Chemistry & Biology, Vol. 9, 1297–1303, December, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S1074-5521(02)00283-1

Trapping Distinct Structural States of a Protein/DNA Interaction through Disulfide Crosslinking

Chuan He[2] and Gregory L. Verdine[1]
Department of Chemistry and Chemical Biology
Harvard University
12 Oxford Street
Cambridge, Massachusetts 02138

Summary

The N-terminal domain of the *Escherichia coli* Ada protein (N-Ada) repairs methyl phosphotriesters in DNA through a zinc-mediated transfer to Cys38 of the protein. Methylation of Cys38 enhances the sequence-specific DNA affinity of N-Ada by ~1000-fold, thereby enabling the protein to activate the genes of a methylation-resistance regulon. It is of interest to understand the structural basis for metalloactivated methyl transfer and methylation-dependent enhancement of DNA binding activity. Although recent progress has been made on the structural front, efforts to develop a complete picture of N-Ada structure/function have been hampered by the inability to prepare homogeneous protein/DNA complexes representing different states of the unmethylated protein. Here, we describe the development of an approach to trap both sequence-specific and nonsequence-specific DNA recognition complexes of N-Ada through formation of an intermolecular disulfide crosslink between the protein and DNA.

Introduction

Cellular DNA is subject to nonenzymatic alkylation by environmental or chemical mutagens, resulting in adducts that are toxic and mutagenic [1, 2]. *Escherichia coli* responds to this threat by activating the so-called adaptive response pathway, the key mediator of which is the Ada protein [3]. Ada possesses two distinct methyltransferase activities: the N-terminal domain (N-Ada) repairs the methyl phosphotriester lesion by direct transfer of the methyl group to the zinc-activated thiolate of Cys38 (C.H. et al., submitted) [4]; the C-terminal domain of Ada (C-Ada) repairs the mutagenic lesion O^6-methylguanine [3, 5, 6] by irreversible methyl transfer to the Cys321 thiolate. In both cases, the lesion is directly reverted to its normal progenitor (phosphodiester and guanine, respectively), but the process is sacrificial with respect to the repair domain in Ada, because methyl transfer results in irreversible loss of repair activity in that domain. Methylation of Cys38 in the N-terminal domain enhances the sequence-specific DNA binding activity of Ada by ~1000-fold [7], enabling it to bind to several promoters and activate transcription of a methylation-resistance regulon [3]. The consensus Ada binding site, 5′-AATNNNNNNGCAA-3′, comprises two conserved sequences, the A box (AAT) and B box (GCAA), separated by a 6-base-pair spacer of variable sequence (Figure 1C) [8]. Binding of Cys38-methylated Ada to this sequence results in recruitment of RNA polymerase to the promoter and hence activation of transcription [9–11].

A truncated version of N-Ada made up of residues 1,2, and 9–139 (N-Ada16) exhibits full activity with respect to both methyl phosphotriester repair and methylation-dependent DNA binding (C.H. et al., submitted) [12]. We have recently determined high-resolution structures of Cys38-methylated N-Ada16 bound to DNA using X-ray and NMR (C.H. et al., submitted). These structures (schematics are shown in Figures 1A and 1B) revealed that N-Ada16 consists of two distinct subdomains, $N\text{-}Ada_N$ (residues 9–75) and $N\text{-}Ada_C$ (residues 80–139), connected by a flexible linker (residues 76–79). $N\text{-}Ada_C$ contains a canonical helix-turn-helix motif that positions itself over the major groove of the B box. $N\text{-}Ada_N$, previously shown to adopt a novel zinc-dependent fold [13, 14], grasps the backbone of the A box and makes several contacts to nearby bases in the minor groove. The $N\text{-}Ada_N$/A box interaction appears to be governed by a zinc-dependent electrostatic switch mechanism which modulates the affinity of the protein for DNA. Briefly, the protein presents the zinc-coordinated thiolates of Cys38 and Cys69 to two successive phosphates in the DNA, thereby creating a repulsive electrostatic interaction in the $N\text{-}Ada_N$/A box interface. Neutralization of negative charge in the DNA backbone resulting from phosphotriester formation alleviates electrostatic repulsion, thereby enabling $N\text{-}Ada_N$ to locate and repair the damage. Transfer of a methyl group to Cys38 renders that residue neutral and also reduces the net charge on Cys69 from $1/2^-$ to $1/3^-$, reducing electrostatic repulsion now from the protein side, thus enhancing the affinity of the $N\text{-}Ada_N$/A box interaction and enabling the protein to locate and bind promoter DNA (C.H. et al., submitted).

