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Biochemistry

Determination of the orientation of a DNA binding motif in a protein–DNA complex by photocrosslinking

(cysteine-specific chemical modification of protein/phenyl azide photoactivatable crosslinking agents/catabolite gene activator protein/cAMP receptor protein/helix–turn–helix motif)

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ABSTRACT **We have developed a straightforward biochemical method to determine the orientation of the DNA binding motif of a sequence-specific DNA binding protein relative to the DNA site in the protein–DNA complex. The method involves incorporation of a photoactivatable crosslinking agent at a single site within the DNA binding motif of the sequence-specific DNA binding protein, formation of the derivatized protein–DNA complex, UV-irradiation of the derivatized protein–DNA complex, and determination of the nucleotide(s) at which crosslinking occurs. We have applied the method to catabolite gene activator protein (CAP). We have constructed and analyzed two derivatives of CAP: one having a phenyl azide photoactivatable crosslinking agent at amino acid 2 of the helix–turn–helix motif of CAP, and one having a phenyl azide photoactivatable crosslinking agent at amino acid 10 of the helix–turn–helix motif of CAP. The results indicate that amino acid 2 of the helix–turn–helix motif is close to the top-strand nucleotides of base pairs 3 and 4 of the DNA half site in the CAP–DNA complex, and that amino acid 10 of the helix–turn–helix motif is close to the bottom-strand nucleotide of base pair 10 of the DNA half site in the CAP–DNA complex. The results define unambiguously the orientation of the helix–turn–helix motif relative to the DNA half site in the CAP–DNA complex. Comparison of the results to the crystallographic structure of the CAP–DNA complex [Schultz, S., Shields, S. & Steitz, T. (1991) *Science* 253, 1001–1007] indicates that the method provides accurate, high-resolution proximity and orientation information.**

For many sequence-specific DNA binding proteins, protein–DNA interaction is mediated by a conserved DNA binding motif: e.g., the helix–turn–helix motif, the homeodomain motif, the C_2C_2 zinc finger motif, the C_2H_2 zinc finger motif, the bZip motif, or the bHLH (helix–loop–helix) motif (reviewed in refs. 1 and 2). We have developed a straightforward biochemical method to determine the orientation of the DNA binding motif of a sequence-specific DNA binding protein relative to the DNA site in the protein–DNA complex. The method is useful in cases in which the three-dimensional structure of the DNA binding motif is known, or can be predicted based on sequence homology, but the three-dimensional structure of the protein–DNA complex is not known. In this paper, we report the method and a test case in which we have applied the method to catabolite gene activator protein [CAP; also referred to as cAMP receptor protein (CRP)].

The method involves covalently attaching a photoactivatable crosslinking agent—one capable of reacting with DNA nucleotides upon UV irradiation—to the DNA binding motif of the sequence-specific DNA binding protein under investigation. The method has two parts: (*i*) One covalently attaches a photoactivatable crosslinking agent to the DNA binding motif of the sequence-specific DNA binding protein at a single amino acid (amino acid *x*). One then forms the protein–DNA complex, UV irradiates the protein–DNA complex, and determines the nucleotide(s) at which crosslinking occurs. The results of *i* identify a nucleotide(s) close to amino acid *x* in the protein–DNA complex. (*ii*) One covalently attaches a photoactivatable crosslinking agent to the DNA binding motif of the sequence-specific DNA binding protein, at a different single amino acid (amino acid *y*). One then forms the protein–DNA complex, UV irradiates the protein–DNA complex, and determines the nucleotide(s) at which crosslinking occurs. The results of *ii* identify a nucleotide(s) close to amino acid *y* in the protein–DNA complex. By combining the results from *i* and *ii*, one determines the orientation of the vector *x*–*y* relative to the DNA site within the protein–DNA complex, and thus the orientation of the DNA binding motif of the sequence-specific DNA binding protein relative to the DNA site within the protein–DNA complex.

