slow cooling to room temperature.

A reaction mixture of 50 μL containing 2.5 nmol of the duplex, 0.1 unit of snake venom phosphodiesterase (Sigma), 75 units of Benzonase (EM Merck), 300 mM Tris-HCl, 20 mM $MgCl_2$, and 0.05 mM $ZnCl_2$ at pH 7.4 was prepared, and equimolar amounts of protein solution were added to the reaction mixture and allowed to incubate at 25 °C for 1 h. One unit of alkaline phosphatase was added, and the mixture was incubated for an additional 1 h. The reaction mixture was filtered with a 0.22 μm filter and then analyzed by reverse-phase high-performance liquid chromatography on a Hewlett-Packard 1090 diode array system. A linear gradient from 100% buffer A (20 mM KH_2PO_4, pH 6.0) to 70% buffer B (100% methanol) was used to separate the digested nucleosides and the T(OMe)T dimer. Authentic standards were used for comparison of UV spectrum and retention times. Blank runs to which the duplex oligonucleotide was omitted served as a control.

Gel Electrophoretic Mobility Shift Assays. For analyzing the DNA binding properties of the unmethylated proteins, Ada17 proteins were incubated at room temperature with an 18mer ^{32}P-labeled duplex oligonucleotide (5′-GCAAATTAAAGCGCAAGA-3′, 3′-CGTTTAATTTCGCGTTCT-5′) in a reaction buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM DTT, and 10% glycerol for 45 min.

For analyzing the DNA binding properties of the *S*-Me-Cys 69 proteins, the unmethylated proteins were added to a mixture of the 18mer ^{32}P-labeled duplex oligonucleotide and the T_{11}OMe substrate (10^4 excess over protein), and the mixture was incubated at room temperature for 2 h.

For analysis of specific and nonspecific competitor duplex DNA, varying amounts of competitor DNA (1−50-fold excess over 18mer duplex) were added to the mixture of Ada17 protein and the 18mer duplex DNA.

For all proteins tested, a total volume of 20 μL of reaction mixture was loaded on a 6% native polyacrylamide gel and electrophoresed for 1 h. Gels were exposed to a phosphoimaging plate and analyzed by a Fuji Bio-Image analyzer.

Quantitation of Zn in Ada. Zinc content was determined by the method of high-resolution inductively coupled plasma mass spectrometry (ICP-MS) (*27*).

NMR Quantitative Titration of the Phosphotriester Duplex and Ada Proteins. ^{15}N-Labeled wild-type Ada17 was purified and exchanged into the NMR buffer. Varying amounts of the 16mer duplex containing one phosphotriester were added to the protein solution at 0 °C, and the mixture was slowly warmed to room temperature over the course of 2 h. ^{15}N−^{1}H heteronuclear single-quantum coherence (HSQC) spectra were taken for the cocomplexes, and the ratios of protein to DNA achieved were 3:1, 2:1, 1:1, 1:2, and 1:3. One additional control spectrum with no DNA added was also taken.

Table 1: Zinc Content of Wild-Type and Mutant N-Ada17 Proteins

proteins[a]	relative Zn^{2+} content (mol %)[b]	standard error (mol %)
wild type	100	5
C38H	129	6
C42H	112	5
C69H	139	5
C72H	126	5
C38D	105	5
C42D	92	5
C72D	111	5

[a] All proteins were prepared from cells grown in LB media supplemented with 0.1 mM $ZnCl_2$ during protein overexpression. Protein concentrations were determined from Bradford protein quantification assays. [b] Percentage values reported for moles of zinc per mole of protein assuming the wild-type protein carries one eqivalent zinc ion.

RESULTS

Cloning, Expression, and Purification of N-Ada17 Mutants. An N-terminal fragment of Ada comprising residues 1−153, hereafter referred to as N-Ada17 (Figure 1B), has been found to possess metal-binding and methylation-dependent DNA recognition properties indistinguishable from those of the full-length protein (*11*). Starting with an N-Ada17 overexpression plasmid (*26*), all of the metal-coordinating residues were mutated individually to His, and all except the nucleophilic Cys 69 were also mutated to Asp. All of these mutant proteins were overexpressed to similar levels as wild-type N-Ada17 upon induction in *E. coli* (data not shown). Furthermore, all of the mutant proteins were present in the soluble fraction upon cell lysis and showed chromatographic behavior similar to that of wild-type N-Ada17 (*12*).

N-Ada17 Mutants Contain One Zinc Ion per Protein Molecule. To test whether the mutant proteins retain the ability to bind zinc, high-resolution inductively coupled plasma resonance mass spectrometry (ICP-MS) (*27*) was performed on samples of the mutant protein and on wild type N-Ada17. These ICP-MS data indicate that the mutant proteins, like the wild type, all contain approximately one zinc per molecule of protein (Table 1).