Although the overall structures of the N-Ada16/DNA complexes solved by X-ray and NMR are quite similar, the two structures exhibit some important differences (C.H. et al., submitted). The solution structure is that of a fully sequence-specific complex, with both the A box and B box in intimate contact with the two subdomains on N-Ada16 (Figure 1A); the base-specific contacts observed in this structure are fully consistent with expectation based on biochemical data [8, 15]. By contrast, in the crystal structure $N\text{-}Ada_N$ is bound specifically to the A box, but $N\text{-}Ada_C$ is bound nonspecifically to DNA (Figure 1B). Indeed, in the crystal structure the orientation of the entire N-Ada16 protein on the *ada* promoter DNA used in crystallization is reversed relative to that expected on the basis of biochemical data and the solution structure, with $N\text{-}Ada_N$ bound to a cryptic A box sequence presented in reverse orientation and $N\text{-}Ada_C$ bound nonspecifically to the opposite end of the neighboring duplex in the crystal (Figures 1A and 1B, compare sequence figures at bottom). Whereas $N\text{-}Ada_C$ in the solution structure inserts deeply into the major groove

[1]Correspondence: verdine@chemistry.harvard.edu
[2]Current address: Department of Chemistry, University of Chicago, Chicago, Illinois 60637.

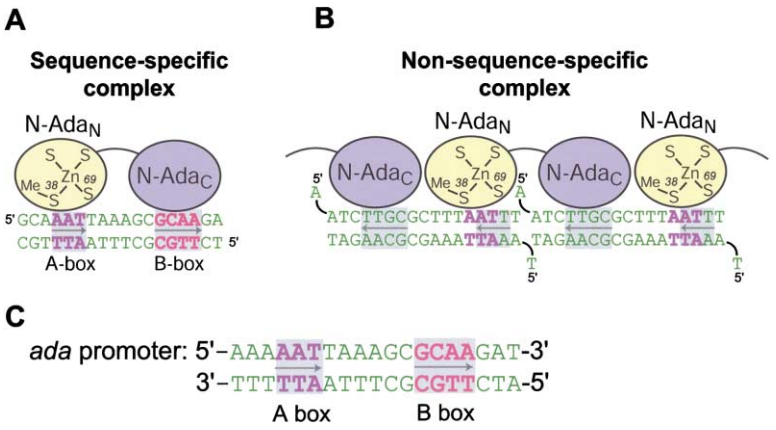

Figure 1. Schematics of Structures of N-Ada/DNA Complexes

(A) NMR structure with the N-Ada$_N$ and N-Ada$_C$ subdomains bound sequence specifically to the A box (purple) and B box (fuschia), respectively, of the *ada* promoter. Arrows in the DNA sequence denote directionality with respect to the startpoint of transcription.

(B) In the X-ray structure, N-Ada$_N$ is bound to a cryptic A box sequence (purple), and N-Ada$_C$ is bound nonsequence specifically to the neighboring DNA duplex in the crystal. Gray boxes and arrows denote the A box and B box of the *ada* promoter sequence, to which the protein is not bound.

to make base-specific contacts, this domain in the X-ray structure retracts from the DNA surface and rotates slightly to maximize its interactions with the DNA backbone. The unexpected binding mode in the X-ray structure is made possible by a combination of the existence of the cryptic A box site, crystal packing interactions, and perhaps relatively strong nonspecific DNA binding by N-Ada$_C$. This nonspecific binding mode may closely resemble that adopted by N-Ada$_C$ when searching the genome for damaged sites or promoters.

Notwithstanding the insights gained from these two structures, many aspects of Ada structure/function remain unelucidated. Of particular interest is the adjustment of the N-Ada$_N$/DNA interface that may occur when a methyl group is removed from Cys38 or is present on the DNA (i.e., a phosphotriester) rather than the protein. With regard to the mechanism of metalloactivated methyl transfer to Cys38, it is of great interest to determine the detailed coordination chemistry of the (Cys$_4$)Zn site in the unmethylated state. We have attempted to crystallize a variety of N-Ada16/DNA complexes that would address these issues but have failed, just as we have not succeeded to date in our efforts to crystallize a fully sequence-specific complex of Cys38-methylated N-Ada16 on *ada* promoter DNA. Such failure may reflect in large part the intrinsic difficulty of crystallizing labile or dynamic states of the protein/DNA interactions; therefore, we sought a method to stabilize various discrete states of Ada/DNA complexes. In previous work, we reported the development of a protein/DNA crosslinking technology that enables the preparation of stabilized homogeneous protein/DNA complexes and thus facilitates their crystallization [16, 17]. Here, we report the use of this disulfide crosslinking technology to trap distinct states of the N-Ada16/DNA interaction.

Results

Crosslinking Strategy for the Sequence-Specific N-Ada16/DNA Complex

Of particular interest is the structural relationship between the methylated and unmethylated forms of N-Ada16, especially with respect to both the ligand environment at the zinc site and to the protein-DNA interface. Because the methylation site resides in the N-Ada$_N$ domain, this subdomain is likely to experience the most significant methylation-dependent structural alterations, hence we elected not to constrain this domain by crosslinking it to DNA. As the DNA-recognition properties of N-Ada$_C$ are not expected to be affected by methylation, we decided to introduce the crosslink via this domain.