One can incorporate a photoactivatable crosslinking agent at a single amino acid within a protein by a two-step procedure consisting of site-directed mutagenesis (3) followed by cysteine-specific chemical modification (4). In step one, one uses site-directed mutagenesis to introduce a unique solvent-accessible cysteine residue at the position of interest. In step two, one derivatizes the resulting protein with a cysteine-specific heterobifunctional photoactivatable crosslinking agent. In the experiments in this paper, we have used a phenyl azide photoactivatable crosslinking agent (review in ref. 5; Fig. 1*A*). We have used a commercially available cysteine-specific heterobifunctional phenyl azide photoactivatable crosslinking agent: i.e., 4-azidophenacyl bromide (ref. 6; Fig. 1*B*). Under defined conditions, reaction of 4-azidophenacyl bromide with a protein having a unique solvent-accessible cysteine residue results in complete and highly selective derivatization of the cysteine residue to yield a conjugate of the form [(4-azidophenacyl)–Cys]protein (Fig. 1*C*).

As a test case, we have applied the method to the well-characterized sequence-specific DNA binding protein CAP (review of CAP in ref. 7). CAP is a helix–turn–helix motif sequence-specific DNA binding protein (reviews of helix–turn–helix motif in refs. 1 and 2). CAP binds to a 22-base-pair 2-fold symmetric DNA site: 5′-AAATGTGATCTAGATCACATTT-3′ (8, 9). The three-dimensional structure of the CAP–DNA complex has been determined to 3.0 Å resolution by x-ray crystallographic analysis (ref. 10; Fig. 2*A*). CAP is a dimer of two identical subunits, each of which is 209 amino acids long. The CAP–DNA complex is 2-fold symmetric: the helix–turn–helix motif of one subunit of CAP interacts with one half of the DNA site; the helix–turn–helix motif of the other subunit of CAP interacts in a 2-fold symmetry-related fashion with the other half of the DNA site.

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Abbreviation: CAP, catabolite gene activator protein.

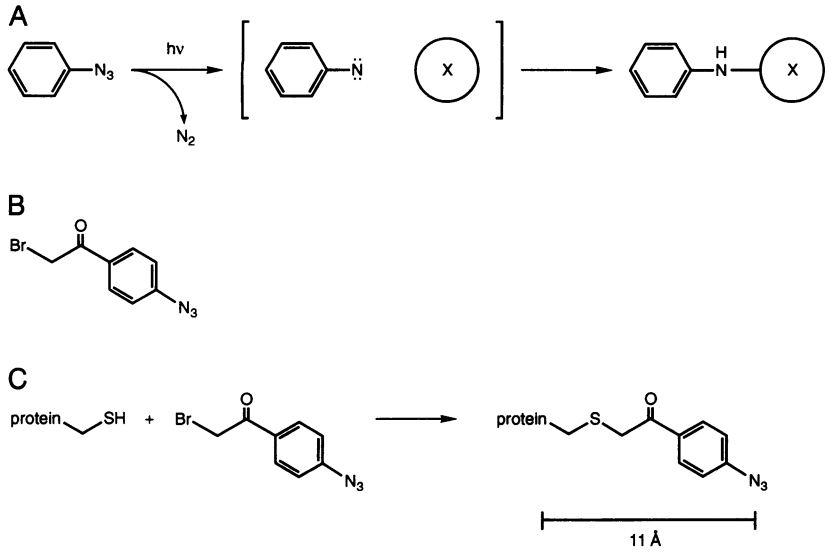

FIG. 1. (*A*) Chemistry of phenyl azide (review in ref. 5). In the dark, phenyl azide is unreactive. However, upon UV irradiation, a highly reactive nitrene is generated. If there is organic material in direct van der Waals contact with the nitrene, covalent bond formation can occur. If there is no organic material in direct van der Waals contact with the nitrene, the nitrene reacts with water and is inactivated. (*B*) 4-Azidophenacyl bromide (6) contains a bromoacetyl moiety, which permits cysteine-specific incorporation into protein, and a phenyl azide moiety, which permits photoactivatable crosslinking. (*C*) Under defined conditions, reaction of 4-azidophenacyl bromide with a protein having a unique solvent-accessible cysteine residue results in complete and highly selective derivatization of the cysteine residue to yield a conjugate of the form [(4-azidophenacyl)–Cys]protein.

Because the orientation of the helix–turn–helix motif of CAP in the CAP–DNA complex is firmly established—both by x-ray crystallographic determination of the structure of the CAP–DNA complex (10) and by experimental identification of two amino acid–base pair contacts in the CAP–DNA complex (12–14)—we reasoned that redetermination of the orientation would constitute a rigorous test of the method. Therefore, we have constructed and analyzed two derivatives of CAP: one having a phenyl azide photoactivatable crosslinking agent at amino acid 10 of the helix–turn–helix motif, and one having a phenyl azide photoactivatable crosslinking agent at amino acid 2 of the helix–turn–helix motif.