Structural Similarity of Mutant Proteins to the Wild Type. The overall structural integrity of the mutant proteins was evaluated using multidimensional NMR. Uniformly ^{15}N-labeled proteins were prepared by overexpressing the N-Ada17 proteins in M9 minimal media with $^{15}NH_4Cl$ as the sole nitrogen source. Two-dimensional $^{15}N-^{1}H$ heteronuclear single quantum coherence spectra (HSQC) were measured for the mutant proteins and compared to the wild-type protein. All of the mutant proteins exhibited HSQC spectra that were nearly identical to that of wild-type N-Ada17 (data not shown). The few observable chemical shift perturbations arose from amide protons of the four zinc ligands or their nearest neighbors (L. J. Sun and G. L. Verdine, unpublished results). On the basis of the close similarities of the physical, spectroscopic, and metal-binding properties of the mutant proteins to those of wild-type N-Ada17, we conclude that mutation of Cys 38, Cys 42, or Cys 72 to His or Asp has negligible effect on global protein structure. The present results also confirm previous findings that mutation of Cys 69 to His has no adverse effect on the structure of the N-terminal domain (*28*).

Mutant N-Ada17 Proteins Do Not Repair Single-Stranded DNA Substrates. To evaluate the methylphosphotriester (MeP) repair activity of the mutant proteins, we employed T_{11}(OMe), a single-stranded thymidine homopolymer containing a single, centrally located MeP, as the repair substrate (*11*). The N-Ada17 proteins were incubated with T_{11}(OMe) at room temperature for 2−48 h, and the products were analyzed by anion-exchange FPLC (Figure 2). In the absence of added protein, two peaks were evident (upper left-hand panel); the major (earlier eluting) arising from a mixture of the S_p and R_p diastereomers of T_{11}(OMe), whereas the minor (later eluting) peak corresponds to T_{11} derived from hydrolysis during ammonia deprotection of T_{11}(OMe) (*23*). After reaction of the phosphotriester substrate with excess wild-type N-Ada17 protein for 1 h, a pronounced shift of peak volume from T_{11}(OMe) to T_{11} was evident, owing to complete repair of the S_p-configurated T_{11}(OMe) to T_{11} and no repair of R_p-configurated T_{11}(OMe). By contrast, none of the mutant N-Ada17 proteins showed any ability to repair T_{11}(OMe), even after a 48 h incubation.

Mutant N-Ada17 Proteins Do Not Repair Methylphosphotriesters in Double-Stranded DNA. We reasoned that the complete lack of phosphotriester repair by the mutant proteins might be due in part to the use of a suboptimal substrate, the single-stranded homopolymer T_{11}(OMe). To evaluate the methylphosphotriester repair activity of the Ada proteins on double-stranded heteropolymeric substrate, we adapted an HPLC-based assay for nucleoside composition (*11*) to the present purpose. First, we synthesized a mixed-sequence DNA containing a single, centrally located methylphosphotriester via solid-phase DNA phosphoramidite coupling chemistry, followed by deprotection using anhydrous 0.05 M K_2CO_3 in methanol at room temperature. This strand was purified by HPLC and annealed to the complementary strand to give a double-stranded 16mer phosphotriester substrate. Following incubation of this duplex with the Ada proteins, the DNA product mixture was digested down to its constituent nucleosides using snake venom phosphodiesterase, Benzonase, and alkaline phosphatase. Whereas the normal phosphodiester and monoester linkages are hydrolyzed to completion under these conditions, both the S_p and R_p methylphosphotriester linkages are completely refractory to cleavage, yielding an undigested dinucleoside methylphosphotriester dimer, T-p(OMe)-T. The four normal deoxynucleosides separate readily from T-p(OMe)-T by reversed-phase HPLC (Figure 3, upper right-hand corner). Moreover, the S_p and R_p diastereomers of T-p(OMe)-T also separate from each other under these chromatographic conditions, making it possible to observe directly the stereochemical course of the repair reaction (see expansion below). In the absence of N-Ada17 protein, an equal amount of the R_p and S_p diastereomers was observed (Figure 3, expansion, top trace). Upon treatment with wild-type N-Ada17 protein for 1 h, only the peak corresponding to the S_p diastereomer disappeared, again due to the complete repair of the S_p methylphosphotriester by the wild-type protein. On the other hand, when the mutant proteins were treated with the double-stranded phosphotriester DNA substrate for 48 h, no statically significant alteration of the S_p/R_p ratio was observed in any