The solution structure of the sequence-specific complex suggested the presence of a hydrogen bonding interaction between the imidazole side chain of His115 in N-Ada$_C$ and the exocyclic 4-NH$_2$ group of the cytosine base at position 9 of the *ada* promoter. Although this contact probably contributes to N-Ada16 recognition of the *ada* promoter, it is not conserved in the *alkA* and *aidB* promoters, because these recognition sites have a base other than cytosine at position 9 [8]. Therefore, we selected position 115 in N-Ada16 and C9 in the DNA as the sites for covalent attachment of the two. The protein/DNA interface could be equipped for crosslinking by mutation of His115 to cysteine (H115C mutant N-Ada16) and attachment of a thiol-bearing tether to the exocyclic amine of C9 (compare Figures 2A and 2B). Modeling suggested that such a crosslink could be accommodated without perturbing the structure of the N-Ada16/DNA complex (D.P.G. Norman and G.L.V., unpublished results).

Formation of the Sequence-Specific Crosslinked Complex

The H115C mutant protein, overexpressed in *E. coli* and purified to apparent homogeneity, exhibited similar chromatographic behavior and repair activity to those of wild-type N-Ada16 (data not shown). A two-carbon thiol tether, protected as the 2-aminoethanethiol mixed disulfide, was introduced at the N^4 position of C9 by convertible nucleoside methodology (Figure 2B) [18–20]. To assay for crosslink formation between the engineered DNA and protein, we incubated DNA1 with (unmethylated) H115C N-Ada16 at 4°C for 1 hr, varying the number of molar equivalents of DNA. The reaction was then quenched by the addition of a large excess of the thiol-capping reagent methyl methanethiolsulfonate, and the products were analyzed using denaturing SDS-polyacrylamide gel electrophoresis under nonreducing conditions (Figure 2D). Control crosslinking reactions with wild-type N-Ada16 were performed in parallel.

In the presence of 1, 2, or 3 molar equivalents of DNA1 (Figure 2D, lanes 2–4), the intensity of the band ascribed

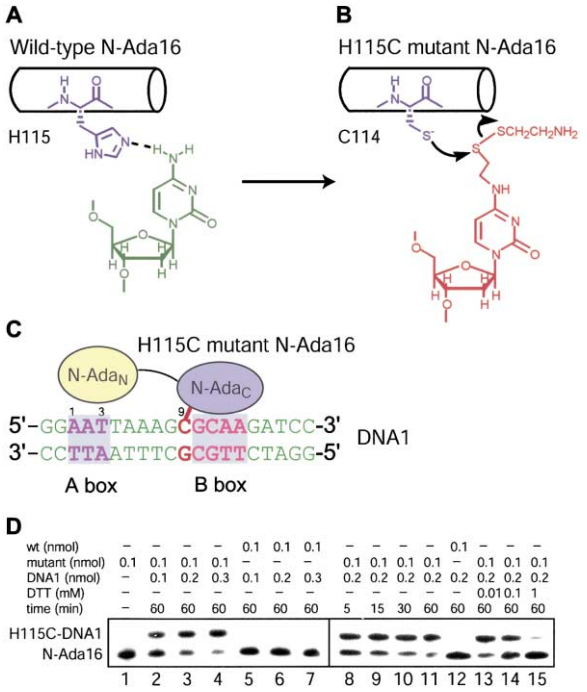

Figure 2. Sequence-Specific Crosslinking

(A) Residue His115 of wild-type N-Ada16 appears to hydrogen bond to the exocyclic amine of the cytosine residue at position 9 of the *ada* promoter.

(B) Replacement of His115 with Cys and addition of a thiol-bearing tether to the exocyclic amine of C-9 provides the reacting partners needed for formation of a disulfide crosslink.

(C) Structure of DNA1, the oligonucleotide used in sequence-specific crosslinking, and notional structure of its crosslink with the N-Ada$_C$ subdomain of N-Ada16 (red line).

(D) SDS gel analysis of the crosslink reaction between H115C and DNA1. Lane 1 is a size standard for N-Ada16 and H115C N-Ada16. Disulfide crosslinking between the protein and DNA1 results in the appearance of a new band having retarded mobility. Lanes 2–4, variation of the stoichiometry of DNA1 relative to H115C N-Ada16; lanes 5–7, controls with wild-type N-Ada16 (note the lack of crosslinking); lanes 8–11, time course of crosslinking; lanes 13–15, effect of external thiol (DTT) on crosslinking efficiency.

to H115C N-Ada16 was reduced relative to that with no added DNA, and this reduction was accompanied by the appearance of a new band having retarded mobility (lanes 1–4). Because the formation of this new band is dependent upon DNA1 in a stoichiometry-dependent manner, we assign the new band as the disulfide crosslinked H115C N-Ada16/DNA1 complex. At a DNA/protein ration of 3/1, greater than 80% of the H115C N-Ada16 became crosslinked to DNA1, as quantified from the intensities of the two bands shown in lane 4 of Figure 2D. Importantly, when wild-type N-Ada16 was used instead of the H115C mutant protein under the same conditions, no formation of a crosslinked product was observed (lanes 5–7). Thus, the crosslinking is highly selective for the engineered cysteine residue in the H115C mutant, despite the presence of three surface-exposed Cys residues (Cys38, Cys69, and Cys91) in wild-type N-Ada16. This selectivity is all the more remarkable considering that Cys38 has been shown to possess high intrinsic nucleophilicity and is located right at the DNA binding surface of N-Ada$_N$ [21].