MATERIALS AND METHODS

[(4-Azidophenacyl)–Cys^{178}]CAP. Reaction mixtures (500 μl) contained 7.5 μM CAP (purified according to the procedure described in ref. 15), 300 μM azidophenacyl bromide (Sigma), 20 mM Tris·HCl (pH 8.0), 200 mM KCl, 0.1 mM EDTA, 5% (vol/vol) glycerol, and 1.7% dimethylformamide. Reactions were carried out in the dark and proceeded for 3 h at 22°C followed by 15 h at 4°C. The product was purified by chromatography on Bio-Gel P-6DG (Bio-Rad). Ellman titration (15, 16) indicated that underivatized CAP had 1 mol of solvent-accessible thiol per mol of subunit and that the product had <0.2 mol of solvent-accessible thiol per mol of subunit.

[(4-Azidophenacyl)–Cys^{170},Ser^{178}]CAP. Reactions were carried out as described above with [Cys^{170},Ser^{178}]CAP (15). Ellman titration (15, 16) indicated that underivatized [Cys^{170},Ser^{178}]CAP had 1 mol of solvent-accessible thiol per mol of subunit and that the product had <0.2 mol of solvent-accessible thiol per mol of subunit.

Formation of Crosslinked Protein–DNA Complexes. Reaction mixtures contained (in 50 μl) 100 nM CAP derivative, 1 nM ^{32}P 5′-end-labeled 40-base-pair DNA fragment having the DNA site for CAP (DNA fragment ICAP of ref. 8; 30 Bq/fmol), 0.2 mM cAMP, 10 mM Mops·NaOH (pH 7.3), 200 mM NaCl, and 50 μg of bovine serum albumin per ml. Reaction mixtures were incubated in the dark for 40 min at 22°C. Reaction mixtures then were UV irradiated in a Rayonet RPR100 photochemical reactor equipped with 16 350-nm tubes and a RMA400 sample holder (Southern New England Ultraviolet, Hamden, CT) for 1 min at 22°C. Reaction vessels were polystyrene microcentrifuge tubes held inside borosilicate glass culture tubes (13 × 100 mm); these reaction vessels exclude wavelengths < 290 nm. UV-irradiated reaction mixtures were mixed with an equal vol of 100 mM Tris·HCl, pH 6.8/4% SDS/20% glycerol/0.001% bromophenol blue, and 20-μl samples were analyzed by electrophoresis through SDS/7.5% polyacrylamide gels. Efficiencies of crosslinking were determined by excision of bands followed by determination of Cerenkov radiation.

Isolation of Crosslinked Protein–DNA Complexes. Crosslinked protein–DNA complexes were prepared as described above, except that the reaction volumes were 100 μl and the DNA fragment concentrations were 5 nM. Crosslinked protein–DNA complexes were purified from uncrosslinked DNA by extraction into phenol followed by ethanol precipitation (cf. ref. 17). The UV-irradiated reaction mixtures were mixed with 1 μl of 10% SDS, heated for 10 min at 70°C, and extracted with 100 μl of phenol/chloroform (4:1). The resulting organic phases were washed twice with 100 μl of 1 M Tris·HCl, pH 8.0/1% SDS. To the resulting washed organic phases were added 9 μl of 3 M sodium acetate (pH 5.2), 2 μl of 500 mM $MgCl_2$, 15 μg of yeast tRNA (type X; Sigma), and 300 μl of ethanol. Precipitates were collected by centrifugation at 10,000 × *g* for 10 min at 4°C and washed twice with 70% ethanol.

Determination of the Nucleotides at Which Crosslinking Occurs. Reaction mixtures contained (in 100 μl) ≈5 fmol of crosslinked protein–DNA complex, 0.1 M NaOH, 20 mM ammonium acetate, 0.1 mM EDTA, and 2% SDS. Reaction components except NaOH were incubated for 15 min at 90°C, NaOH was added, and the reaction mixtures were incubated for 30 min at 90°C. Reactions were quenched by addition of 100 μl of 20 mM Tris·HCl (pH 8.0) and 6.5 μl of 2 M HCl, and reaction products were ethanol precipitated. Reaction products were redissolved in 20 μl of 80% formamide/0.001% bromophenol blue/0.001% xylene cyanol and analyzed by gel electrophoresis through 20% polyacrylamide/8.3 M urea slab gels (18).

