Incorporation of an EDTA–Metal Complex at a Rationally Selected Site within a Protein: Application to EDTA–Iron DNA Affinity Cleaving with Catabolite Gene Activator Protein (CAP) and Cro†

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ABSTRACT: We have developed a simple procedure to incorporate an EDTA–metal complex at a rationally selected site within a full-length protein. Our procedure has two steps: In step 1, we use site-directed mutagenesis to introduce a unique solvent-accessible cysteine residue at the site of interest. In step 2, we derivatize the resulting protein with *S*-(2-pyridylthio)cysteaminyl-EDTA–metal, a novel aromatic disulfide derivative of EDTA–metal. We have used this procedure to incorporate an EDTA–iron complex at amino acid 2 of the helix–turn–helix motif of each of two helix–turn–helix motif sequence-specific DNA binding proteins, catabolite gene activator protein (CAP) and Cro, and we have analyzed EDTA–iron-mediated DNA affinity cleavage by the resulting protein derivatives. The CAP derivative cleaves DNA at base pair 2 of the DNA half-site in the protein–DNA complex, and the Cro derivative cleaves DNA at base pairs −3 to 5 of the DNA half-site in the protein–DNA complex. We infer that amino acid 2 of the helix–turn–helix motif of CAP is close to base pair 2 of the DNA half-site in the CAP–DNA complex in solution and that amino acid 2 of the helix–turn–helix motif of Cro is close to base pairs −3 to 5 of the DNA half-site in the Cro–DNA complex in solution. The results are in excellent agreement with the crystallographic structures of the CAP–DNA and Cro–DNA complexes [Schultz, S., Shields, S., & Steitz, T. (1991) *Science 253*, 1001; Brennan, R., Roderick, S., Takeda, Y., & Matthews, B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8165]. In addition to EDTA–iron affinity cleaving with sequence-specific DNA binding proteins, the procedure of this report has potential applications in site-specific radioactive labeling of protein, site-specific fluorescent labeling of protein, and site-specific heavy-atom labeling of protein.

EDTA forms complexes with a wide range of metal ions, including radioactive, fluorescent, and reactive metal ions (Bjerrum et al., 1957; Bell, 1977). EDTA–metal complexes can be conjugated to proteins having specificity for target biomolecules [e.g., monoclonal antibodies and sequence-specific DNA binding proteins (Sundberg et al., 1974; Leung & Meares, 1977; Leung et al., 1978; Yeh et al., 1979a,b; Scheinberg et al., 1982; Wensel & Meares, 1983; Meares & Wensel, 1984; Meares et al., 1984; Hemmilä et al., 1984; Brechbiel et al., 1986; Sluka et al., 1987, 1990; Mack et al., 1990; Graham & Dervan, 1990; Oakley & Dervan, 1990; Rana & Meares, 1990a,b, 1991a,b; Diamandis & Christopoulos, 1990; Shin et al., 1991; Dervan, 1991; Arya & Gariépy, 1991)]. The resulting (EDTA–metal)–protein conjugates have applications as radiodiagnostics, radiotherapeutics, and affinity cleaving reagents.

For certain applications, it is essential to incorporate an EDTA–metal complex at a rationally selected site within a protein. One such application is EDTA–iron DNA affinity cleaving to determine the location and orientation of the DNA site for a sequence-specific DNA binding protein (Sluka et al., 1987; Mack et al., 1990; Graham & Dervan, 1990; Oakley & Dervan, 1990; Shin et al., 1991; Dervan, 1991). In EDTA–iron DNA affinity cleaving, one incorporates EDTA–iron at an amino acid not critical for protein–DNA complex formation but nevertheless close to DNA in the protein–DNA complex, one forms the derivatized protein–DNA complex, and one determines the nucleotide(s) at which EDTA–iron-mediated DNA cleavage occurs.

Previously, incorporation of an EDTA–metal complex at a rationally selected site within a protein has been possible only by total synthesis of the protein (Sluka et al., 1987, 1990; Mack et al., 1990; Graham & Dervan, 1990; Oakley & Dervan, 1990; Shin et al., 1991; Dervan, 1991; Arya & Gariépy, 1991)—a procedure usually limited to proteins of less than 60 amino acids. In this report, we describe a procedure to incorporate an EDTA–metal complex at a rationally selected site within any protein not having a preexisting essential solvent-accessible cysteine residue. Our procedure has two steps: In step 1, we use site-directed mutagenesis (Kunkel et al., 1991) to introduce a unique solvent-accessible cysteine residue at the site of interest. In step 2, we derivatize the resulting protein with *S*-(2-pyridylthio)cysteaminyl-EDTA–metal, a novel aromatic disulfide derivative of EDTA–metal (Figure 1). In addition, in this report, we describe application of our procedure to EDTA–iron affinity DNA cleaving with each of two sequence-specific DNA binding proteins: *Escherichia coli* catabolite gene activator protein (CAP; also referred to as the cAMP receptor protein, CRP) and bacteriophage λ Cro.