Next, we analyzed the time course of the crosslinking reaction. Two equivalents of DNA1 were incubated at 4°C with one equivalent of H115C, and the reaction was quenched after 5, 15, 30, and 60 min by the addition of methyl methanethiolsulfonate (Figure 2D, lanes 8–11). Nonreducing SDS-PAGE analysis of the time-course samples established that the crosslinking reaction is essentially complete in 30 min. To probe the stability of the disulfide crosslink, we carried out the crosslinking reaction in the presence of varying amounts of dithiothreitol (DTT), a potent disulfide-reducing agent, then quenched the reaction with methyl methanethiolsulfonate and analyzed the products by nonreducing SDS-PAGE (lanes 13–15). The crosslink appears to withstand low concentrations of DTT (0.01 and 0.1 mM; lanes 13 and 14, respectively). At a higher concentration of DTT (1.0 mM, lane 15), the extent of crosslinking is substantially diminished.

Crosslinking Strategy for the Nonsequence-Specific N-Ada16/DNA Complex

A further goal of our ongoing structural studies on Ada is to understand how the protein scans DNA while searching for methyl phosphotriesters and promoter DNA. Such studies require the preparation of homogeneous protein/DNA complexes in which N-Ada16 or its Cys38-methylated form is bound nonsequence specifically to DNA. To stabilize this kind of complex, we inspected the X-ray structure of the methylated N-Ada16/DNA complex, in which the N-Ada$_C$ subdomain is bound to DNA nonsequence specifically. The N-Ada$_C$//DNA interface in the X-ray structure reveals only one amino acid/DNA base contact, that being between the aryl ring of Phe114 side chain and the methyl group of a thymine base (Figure 3A). We envisioned that we might be able to trap a sequence-nonspecific complex by introducing crosslinking moieties that would replace this noncovalent contact with a covalent linkage (Figure 3B). To investigate this possibility, we overexpressed and purified an N-Ada16 mutant protein having Phe114 changed to cysteine (F114C N-Ada16). To equip the DNA suitably for crosslink formation, we started with the DNA sequence used in our prior X-ray studies and mutated the T:A pair involved in the Phe114 contact to a C:G pair (Figure 3C, red). Thioalkyl tethers of varying lengths (2–4 carbons, present as the respective mixed disulfides) were attached to the N^4 position of this C residue, again using convertible nucleoside chemistry.

Formation of the Nonsequence-Specific Crosslinked Complex

Equimolar amounts of F114C N-Ada16 and DNA2 were incubated at 4°C for 1 hr. After capping the free thiol groups with methyl methanethiosulfonate, we analyzed the reaction mixtures by nonreducing SDS-PAGE (Figure 3D). Again, the wild-type N-Ada16 protein was analyzed in parallel to gauge the specificity of crosslinking for the engineered Cys at position 114. While the sample containing only F114C N-Ada16 showed a single protein band at the position of the free protein (lane 1), addition of DNA2 with a two-carbon (C2) thiol tether yielded a covalently linked complex having retarded mobility (lane

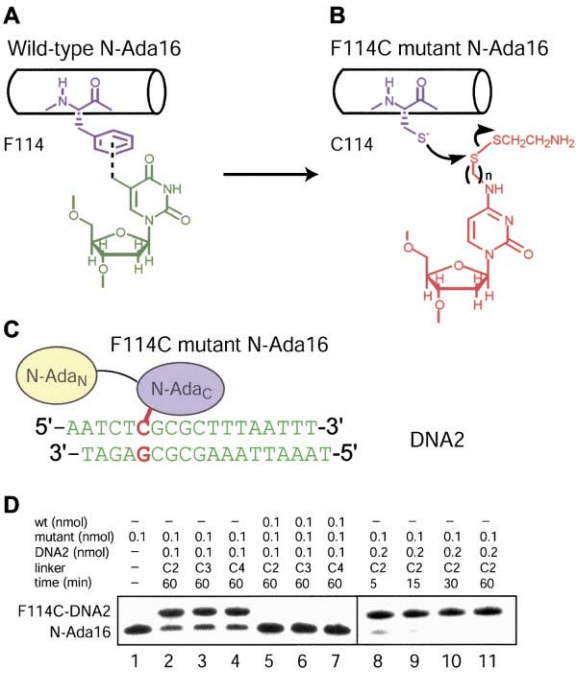

Figure 3. Nonsequence-Specific Crosslinking

(A) Residue Phe114 of wild-type N-Ada16 appears to make a hydrophobic interaction with the methyl group of a thymine base in the DNA. In the X-ray structure, this is the only base-specific contact between N-Ada$_C$ and the DNA.