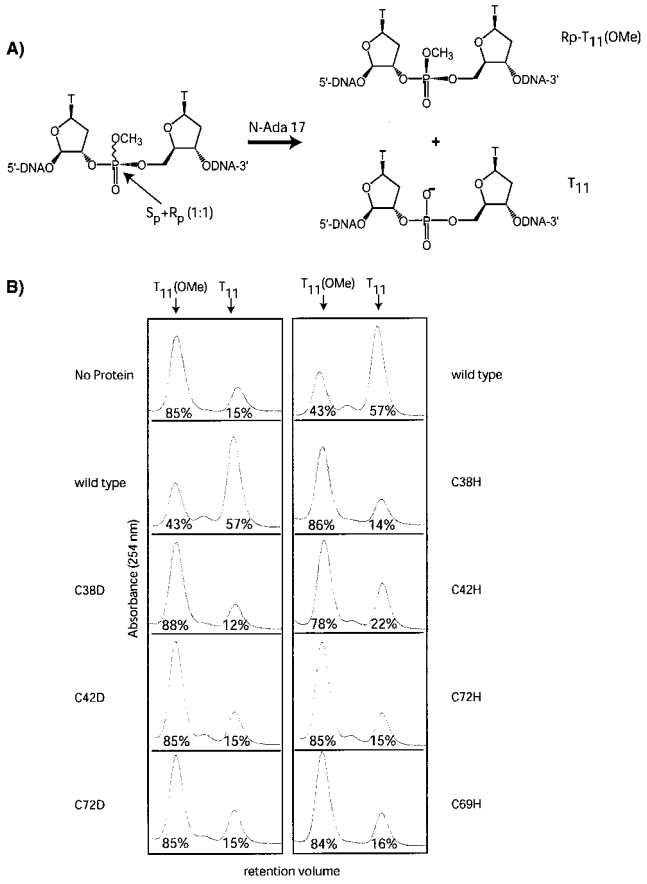

FIGURE 2: Assay for repair of methylphosphotriesters in the single-stranded substrate T_{11}(OMe). (A) Overall repair reaction. The T_{11}-(OMe) substrate exists as a roughly equal mixture of the S_p and R_p diastereomers at the chiral phosphorus atom of the methylphosphotriester. N-Ada17 is capable of repairing only the S_p diastereomer, converting it stoichiometrically to the unmethylated homopolymer T_{11}. The R_p diastereomer is unaffected by treatment with Ada. (B) FPLC elution profiles monitoring the progress of DNA repair by N-Ada17. Anion-exchange FPLC separates T_{11} from the diastereomeric mixture of phosphotriesters but does not separate the S_p and R_p diastereomers of T_{11}(OMe). As is evident in the top left panel, T_{11}(OMe) contains ~15% T_{11}, as a byproduct of the synthesis. Treatment with wild-type N-Ada17 causes a decrease in the T_{11}(OMe) peak and a proportionate increase in the T_{11} peak (top right panel). The ratio of peak areas is unaffected by the mutant proteins, indicating a lack of phosphotriester repair activity.

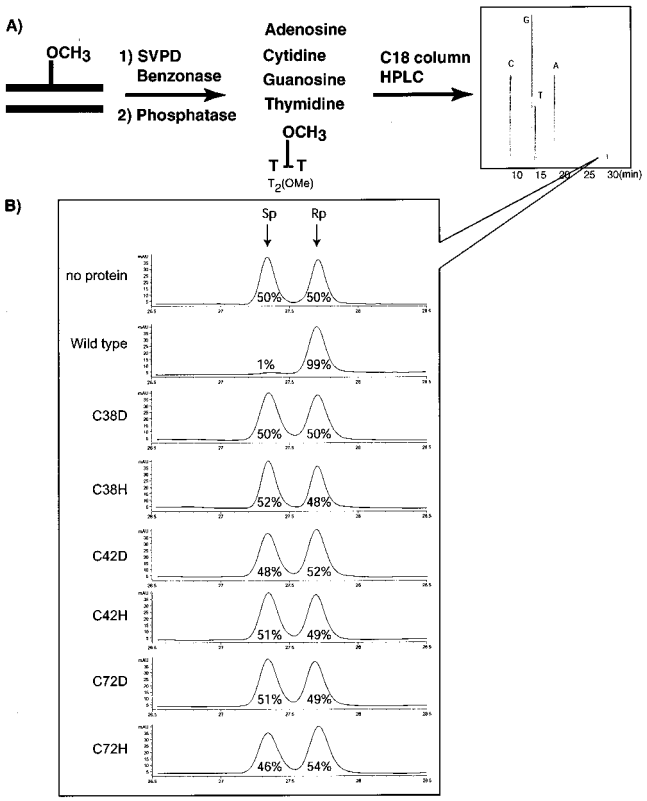

FIGURE 3: Repair of methylphosphotriesters in mixed-sequence duplex DNA by wild-type versus mutant N-Ada17 proteins. (A) Scheme for duplex DNA repair by Ada proteins. A duplex 16mer DNA containing one central backbone methylphosphotriester was incubated with Ada proteins and then subjected to exhaustive digestion by snake venom phosphodiesterase (SVPD), Benzonase, and alkaline phosphatase to obtain individual nucleosides plus the phosphotriester-linked dinucleotide T_2(OMe), which is completely resistant to further digestion. The mixture of nucleosides was analyzed by high-performance liquid chromatography (HPLC) using a reversed-phase C18 column. The chromatograms separate the four normal deoxynucleosides from the later eluting S_p and R_p diastereomers of T_2(OMe). (B) Expanded views of the HPLC elution profiles of nucleotide mixtures resulting from DNA digestion, in which the separate peaks for the S_p T_2(OMe) and R_p T_2(OMe) are clearly visible. In the top panel, it can be seen that the two diastereomers are present in equal amounts in T_{11}(OMe). Treatment with wild-type N-Ada17 results in specific loss of the peak corresponding to S_p T_2(OMe). The diastereomer ratio is unaffected by exposure of the DNA substrate to the mutant proteins, indicating a lack of DNA repair activity.

case, thus indicating that the mutant proteins are virtually devoid of repair activity (Figure 3).