RESULTS

CAP Derivative Having a Phenyl Azide Photoactivatable Crosslinking Agent at Amino Acid 10 of the Helix–Turn–Helix Motif: [(4-Azidophenacyl)–Cys^{178}]CAP. Fortuitously, CAP contains a preexisting unique, solvent-accessible cysteine residue at amino acid 10 of the helix–turn–helix motif (i.e., Cys^{178}; ref. 19). Inspection of the three-dimensional structure of the CAP–DNA complex (10) suggested that covalent attachment of phenyl azide at amino acid 10 of the helix–turn–helix motif of CAP would place the phenyl azide moiety in close proximity to DNA in the protein–DNA complex—potentially close enough to crosslink (Fig. 2*B*). We have

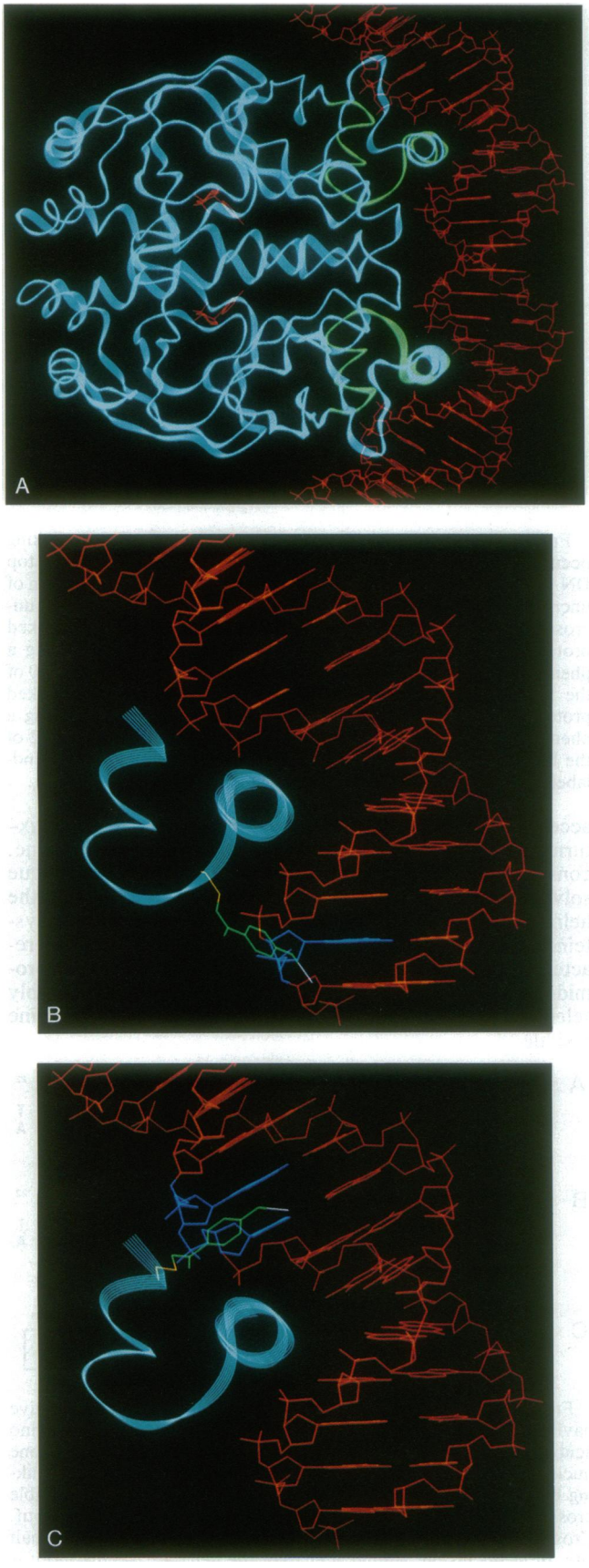

FIG. 2. (*A*) Structure of the CAP–DNA complex (10). The helix–turn–helix motif of each subunit of CAP is illustrated in green. (*B*) Model for the structure of the complex by the CAP derivative having a phenyl azide photoactivatable crosslinking agent at amino acid 10 of the helix–turn–helix motif: [(4-azidophenacyl)–Cys^{178}]CAP. For clarity, only one helix–turn–helix motif and one DNA half site are illustrated. The side chain of amino acid 10 of the helix–turn–helix motif is illustrated in yellow (i.e., Cys^{178}). The 4-azidophenacyl moiety is illustrated in green. The nucleotide at which crosslinking occurs is illustrated in blue. (*C*) Model for the structure of the complex by the CAP derivative having a phenyl azide photoactivatable crosslinking agent at amino acid 2 of the helix–turn–helix motif: [(4-azidophenacyl)–Cys^{170},Ser^{178}]CAP. For clarity, only one helix–turn–helix motif and one DNA half site are illustrated. The side chain of amino acid 2 of the helix–turn–helix motif is illustrated in yellow (i.e., Cys^{170}). The 4-azidophenacyl moiety is illustrated in green. The nucleotides at which crosslinking occurs are illustrated in blue. Method: Coordinates for the structure of the "closed" subunit of CAP were from the Brookhaven Protein Data Bank (ref. 11; accession code 3GAP). Coordinates for the structure of the 4-azidophenacyl moiety were generated using the program INSIGHT. Coordinates for the structure of DNA were generated using the program DNA FIT MAN, written by J. Warwicker (Yale University).