(B) Replacement of Phe114 with Cys and replacement of the thymine with an N4-thioalkyl-dC (and corresponding replacement of the complementary bases) provides the reacting partners needed for formation of a disulfide crosslink.

(C) Structure of DNA2, the oligonucleotide used in nonsequence-specific crosslinking and notional structure of its crosslink with the N-Ada$_C$ subdomain of N-Ada16 (red line).

(D) SDS gel analysis of the crosslink reaction between F114C and DNA2. Lane 1, size standard; lanes 2–4, variation of the number of methylene units in the hydrocarbon chain of the thiol tether from 2 (as in B, $n = 2$; C2 tether) to 4 (as in B, $n = 4$; C4 tether); lanes 5–7, controls with wild-type N-Ada16 (note the lack of crosslinking); lanes 8–11, time course with F114C N-Ada16.

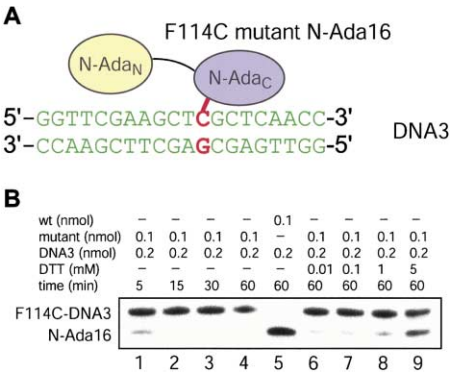

Figure 4. Nonsequence-Specific Crosslinking of F114C N-Ada16 with DNA3

(A) Structure of DNA3, the sequence of which was randomly generated.

(B) SDS gel analysis of the crosslink reaction between F114C and DNA3. Lanes 1–4, time course of crosslink formation; lane 5, wild-type N-Ada16 control; lanes 6–9, effect of external thiol (DTT) on crosslinking efficiency.

2). Similar reactions of F114C N-Ada16 with C3- and C4-tethered DNA2 were performed (lane 3, C3; lane 4, C4). No significant difference in crosslinking efficiency was observed for all three tethers tested, thus indicating that the crosslinking reaction is tolerant to variation of the linkage length between the protein and DNA. Control crosslinking reactions with wild-type N-Ada16 and all three thiol-tethered oligonucleotides gave no crosslinked complex (lanes 5–7). Thus, even the nonsequence-specific crosslinking reaction is highly selective for position 114 over the other surface-exposed Cys residues. We further investigated the time course of crosslink formation between DNA2 (C2 tether length) and F114C N-Ada16 at a DNA/protein ratio of 2/1. The reactions were quenched with methyl methanethiosulfonate at time intervals of 5, 15, 30, and 60 min (lanes 8, 9, 10, and 11, respectively), and the results were analyzed by nonreducing SDS-PAGE. After 15 min incubation at 4°C, the protein had undergone virtually quantitative crosslinking to DNA2 under these conditions. Such efficient crosslinking is unprecedented in our experience.

The results with DNA2 suggested that F114C N-Ada16 might undergo efficient crosslinking to a randomly selected sequence to which it would bind nonsequence specifically. To assess this possibility, we generated oligonucleotide DNA3 (C2 tether only) and analyzed its crosslinking to F114C N-Ada16 (Figure 4A). Incubation of one equivalent of F114C N-Ada16 with two equivalents of DNA3 at 4°C resulted in virtually quantitative crosslinking of the mutant protein to the DNA within 15 min (Figure 4B). Wild-type N-Ada16 failed to form a crosslinked product in the presence of DNA 3 (lane 5), again indicating the high positional selectivity of the crosslinking reaction for the engineered Cys residue at position 114. Next, the ability of the crosslinking reaction to compete against DTT was examined. The disulfide bond was found to exhibit a remarkable degree of stability to DTT up to concentrations of 1 mM (lanes 6–8). Even at 5 mM DTT (lane 9), greater than 50% of crosslinked complex was still formed.

Isolation of the Covalently Crosslinked Complex

For the present crosslinking system to be useful in structural studies, it is important that the crosslink should be straightforward to purify. To assess this, we performed preparative-scale (0.04 mmol DNA and 0.08 mmol protein) crosslinking reactions to produce both sequence-specific and nonsequence-specific complexes and subjected both to anion-exchange chromatography using a Mono Q HR 5/5 column (Amersham Pharmacia) eluted with a linear NaCl gradient. The covalently crosslinked complexes eluted at ~550 mM NaCl and baseline separated from the free DNA at ~600 mM; unreacted protein was present in the void volume fractions. The fractions containing the crosslinked species were collected and analyzed by nonreducing SDS-PAGE, which revealed that the preparations were essentially homogeneous (see Supplemental Data). The purified complexes could

be concentrated to 0.5–0.7 mM by centrifugal dialysis (Millipore 0.5 centrifugal filter, 5 K membrane), concentrations suitable for structural studies.