Mutant N-Ada17 Proteins Bind DNA Sequence-Specifically but with Lower Affinity. The unmethylated form of wild-type N-Ada is able to bind the *E. coli ada* promoter specifically, with an equilibrium dissociation constant (K_d) of ~120 nM (*11*). This sequence-specific DNA binding of the unmethylated protein provides an independent test of activity for those mutant forms that are unable to repair DNA. Using an 18mer duplex DNA containing the core Ada recognition site from *ada* promoter, we performed electrophoretic mobility shift assays to test the sequence-specific DNA binding activity of the mutant proteins. The Cys-to-His mutant proteins C38H, C42H, and C72H bound the *ada* promoter DNA only 2−4-fold more weakly than did the wild-type protein (Figure 4A). However, the Cys-to-Asp mutants C38D, C42D, and C72D showed greatly diminished

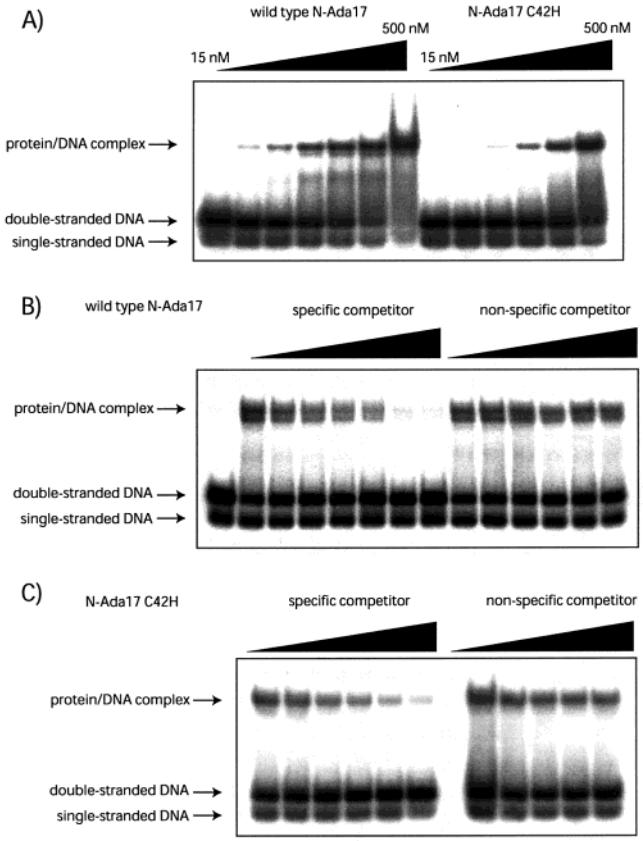

FIGURE 4: Binding of N-Ada17 proteins to oligonucleotides containing the consensus Ada binding sequence. (A) Gel mobility shift assay of N-Ada17 wild-type protein and N-Ada17 C42H mutant binding to an 18mer double-stranded duplex containing the consensus Ada binding sequence in the absence of any methylating agent. The protein concentration ranges of both the wild-type and the mutant proteins were varied from 15 to 500 nM. (B) Competition assay of specific and nonspecific duplex oligonucleotides binding to the wild-type N-Ada17 protein. The concentrations of unlabeled competitor duplex DNAs were varied from a 1.5- to 50-fold excess of ^{32}P-labeled DNA. The specific competitor DNA has the same sequence as the labeled DNA. The nonspecific competitor DNA is a sequence that does not bind Ada proteins specifically. (C) Competition assay of specific and nonspecific duplex oligonucleotides binding to N-Ada17 C42H protein. The conditions are the same as in (B).

DNA-binding activity, with dissociation constants in the range consistent with nonspecific binding (>2 μM) (data not shown). To further assess the specificity of the DNA interaction, the protein/DNA complexes were challenged with increasing amounts of either a specific or nonspecific unlabeled competitor oligonucleotide. With both wild-type N-Ada17 and the C38H, C42H, and C72H mutants, stoichiometric competition was observed for the specific competitor, while the nonspecific competitor had no effect (Figure 4B,C), consistent with their being bound to DNA specifically. The Cys to Asp mutants were not tested in this competition assay, owing to their weak affinity for DNA.

Mutant N-Ada17 Proteins Do Not Exhibit Methylation-Dependent Enhancement of DNA-Binding Affinity. The affinity of N-Ada is enhanced ~10^3-fold upon methylation of Cys 69 (*11*). We therefore tested the mutant proteins for enhancement of DNA-binding affinity in the presence of a methyl-donor DNA substrate, T_{11}(OMe). For these assays, we used an 18mer duplex DNA containing the *ada* operator as the specific DNA sequence (*29*). As shown in Figure 5A,