constructed and analyzed a CAP derivative having phenyl azide at amino acid 10 of the helix–turn–helix motif: i.e., [(4-azidophenacyl)–Cys^{178}]CAP.

To construct this CAP derivative, we reacted CAP with 4-azidophenacyl bromide under conditions that resulted in complete and highly selective derivatization of the solvent-accessible cysteine residue.

To assess protein–DNA crosslinking with this CAP derivative, we incubated the CAP derivative with a ^{32}P end-labeled 40-base-pair DNA fragment having the consensus DNA site for CAP, UV-irradiated the reaction mixture, and analyzed the reaction products by SDS/PAGE. The results in Fig. 3*A* indicate that UV irradiation results in formation of a crosslinked protein–DNA complex. The efficiency of crosslinking is ≈1%. The electrophoretic mobility of the crosslinked protein–DNA complex is substantially less than that of the uncrosslinked DNA fragment and is slightly less than that of uncrosslinked CAP. The stability properties of the crosslinked protein–DNA complex establish that the crosslinked protein–DNA complex contains a covalent crosslink. Thus, the crosslinked protein–DNA complex is stable to high salt (1 M NaCl), high temperature (70°C), 2% SDS, and phenol. Treatment of the crosslinked protein–DNA complex with proteinase K (2.5 μg in 0.5% SDS for 1 h at 50°C) results in digestion of the crosslinked protein–DNA complex and formation of a limit-digest product having an electrophoretic mobility slightly less than that of the uncrosslinked DNA fragment. Control experiments establish that formation of the crosslinked protein–DNA complex requires UV-irradiation, the CAP derivative, and cAMP (the allosteric effector required for DNA binding by CAP; ref. 7). Underivatized CAP is not able to substitute for the CAP derivative. Prebound underivatized CAP blocks formation of the crosslinked protein–DNA complex by subsequently added CAP derivative. Additional control experiments establish that formation of the crosslinked protein–DNA complex requires formation of the specific protein–DNA complex. Thus, formation of the crosslinked protein–DNA complex does not occur with 40-base-pair DNA fragments not containing the consensus DNA site for CAP (DNA fragments ICAP-7A-SYM and ICAP-7C-SYM of ref. 9).