Conclusions

In previous work on HIV reverse transcriptase, we developed the technique of protein-DNA crosslinking to stabilize a stalled catalytic enzyme/DNA complex and to prevent its interconversion with alternative, nonspecific modes of enzyme/DNA interaction [16, 17]. We chose disulfide bond formation as the crosslinking chemistry because thiol-disulfide interchange is an equilibrium process [22, 23], and hence crosslinking is sensitive to the imposition of strain. By the same token, the efficiency of disulfide crosslinking is also affected by the strength of intermolecular interactions between the protein and DNA outside the vicinity of the crosslink [24]. These chemical attributes make disulfide crosslinking intrinsically less likely to trap strained or otherwise irrelevant complexes than, for example, kinetically controlled methods such as photo-crosslinking. Consistent with this notion, in the case of RT we found that the efficiency of disulfide crosslinking was exquisitely sensitive to the relative positions of the Cys residue in the protein and the thiol tether in the DNA; indeed, the crosslinking reaction was strongly influenced by even seemingly subtle factors, such as the conformational difference between a DNA:DNA versus a DNA:RNA primer:template [16, 17]. In other work, we have demonstrated that disulfide crosslinking can be used to stabilize the minimal structural components of a protein-DNA interaction, such as a single α-helical peptide bound sequence specifically to DNA [24] (P. Zhou, L. J. Sun, and G.L.V., submitted). Again, in this system the extent of disulfide crosslinking was strongly affected by mutations that abrogate sequence-specific interactions between the peptide and DNA [24].

In the work described here, we have used disulfide crosslinking to address a different set of problems, namely to trap distinct structural states of a protein/DNA interaction. In the course of performing its normal biologic function, the N-terminal domain of Ada must scan the genome in search of methyl phosphotriesters, and upon locating such a lesion, transfer its methyl group to Cys38, again search the genome for cognate promoters, and finally form a sequence-specific complex that can recruit RNA polymerase. We are interested to know the structural relationship between these distinct states of the protein/DNA interaction, all of which are functionally relevant. An advantage of conventional crystallography is that it can provide detailed images of macromolecular interactions, but a limitation is that it requires the preparation of structurally homogeneous complexes. This limitation has made it nearly impossible to observe protein/DNA search complexes, because these typically exist as labile, rapidly equilibrating mixtures of nearly isoenergetic complexes, even with short oligonucleotides. Even many sequence-specific or structure-specific protein/DNA complexes, such as RT/DNA, have proven difficult to crystallize, perhaps owing to kinetic lability or structural interconversion. For example, we have thus far been unable to crystallize the noncovalent intermolecular complex formed between N-Ada16 and *ada* promoter DNA, despite the fact that the protein forms a specific recognition complex with this DNA sequence. Rather than pursue exhaustive screening of crystallization conditions, we decided to develop a crosslinking system that would provide access to both stabilized sequence-specific recognition complexes and nonspecific scanning complexes. In the work described above, we have demonstrated highly efficient formation of sequence-specific crosslinks between the H115C mutant of N-Ada16 and DNA having a thiol-tethered C at position 9 of the *ada* promoter. Perhaps more unexpectedly, we also found that the F114C mutant of N-Ada16 undergoes rapid and nearly quantitative crosslinking to an oligonucleotide having no specific recognition sequence for the protein. In both cases, crosslink formation was completely dependent upon the presence of the engineered Cys residue, despite the presence of nucleophilic (Cys38) and surface-exposed (Cys38 and Cys69) residues located directly in the protein/DNA interface (C.H. et al., submitted). Thus, here too disulfide crosslinking has proven to be highly selective for Cys residues that are properly juxtaposed with the thiol tether in DNA.

A standard procedure in crystallization of protein/DNA complexes is screening of oligonucleotides having different lengths and end structures. A concern is that the addition of a disulfide crosslinking step might present a burdensome complication to the screening process. This concern is mitigated by several considerations: (1) the synthesis of the thiol-tethered DNA is no more difficult or time consuming than that of normal DNA; (2) both of the crosslinking systems described here provide sufficiently high yields of crosslink that it should not be necessary to purify the crosslinked complexes prior to crystallization, provided that the DNA is present in the usual 2-fold excess; and (3) even if purification is necessary, we have shown here that it can be performed by a simple one-step anion-exchange chromatography run. On the other hand, in unpublished work we have found that crosslinking to DNA provides such a powerful chromatographic handle that it can obviate the need for extensive protein purification prior to crosslinking.

We are intrigued by the notion that the C-terminal subdomain of N-Ada16, crosslinked to DNA, might be used as module to facilitate crystallization of heterologous fusion partners. In this scenario, N-Ada$_C$ would be fused to the protein of interest and then crosslinked to DNA, thereby allowing the fusion protein to utilize coaxial stacking of the DNA helix in order to form ordered arrays that would form crystals. Obviously, this fusion system offers promise for studies of DNA recognition. More intriguing is the possibility that this crystal engineering scheme would enable crystallization of proteins having no physiological interaction with DNA.