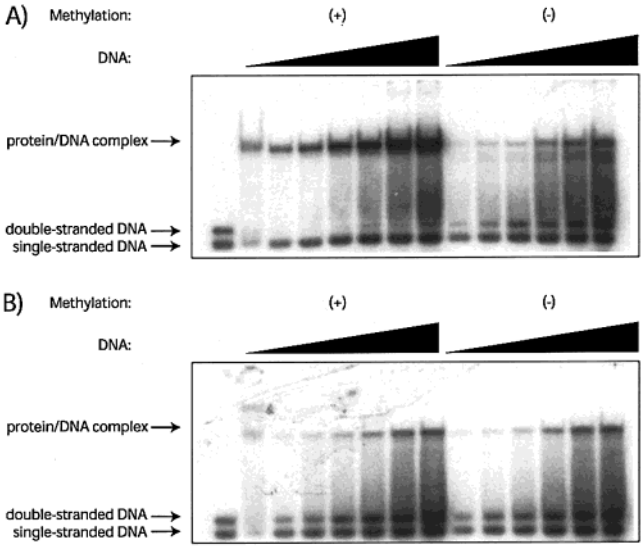

FIGURE 5: Native gel electrophoresis assays for the binding of N-Ada17 proteins upon treatment with the methylating agent T_{11}-(OMe). (A) Gel mobility shift assay of wild-type N-Ada17 protein in the presence of T_{11}(OMe) as the methylation substrate (left panel) and in the absence of methylation (right panel). An 18mer DNA containing the Ada consensus binding site was used, and the concentration was varied from 100 pM to 6 nM. The protein concentration was kept at a constant 100 nM. (B) Gel mobility shift assay of N-Ada17 C42H protein in the presence of T_{11}(OMe) as the methylation substrate (left panel) and in the absence of methylation (right panel).

the presence of T_{11}(OMe) caused the expected increase in the affinity of wild-type N-Ada17 for DNA. On the other hand, the C42H mutant protein exhibited no detectable enhancement of DNA binding when treated with T_{11}(OMe) (Figure 5B). This behavior is consistent with the results above showing that the C42H mutant protein fails to undergo methylation by T_{11}(OMe).

Mutant N-Ada17 Proteins Are Unreactive toward Methyl Iodide. With wild-type Ada, Cys 69 can be regiospecifically methylated by methyl iodide, a reaction that can be conveniently monitored by isotope-edited NMR using $^{13}CH_3I$. These assays are performed in the presence of a specific duplex oligonucleotide, which stabilizes the Cys 69-methylated form of the protein. The 2D $^1H-^{13}C$ heteronuclear single-quantum coherence (HSQC) spectrum of wild-type N-Ada17 methylated with $^{13}CH_3I$ showed a predominant cross-peak at 2.72 ppm (^1H)/ 21.2 ppm (^{13}C), which as reported previously (*21*) is due to the Cys 69-S-$^{13}CH_3$ group in the methylated protein/DNA complex (Figure 6A). The corresponding spectrum obtained with the C42H Ada/DNA complex showed no detectable cross-peak in this region of the spectrum, thus indicating that the mutant protein is refractory to methylation by even the potent methyl donor $^{13}CH_3I$ (Figure 6B).

DISCUSSION

Ada is the prototypical example of a protein that uses metal coordination to activate the nucleophilicity of a Cys residue toward alkylation. Previous studies have established the indispensability of the metal ion and of Cys 69 for DNA repair activity. Specifically, removal of the divalent metal ion or mutation of Cys 69 to His or Asp results in a complete loss of DNA phosphotriester repair activity (*28*, *30*).

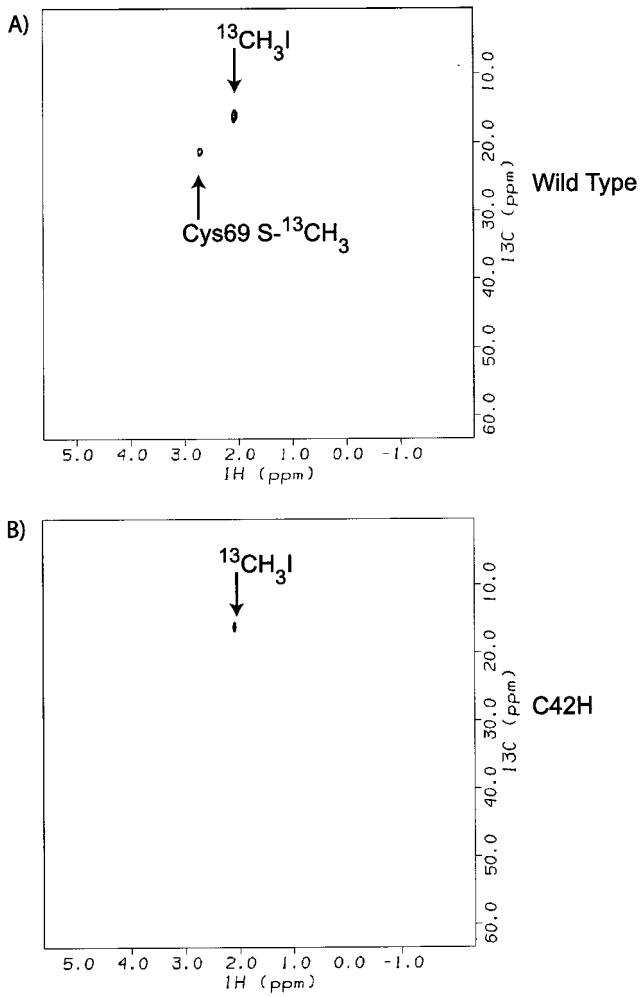

FIGURE 6: NMR spectra from a 2D $^1H-^{13}C$ HSQC experiment on (A) a Cys 69-S-$^{13}CH_3$-N-Ada17/DNA cocomplex and (B) an N-Ada17 (C42H)/DNA cocomplex formed using [*methyl*-^{13}C]MeI as the methylating agent. The major cross-peak arising from the Cys 69-S-$^{13}CH_3$ is denoted in (A). The only other peak in the spectrum corresponds to [*methyl*-^{13}C]MeI, which is present due to incomplete buffer exchange. No cross-peak for Cys 69-S-$^{13}CH_3$ is seen in (B), indicating a lack of methylation by methyl iodide under these conditions.