To determine the nucleotide(s) at which crosslinking occurs, we isolated the crosslinked protein–DNA complex in fmol quantities, treated the isolated crosslinked protein–DNA complex with 0.1 M NaOH at 90°C, and analyzed the products by urea/PAGE. Treatment of the crosslinked protein–DNA complex with NaOH at high temperature results in DNA scission at the crosslinked nucleotide(s); from the lengths of the resulting DNA fragments, it is possible to determine the positions of the nucleotide(s) at which crosslinking occurred (cf. refs. 18, 20, and 21). The results in

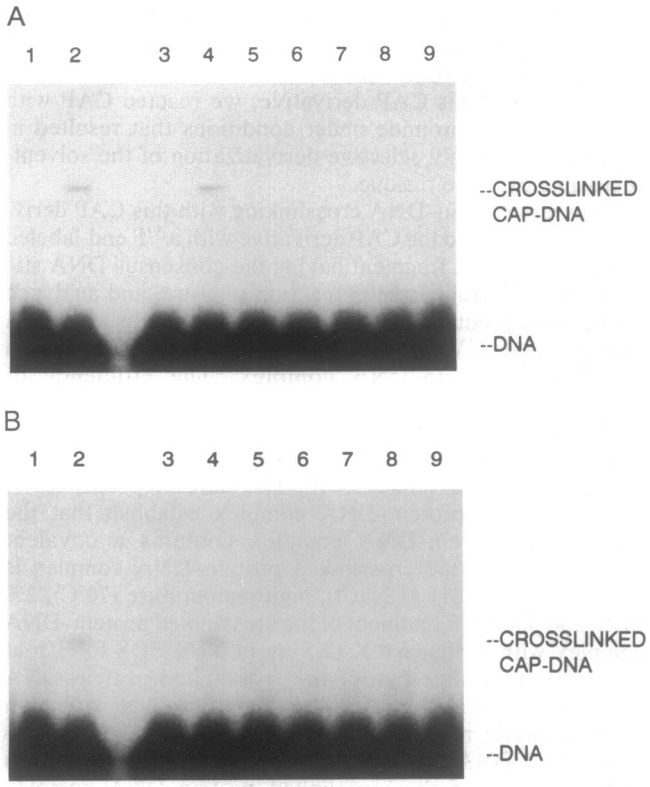

FIG. 3. Formation of crosslinked protein–DNA complex. (*A*) Data for the CAP derivative having a phenyl azide photoactivatable crosslinking agent at amino acid 10 of the helix–turn–helix motif. Lanes: 1 and 3, uncrosslinked DNA fragment; 2 and 4, crosslinking reaction; 5, control reaction omitting UV irradiation; 6, control reaction omitting the CAP derivative; 7, control reaction omitting cAMP; 8, control reaction using underivatized CAP in place of the CAP derivative; 9, control reaction with 10 nM underivatized CAP incubated for 10 min at 22°C before addition of the CAP derivative. (*B*) Data for the CAP derivative having a phenyl azide photoactivatable crosslinking agent at amino acid 2 of the helix–turn–helix motif. Lanes are the same as in *A*.

Fig. 4 indicate that crosslinking occurs at one nucleotide of the top DNA strand and at one nucleotide of the bottom DNA strand. The nucleotides at which crosslinking occurs occupy 2-fold symmetry-related positions within the 2-fold symmetric DNA site for CAP: i.e., crosslinking occurs at the bottom-strand nucleotide of base pair 10 of each DNA half site (Fig. 5*A*). We conclude from the photocrosslinking results that the bottom-strand nucleotide of base pair 10 of the DNA half site is close to amino acid 10 of the helix–turn–helix motif in the CAP–DNA complex. This conclusion is in agreement with the crystallographic structure of the CAP–DNA complex (10), which indicates that the bottom-strand nucleotide of base pair 10 of the DNA half site is *the nucleotide closest* to amino acid 10 of the helix–turn–helix motif in the CAP–DNA complex (Fig. 2*B*).

CAP Derivative Having a Phenyl Azide Photoactivatable Crosslinking Agent at Amino Acid 2 of the Helix–Turn–Helix Motif: [(4-Azidophenacyl)–Cys^{170},Ser^{178}]CAP. Inspection of the three-dimensional structure of the CAP–DNA complex (10) suggested that covalent attachment of phenyl azide at amino acid 2 of the helix–turn–helix motif of CAP would place the phenyl azide moiety in close proximity to DNA in the protein–DNA complex—potentially close enough to crosslink (Fig. 2*C*). We have constructed and analyzed a CAP derivative having phenyl azide at amino acid 2 of the helix–turn–helix motif: i.e., [(4-azidophenacyl)–Cys^{170},Ser^{178}]CAP.