Significance

The weak association of the unmethylated form of N-Ada with DNA is an impediment to structural characterization. In an effort to develop a complete picture of N-Ada structure/function, a disulfide crosslinking

technique was used to trap distinct states of unmethylated N-Ada/DNA complexes. Highly efficient formation of sequence-specific crosslinks between the H115C mutant of N-Ada16 and DNA having a thiol-tethered C at position 9 of the *ada* promoter was observed. Unexpectedly, we also found that a nonsequence-specific complex of N-Ada16 (F114C mutant) also undergoes rapid and essentially quantitative crosslinking to DNA. By this strategy, homogeneous samples of both sequence-specific and nonsequence-specific DNA recognition complexes of N-Ada have been trapped for future structural characterization. This methodology could potentially be broadly applicable to the study of otherwise unstable or transient states of protein DNA interactions.

Experimental Procedures

Construction, Expression, and Purification of N-Ada16

Wild-type and mutant versions of N-Ada16 were overexpressed and purified as described (C.H. et al., submitted). Point mutations in N-Ada16 (H115C and F114C) were introduced by megaprimer mutagenesis and were confirmed by sequencing of the entire coding sequence.

Synthetic Oligonucleotides

Oligodeoxynucleotides were synthesized on an Applied Biosystems 392 DNA synthesizer. Thiol-tethered oligonucleotides were prepared by incorporation of O4-triazolyl-dU-CE phosphoramidite (Glen Research) at the modified positions during solid-phase synthesis, followed by postsynthetic modification/deprotection by treatment with diamine disulfides as described previously [18–20]. Modified and unmodified oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. Concentrations of the oligonucleotides were estimated by UV at 260 nm.

Crosslinking Reaction and Analysis

Purified N-Ada16 mutant proteins were dialyzed into a buffer containing 10 mM Tris-HCl (pH 7.4), 100 mM NaCl. The same buffer was used for crosslinking reactions. In a typical reaction (20–100 μl), the mutant N-Ada16 protein (10 μM) and the thiol-tethered duplex DNA (20 μM) were incubated at 4°C for varying periods of time. The reaction was then quenched by the addition of a thiol-capping reagent, methyl methanethiolsulfonate, to a final concentration of 20 mM for 10 min at room temperature. SDS-PAGE loading dye free of reducing agents was added, and the samples were heated at 95°C for 5 min, then analyzed by SDS-PAGE as in [17]. In experiments analyzing disulfide stability, varying concentrations of DTT were present throughout the crosslinking reaction, and the final concentration of thiol-capping reagent was elevated to 100 mM to ensure complete capping of all the free thiol groups. The reaction was analyzed by SDS-PAGE under nonreducing conditions.

Purification of the Crosslinked Complex

The crosslinking reaction was carried out on a 2 ml volume (0.04 mmol DNA and 0.08 mmol protein) under the same conditions as above for 1 hr. The reaction mixture was loaded onto a Mono Q HR 5/5 column (Amersham Pharmacia) preequilibrated with buffer A (10 mM Tris-HCl [pH 7.4]). At a flow rate of 1 ml/min, a linear gradient from 30% to 70% buffer B (10 mM Tris-HCl, 1 M NaCl [pH 7.4]) was run. The covalently linked complexes typically eluted at ~550 mM NaCl concentration. Purity of the complex was determined by SDS-PAGE.

Supplemental Data

Figure S1 shows SDS gel analysis of FPLC fractions of a preparative-scale crosslinking reaction between H115C N-Ada16 and DNA1. Please write to chembiol@cell.com for a PDF.

Acknowledgments

This research was supported by a grant from the NIH (GM-51330). This research is partially supported by Damon Runyon Fellowship DRG-1628 from the Damon Runyon Cancer Research Foundation. We thank Mr. Anirban Banerjee for providing 3-aminopropane disulfide and 4-aminobutane disulfide, Mr. D.P.G. Norman for sharing modeling results, and Dr. L.J. Sun for helpful discussions.

Received: September 24, 2002
Revised: November 6, 2002
Accepted: November 6, 2002