Furthermore, replacement of Zn^{2+} with Cd^{2+} causes a 4-fold decrease in the rate of DNA repair, whereas replacement with Hg^{2+} abolishes repair activity (K. Wetterhahn and G. L. Verdine, unpublished results). Although these results clearly establish the importance of the interaction between Cys 69 and Zn^{2+}, they leave open the question of whether the other three Cys ligands that share the Zn^{2+} with Cys 69 have any influence on metalloactivated DNA repair. Here we have examined the role of the three "spectator" ligands in Ada through mutation to alternative metal-coordinating residues and characterization of the biochemical properties of the resulting Ada variants. We find that these mutant proteins all fold into a nativelike structure with zinc bound, and some of them even retain the ability to bind DNA sequence-specifically, but none possess detectable levels of phosphotriester repair activity. Thus, Ada depends critically upon the presence of a $(Cys)_4$ metal-coordination sphere to execute DNA repair through sacrificial methyl transfer to Cys 69. Consistent with these results, the $(Cys)_4$ cluster is completely conserved in all N-Ada homologues identified to date (*44*), including the *Bacillus subtilis* AdaA protein,

which shares only 46% amino acid identity with *E. coli* N-Ada (*32*).

Why does ligand replacement have such a pronounced effect on metalloactivated methyl transfer to Ada? The trivial possibility that these mutations cause some dramatic effect on the structure of Ada is ruled out by our observations that all of the mutant proteins bind 1 equiv of Zn^{2+}, and the mono-His mutants even retain the majority of their sequence-specific DNA-binding activity. Furthermore, the HSQC spectra of all the mutant proteins are nearly identical to those of the wild-type protein, with the exception of a few residues comprising the ligand cluster and its neighbors, thus ruling out any gross alteration of backbone structure. Nevertheless, the possibility remains that local adjustments in the ligand cluster lead to an unproductive geometry for methyl transfer. Equally likely, we believe, is the possibility that the spectator ligands communicate electronically or sterically through the metal to Cys 69, and perturbation of this coupling decreases the reactivity of Cys 69. The precise reasons for the activation of Cys 69 by Zn^{2+} in Ada are not well understood, but the observation that CH_3I regioselectively alkylates Cys 69 in overwhelming preference to the equally exposed spectator ligand Cys 72 strongly suggests that electronic factors play an important role in accentuating the reactivity of Cys 69 uniquely. This hypothesis is strengthened by the fact that the β-protons of Cys 69 are not scalar-coupled to the metal in the NMR spectra of $^{113}Cd^{2+}$-Ada, whereas those of Cys 38, Cys 42, and Cys 72 are coupled (L. C. Myers, G. Wagner, and G. L. Verdine, unpublished results) (*31*). Weak coordination of Cys 69 to the metal may also explain the unusually low chemical shift of ^{113}Cd in Ada, as compared with reference Cys_4-coordinated metalloproteins in which the metal plays a purely structural role (*23*, *28*). One attractive explanation would be that Ada binds the metal in a distorted tetrahedral geometry, perhaps closer to a truncated trigonal bipyramid, with Cys 69 adopting an apical position. This would focus the electrostatic repulsion from the other three thiolate ligands on Cys 69, thus weakening its bond to the cationic metal. Were this the case, then substitution of the spectator ligands to His would decrease the net charge on the ligand sphere, increasing the electron demand of the metal center, consequently strengthening the metal−Cys 69 bond. Although Cys-to-Asp mutations would cause no change in the overall formal charge on the ligand sphere, the charge on Asp is more delocalized than that on Cys, and Asp is invariably a weaker Zn^{2+} ligand than Cys; hence, it too is a weaker electron donor than Cys. It is perhaps equally likely that the steric adjustments about the metal center caused by mutating ligand residues perturb the interaction of the Zn^{2+} with Cys 69. At this stage, it is not possible to discriminate among the various alternative explanations for the nucleophilicity-suppressing effect of mutating spectator ligands, in part owing to the lack of structural data at a sufficiently high resolution to discern subtle aspects of ligand coordination in Ada.

The loss of thiolate nucleophilicity upon replacement of spectator thiolate ligands seen here with Ada has also been observed in small-molecule zinc-coordination complexes (*24*, *25*). Specifically, a tetra(benzenethiolate)−Zn^{2+} complex underwent rapid methyl transfer when treated with trimethyl phosphate, but the rate constant for the S-alkylation reaction was diminished 10-fold upon replacement of one benzenethiolate ligand with *N*-methylimidazole (analogous to Cys_3-His) and 1000-fold upon replacement of two benzenethiolate ligands with *N*-methylimidazoles (analogous to Cys_2His_2). The overall trends of reactivity changes are similar in Ada and in the small-molecule complexes, however, the protein shows a more pronounced response to ligand replacement. In principle, the ablation of Ada phosphotriester repair activity caused by ligand replacement might result from some disturbance of noncovalent docking interactions with its DNA substrate, but this seems unlikely in light of the fact that even CH_3I fails to alkylate these mutant proteins efficiently, and at least the His-spectator mutants retain nearly all of their sequence-specific DNA-binding activity. Thus the differences in sensitivity of Ada versus small molecule coordination complexes appear to be due to the chemistry of the metal−ligand clusters. For example, the electronic properties of the alkylthiolate ligands in Ada are not expected to be identical to those of the arylthiolates in the models. Alternatively, the more constrained steric environment of the protein may be less adaptable to changes in ligand structure than the relatively open environment of four unconnected small-molecule ligands.