To construct this CAP derivative, we used site-directed mutagenesis to eliminate the preexisting unique solvent-

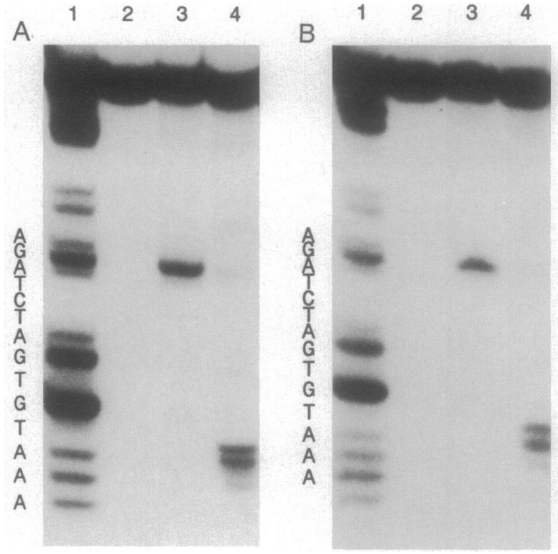

FIG. 4. Determination of the nucleotide(s) at which crosslinking occurs. (*A*) Data for DNA fragment ^{32}P 5′-end-labeled on the top DNA strand. Lanes: 1, Maxam–Gilbert G>A sequencing reaction of uncrosslinked DNA fragment (18); 2, alkali/heat reaction of uncrosslinked DNA fragment; 3, alkali/heat reaction of crosslinked protein–DNA complex formed with the CAP derivative having a phenyl azide photoactivatable crosslinking agent at amino acid 10 of the helix–turn–helix motif; 4, alkali/heat reaction of crosslinked protein–DNA complex formed with the CAP derivative having a phenyl azide photoactivatable crosslinking agent at amino acid 2 of the helix–turn–helix motif. (*B*) Data for DNA fragment ^{32}P 5′-end-labeled on the bottom DNA strand. Lanes are the same as in *A*.

accessible cysteine residue at amino acid 10 of the helix–turn–helix motif (by replacement of Cys^{178} with serine, constructing [Ser^{178}]CAP; ref. 15) and to introduce a unique solvent-accessible cysteine residue at amino acid 2 of the helix–turn–helix motif (by replacement of Gln^{170} with cysteine, constructing [Cys^{170},Ser^{178}]CAP; ref. 15), and we reacted the resulting mutant CAP with 4-azidophenacyl bromide under conditions that resulted in complete and highly selective derivatization of the solvent-accessible cysteine residue.

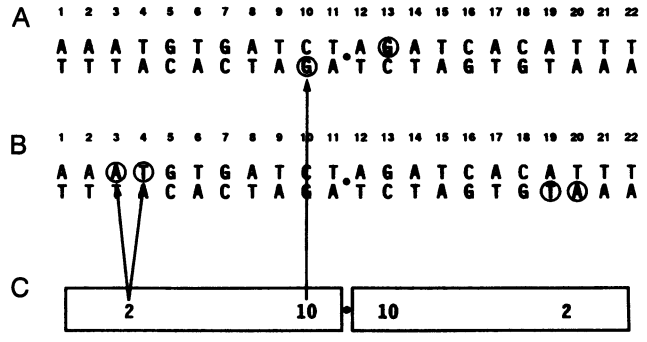

FIG. 5. (*A*) Summary of crosslinking by the CAP derivative having a phenyl azide photoactivatable crosslinking agent at amino acid 10 of the helix–turn–helix motif. Crosslinking occurs at one nucleotide (circled) in each DNA half site. (*B*) Summary of crosslinking by the CAP derivative having a phenyl azide photoactivatable crosslinking agent at amino acid 2 of the helix–turn–helix motif. Crosslinking occurs at two nucleotides (circled) in each DNA half site. (*C*) Conclusion: Amino acid 10 of the helix–turn–helix motif is close to the bottom-strand nucleotide of base pair 10 of the DNA half site in the CAP–DNA complex, and amino acid 2 of the helix–turn–helix motif is close to the top-strand nucleotides of base pairs 3 and 4 of the DNA half site in the CAP–DNA complex. Note: Positions within the DNA site are numbered as in refs. 7–9, 12–15, and 19. A different numbering convention is used in ref. 10.