References

1. Wood, R.D., Mitchell, M., Sgouros, J., and Lindahl, T. (2001). Human DNA repair genes. Science *291*, 1284–1289.
2. Friedberg, E.C., Walker, G.C., and Siede, W. (1995). DNA Repair and Mutagenesis (Washington, D.C.: ASM Press).
3. Lindahl, T., Sedgewick, B., Sekiguchi, M., and Nakabeppu, Y. (1988). Regulation and expression of the adaptive response to alkylating agents. Annu. Rev. Biochem. *57*, 133–157.
4. Myers, L.C., Terranova, M.P., Ferentz, A.E., Wagner, G., and Verdine, G.L. (1993). Repair of DNA methylphosphotriester through a metalloactivated cysteine nucleophile. Science *261*, 1164–1167.
5. Demple, B., Sedgwick, B., Robin, P., Totty, N., Waterfield, M.D., and Lindahl, T. (1985). Active site and complete sequence of the suicidal methyltransferase that counters alkylation mutagenesis. Proc. Natl. Acad. Sci. USA *82*, 2688–2692.
6. Nakabeppu, Y., Kondo, H., Kawabata, S., Iwanaga, S., and Sekigichui, M. (1985). Purification and structure of the intact Ada regulatory protein of *Escherichia coli* K12, O6-methylguanine-DNA methyltransferase. J. Biol. Chem. *260*, 7281–7288.
7. Myers, L.C., Jackow, F., and Verdine, G.L. (1995). Metal dependence of transcriptional switching in *Escherichia coli* Ada. J. Biol. Chem. *270*, 6664–6670.
8. Landini, P., and Volkert, M.R. (1995). Transcriptional activation of the *Escherichia coli* adaptive response gene *aidB* is mediated by binding of methylated Ada protein. Evidence for a new consensus sequence for Ada-binding sites. J. Biol. Chem. *270*, 8285–8289.
9. Landini, P., and Volkert, M.R. (1995). RNA polymerase alpha subunit binding site in positively controlled promoters: a new model for RNA polymerase-promoter interaction and transcriptional activation in the *Escherichia coli ada* and *aidB* genes. EMBO J. *14*, 4329–4335.
10. Landini, P., Bown, J.A., Volkert, M.R., and Busby, S.J. (1998). Ada protein-RNA polymerase sigma subunit interaction and alpha subunit- promoter DNA interaction are necessary at different steps in transcription initiation at the *Escherichia coli ada* and *aidB* promoters. J. Biol. Chem. *273*, 13307–13312.
11. Landini, P., and Busby, S.J.W. (1999). The *Escherichia coli* Ada protein can interact with two distinct determinants in the sigma70 subunit of RNA polymerase according to promoter architecture: indentification of the target of Ada activation at the *alkA* promoter. J. Biol. Chem. *181*, 1524–1529.
12. Sakashita, H., Sakuma, T., Ohkubo, T., Kainosho, M., Sakumi, K., Sekiguchi, M., and Morikawa, K. (1993). Folding topology and DNA binding of the N-terminal fragment of Ada protein. FEBS Lett. *323*, 252–256.
13. Lin, Y., Dötsch, V., Wintner, T., Peariso, K., Myers, L.C., Penner-Hahn, J.E., Verdine, G.L., and Wagner, G. (2001). Structural basis for the functional switch of the *E. coli* Ada protein. Biochemistry *40*, 4261–4271.
14. Myers, L.C., Verdine, G.L., and Wagner, G. (1993). Solution structure of the DNA methyl phosphotriester repair domain of *Escherichia coli* Ada. Biochemistry *32*, 14089–14094.
15. Storek, M.J., Ernst, A., and Verdine, G.L. (2002). High-resolution footprinting of sequence-specific protein-DNA contacts. Nat. Biotechnol. *20*, 183–186.
16. Huang, H., Chopra, R., Verdine, G.L., and Harrison, S.C. (1998). Structure of a covalently trapped catalytic complex of HIV-1

reverse transcriptase at 2.7 A resolution: implications of comformational changes for polymerization and inhibition mechanism. Science *282*, 1669–1675.

17. Huang, H., Harrison, S.C., and Verdine, G.L. (2000). Trapping of a catalytic HIV reverse transcriptase-template:primer complex through a disulfide bond. Chem. Biol. *7*, 355–364.
18. Xu, Y.Z., Zheng, Q., and Swann, P.F. (1992). Synthesis of DNA containing modified bases by postsynthetic substitution. Synthesis of oligomers containing 4-substituted thymine: O^4Alkylthyine, 5-methylcytosine, N^4-(dimethylamino)-5-methylcytosine and 4-thiothymine. J. Org. Chem. *57*, 3839–3845.
19. MacMillan, A.M., and Verdine, G.L. (1990). Synthesis of functionally tethered oligonucleotides by the convertible nucleoside approach. J. Org. Chem. *55*, 5931–5933.
20. MacMillan, A.M., and Verdine, G.L. (1991). Engineering tethered DNA molecules by the convertible nucleoside approach. Tetrahedron *47*, 2603–2616.
21. Myers, L.C., Wagner, G., and Verdine, G.L. (1995). Direct activation of the methyl chemosensor protein N-Ada by CH_3I. J. Am. Chem. Soc. *117*, 10749–10750.
22. Houk, J., Singh, R., and Whiteside, G.M. (1987). Measurement of thiol-disulfide interchange reactions and thiol pKa values. Methods Enzymol. *143*, 129–140.
23. Creighton, T.E., Zapun, A., and Darby, N.J. (1995). Mechanisms and catalysis of disulfide bond formation in proteins. Trends Biotechnol. *13*, 18–23.
24. Stanojevic, D., and Verdine, G.L. (1995). Deconstruction of GCN4/GCRE into a monomeric peptide-DNA complex. Nat. Struct. Biol. *2*, 450–457.