It is interesting to note that although a $(Cys)_4$ coordination cluster is required in *E. coli* Ada and is invariant in all known Ada orthologues, not all proteins that carry out zinc-activated alkyl transfer reactions possess this coordination scheme. For example, farnesyltransferases utilize $(Cys)_2$HisAsp coordination, with the reactive Cys being donated by the prenylation substrate (*33*, *34*). Geranylygeranyltransferases are also believed to use $(Cys)_2$HisAsp coordination, again with one Cys coming from the substrate (*35*). Mutation of either the His or Asp zinc-binding ligand results in marked reduction of zinc affinity and enzymatic activity (*36*). Cobalamin-independent methionine synthetases are believed to use $(Cys)_2$HishCys, where hCys is the substrate homocysteine; interestingly, this ligand sphere is isoelectronic with our inactive $(Cys)_3$His Ada mutant Ada proteins (*37*, *38*). Betaine−homocysteine methyltransferases and colbalamin-dependent methionine synthase use $(Cys)_3$hCys (*38*−*40*). Zinc coordination is thought to lower the pK_a of thiol substrates through a Lewis acid effect. Interestingly, the zinc sites in both cobalamin-independent and cobalamin-dependent methionine synthase exhibit the same Lewis acidity toward hCys, despite the fact that the zinc coordination spheres of the two enzymes differ by the substitution of a Cys (latter) for His (former) (*41*). In these systems, it is believed that small differences in the metal−ligand bond lengths compensate for the differences in electronic character of the ligands, analogous to the valence buffering seen in cytidine deaminases (*42*). It is thus clear that different metalloactivated thiol-alkylating proteins have evolved unique strategies for communicating the ligand-coordination environment to the reactive thiol moiety. This may reflect in part differences in the transition state electronic demands of the reacting thiolate nucleophile and alkylating electrophile, especially with regard to the extent of the associative (SN_2-like) versus dissociative (SN_1-like) character of the overall reaction. The Ada methylation reaction involves a very weakly electrophilic substrate and thus depends strongly on the nucleophilicity of Cys 69; prenyltransferases employ a more electrophilic alkyl donor and therefore are not as dependent on the electron richness of the Cys thiolate.

In addition to the mechanistic insights into metalloactivated alkylation provided by the present studies, this work suggests that it might be possible to obtain a stable recognition complex formed between an alkylation-defective mutant of Ada and duplex DNA containing a phosphotriester. Although we were unable to detect the formation of such a complex using gel-shift assays, methods have been developed in our laboratory for trapping such weak recognition complexes (*43*), and high-resolution structures have resulted from such efforts. Preliminary results indicate the feasibility of trapping a recognition-competent, repair-deficient complex of Ada bound over a DNA phosphotriester (C. He, L. J. Sun, and G. L. Verdine, unpublished results).

ACKNOWLEDGMENT

We thank Dr. Pei Zhou for help with the NMR instrumentation, Drs. Chuan He and Steven Bruner for critical reading of the manuscript, and members of the Verdine laboratory for discussions.