To assess protein–DNA crosslinking with this CAP derivative, we used procedures identical to those used with the preceding CAP derivative. The results in Fig. 3*B* indicate that UV irradiation results in formation of a crosslinked protein–DNA complex. The efficiency of crosslinking is ≈0.5%. The electrophoretic mobility of the crosslinked protein–DNA complex with this CAP derivative, the stability properties of the crosslinked protein–DNA complex with this CAP derivative, and the requirements for formation of the crosslinked protein–DNA complex with this CAP derivative are as described for the preceding CAP derivative.

The results in Fig. 4 indicate that crosslinking with this CAP derivative occurs at two adjacent nucleotides of the top DNA strand and at two adjacent nucleotides of the bottom DNA strand. The nucleotides at which crosslinking occurs occupy 2-fold symmetry-related positions within the 2-fold symmetric DNA site for CAP: i.e., crosslinking occurs at the top-strand nucleotides of base pairs 3 and 4 of each DNA half site (Fig. 5*B*). We conclude from the photocrosslinking results that the top-strand nucleotides of base pairs 3 and 4 of the DNA half site are close to amino acid 2 of the helix–turn–helix motif in the CAP–DNA complex. This conclusion is in agreement with the crystallographic structure of the CAP–DNA complex (10), which indicates that the top-strand nucleotides of base pairs 3 and 4 of the DNA half site are *the nucleotides closest* to amino acid 2 of the helix–turn–helix motif in the CAP–DNA complex (Fig. 2*C*).

DISCUSSION

The photocrosslinking results indicate that amino acid 10 of the helix–turn–helix motif is close to the bottom-strand nucleotide of base pair 10 of the DNA half site in the CAP–DNA complex, and that amino acid 2 of the helix–turn–helix motif is close to the top-strand nucleotides of base pairs 3 and 4 of the DNA half site in the CAP–DNA complex. The photocrosslinking results are sufficient to define unambiguously the orientation of the helix–turn–helix motif relative to the DNA half site in the CAP–DNA complex (Fig. 5*C*) and, in conjunction with the three-dimensional structure of the helix–turn–helix motif, are sufficient to define a three-dimensional model for the interaction between the helix–turn–helix motif and the DNA half site. The amino acid–nucleotide proximities deduced from the photocrosslinking results, and the orientation of the helix–turn–helix motif relative to the DNA half site deduced from the photocrosslinking results, are in complete agreement with the crystallographic structure of the CAP–DNA complex (ref. 10; Fig. 2). We conclude that the photocrosslinking method yields accurate, reliable proximity and orientation information. We conclude further that the photocrosslinking method yields high-resolution proximity and orientation information: *single-nucleotide resolution*.

It is noteworthy that crosslinking occurred both with phenyl azide incorporated at amino acid 10 of the helix–turn–helix motif of CAP, an amino acid near the DNA minor groove in the CAP–DNA complex (10), and with phenyl azide incorporated at amino acid 2 of the helix–turn–helix motif of CAP, an amino acid near the DNA major groove in the CAP–DNA complex (10). We conclude that phenyl azide photoactivatable crosslinking agents are capable of reacting both with determinants in the DNA minor groove and with determinants in the DNA major groove. It also is noteworthy that crosslinking was observed at an adenine, a thymine, and a guanine. We conclude that phenyl azide photoactivatable crosslinking agents are capable of reacting to yield alkali/heat-labile adducts with at least three of the four DNA nucleotides.

We believe that the photocrosslinking method of this report is the most effective biochemical method to determine the orientation of a DNA binding motif in a protein–DNA complex. The alternative biochemical method to determine the orientation of a DNA binding motif in a protein–DNA complex is to incorporate a nucleolytic chelator–metal complex (e.g., 1,10-phenanthroline–copper or EDTA–iron) at a single site within the DNA binding motif of the sequence-specific DNA binding protein under investigation, to form the derivatized protein–DNA complex, and to determine the nucleotides at which DNA cleavage occurs (19, 22–28). The alternative biochemical method has the disadvantage that reaction can occur through a diffusible intermediate: i.e., hydroxyl radical (22–28). Reaction through a diffusible intermediate significantly reduces resolution and can complicate interpretation of results. [DNA cleavage by (1,10-phenanthroline–copper)–protein conjugates typically occurs over 3–9 nucleotide pairs (19, 22); DNA cleavage by (EDTA–iron)–protein conjugates typically occurs over 6–14 nucleotide pairs (23–28).]

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