REFERENCES

1. Karran, P., Hjelmgren, T., and Lindahl, T. (1982) *Nature 296*, 770−773.
2. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA Repair and Mutagenesis*, ASM Press, Herndon, VA.
3. Lindahl, T., Sedgwick, B., Sekiguchi, M., and Nakabeppu, Y. (1988) *Annu. Rev. Biochem. 57*, 133−157.
4. Demple, B., Sedgwick, B., Robins, P., Totty, N., Waterfield, M. D., and Lindahl, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2688−2692.
5. Teo, I., Sedgwick, B., Demple, B., Li, B., and Lindahl, T. (1984) *EMBO J. 3*, 2151−2157.
6. Nakabeppu, Y., Kondo, H., Kawabata, S., Iwanaga, S., and Sekiguchi, M. (1985) *J. Biol. Chem. 260*, 7281−7288.
7. Sedgwick, B., Robins, P., Totty, N., and Lindahl, T. (1988) *J. Biol. Chem. 263*, 4430−4433.
8. Teo, I., Sedgwick, B., Kilpatrick, M. W., McCarthy, T. V., and Lindahl, T. (1986) *Cell 45*, 315−324.
9. Sakumi, K., and Sekiguchi, M. (1989) *J. Mol. Biol. 205*, 373−385.
10. Akimaru, H., Sakumi, K., Yoshikai, T., Anai, M., and Sekiguchi, M. (1990) *J. Mol. Biol. 216*, 261−273.
11. Myers, L. C., Jackow, F., and Verdine, G. L. (1995) *J. Biol. Chem. 270*, 6664−6670.
12. Myers, L. C., Terranova, M. P., Nash, H. M., Markus, M. A., and Verdine, G. L. (1992) *Biochemistry 31*, 4541−4547.
13. Myers, L. C., Verdine, G. L., and Wagner, G. (1993) *Biochemistry 32*, 14089−14094.
14. Habazettl, J., Myers, L. C., Yuan, F., Verdine, G. L., and Wagner, G. (1996) *Biochemistry 35*, 9335−9348.
15. Matthews, R. G., and Goulding, C. W. (1997) *Curr. Opin. Chem. Biol. 1*, 332−339.
16. Hightower, K. E., and Fierke, C. A. (1999) *Curr. Opin. Chem. Biol. 3*, 176−181.
17. Saderholm, M. J., Hightower, K. E., and Fierke, C. A. (2000) *Biochemistry 39*, 12398−12405.
18. Peariso, K., Goulding, C. W., Huang, S., Matthews, R. G., and Penner-Hahn, J. E. (1998) *J. Am. Chem. Soc. 120*, 8410−8416.
19. Zhou, Z. S., Peariso, K., Penner-Hahn, J. E., and Matthews, R. G. (1999) *Biochemistry 38*, 15915−15926.
20. Allen, J. R., Clark, D. D., Krum, J. G., and Ensign, S. A. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 8432−8437.
21. Myers, L. C., Wanger, G., and Verdine, G. L. (1995) *J. Am. Chem. Soc. 117*, 10749−10750.
22. Takahashi, K., Kawazoe, Y., Sakumi, K., Nakabeppu, Y., and Sekiguchi, M. (1988) *J. Biol. Chem. 263*, 13490−13492.
23. Myers, L. C., Cushing, T. D., Wagner, G., and Verdine, G. L. (1994) *Chem. Biol. 1*, 91−97.
24. Wilker, J. J., and Lippard, S. J. (1995) *J. Am. Chem. Soc. 117*, 8682−8683.
25. Wilker, J. J., and Lippard, S. J. (1997) *Inorg. Chem. 36*, 969−978.
26. Sodeoka, M., Larson, C. J., Chen, L., LeClair, K. P., and Verdine, G. L. (1993) *Bioorg. Med. Chem. Lett. 3*, 1089−1094.
27. Zamble, D. B., McClure, C. P., Penner-Hahn, J. E., and Walsh, C. T. (2000) *Biochemistry 39*, 16190−16199.
28. Myers, L. C., Terranova, M. P., Ferentz, A. E., Wagner, G., and Verdine, G. L. (1993) *Science 261*, 1164−1167.
29. Landini, P., and Volkert, M. R. (1995) *J. Biol. Chem. 270*, 8285−8289.
30. Taketomi, A., Nakabeppu, Y., Ihara, K., Hart, D. J., Furuichi, M., and Sekiguchi, M. (1996) *Mol. Gen. Genet. 250*, 523−532.
31. Ohkubo, T., Sakashita, H., Sakuma, T., Kainosho, M., Sekiguchi, M., and Morikawa, K. (1994) *J. Am. Chem. Soc. 116*, 6035−6036.
32. Morohoshi, F., Hayashi, K., and Munakata, N. (1990) *Nucleic Acids Res. 18*, 5473−5480.
33. Park, H. W., Boduluri, S. R., Moomaw, J. F., Casey, P. J., and Beese, L. S. (1997) *Science 275*, 1800−1804.
34. Strickland, C. L., Windsor, W. T., Syto, R., Wang, L., Bond, R., Wu, Z., Schwartz, J., Le, H. V., Beese, L. S., and Weber, P. C. (1998) *Biochemistry 37*, 16601−16611.
35. Zhang, F. L., Diehl, R. E., Kohl, N. E., Gibbs, J. B., Giros, B., Casey, P. J., and Omer, C. A. (1994) *J. Biol. Chem. 269*, 3175−3180.
36. Fu, H. W., Beese, L. S., and Casey, P. J. (1998) *Biochemistry 37*, 4465−4472.
37. Gonzalez, J. C., Peariso, K., Penner-Hahn, J. E., and Matthews, R. G. (1996) *Biochemistry 35*, 12228−12234.
38. Peariso, K., Goulding, C. W., Huang, S., Matthews, R. G., and Penner-Hahn, J. E. (1998) *J. Am. Chem. Soc. 120*, 8410−8416.
39. Millian, N. S., and Garrow, T. A. (1998) *Arch. Biochem. Biophys. 356*, 93−98.
40. Breksa, A. P., III, and Garrow, T. A. (1999) *Biochemistry 38*, 13991−13998.
41. Peariso, K., Zhou, Z., Smith, A. E., Matthews, R. G., and Penner-Hahn, J. E. (2001) *Biochemistry 40*, 987−993.
42. Carlow, D. C., Carter, C. W. J., Mejlhede, N., Neuhard, J., and Wolfenden, R. (1999) *Biochemistry 38*, 12258−12265.
43. Huang, H., Chopra, R., Verdine, G. L., and Harrison, S. C. (1998) *Science 282*, 1669−1675.
44. Sun, L. J. (2001) Ph.D. Thesis, Harvard University, Cambridge, MA.

BI011001M