MATERIALS AND METHODS

S-(2-Pyridylthio)cysteamine Hydrochloride. Pyridyl disulfide (Aldrich; 4.41 g, 20.0 mmol) was dissolved in 20 mL of methanol and 0.8 mL of acetic acid. Into this solution was added dropwise over a period of 0.5 h 2-aminoethanethiol hydrochloride (Aldrich; 1.14 g, 10.0 mmol) in 10 mL of methanol. The reaction mixture was stirred for an additional 48 h and was then evaporated under high vacuum to a yellow

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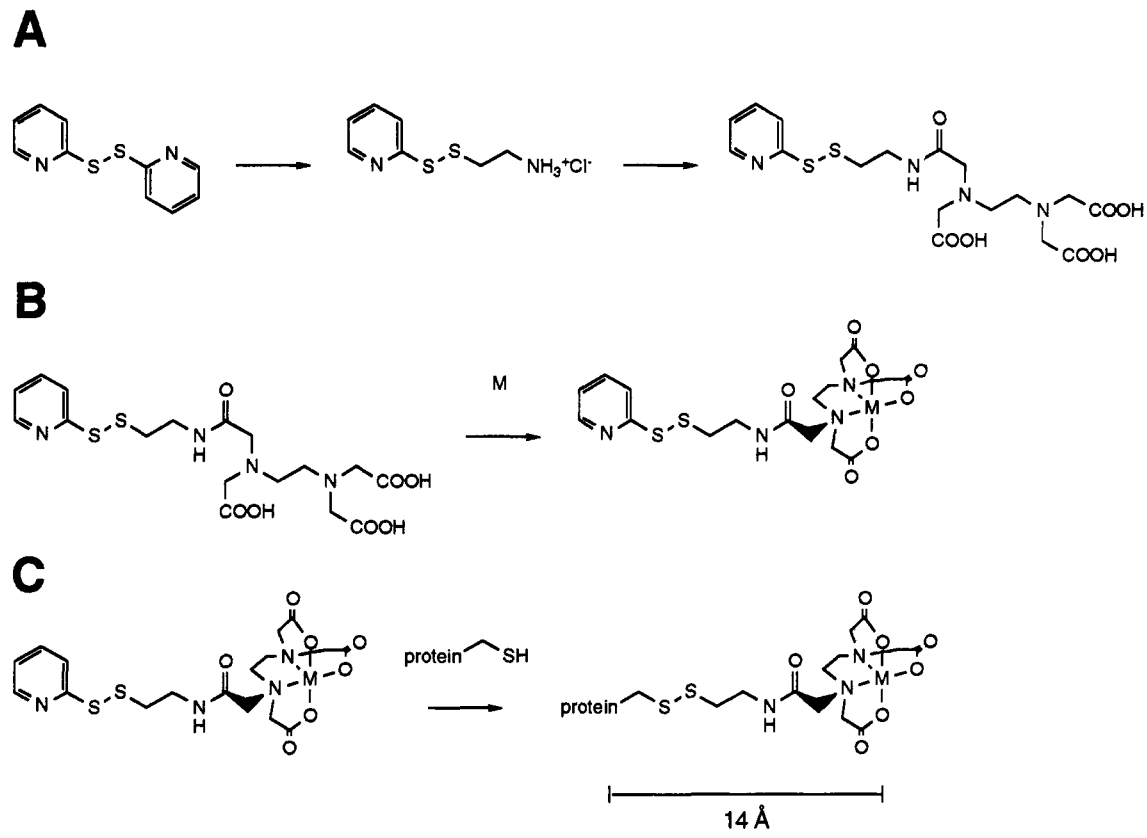

FIGURE 1: (A) Synthesis of *S*-(2-pyridylthio)cysteaminyl-EDTA. (B) Reaction of *S*-(2-pyridylthio)cysteaminyl-EDTA with a metal ion (M), yielding *S*-(2-pyridylthio)cysteaminyl-EDTA–metal. (C) Reaction of *S*-(2-pyridylthio)cysteaminyl-EDTA–metal with a protein having a unique solvent-accessible cysteine residue, yielding a conjugate of the form [(cysteaminyl-EDTA–metal)-Cys]protein. If fully extended, the linker arm between the α carbon of the cysteine residue and the metal ion is 14 Å.

oil. The product was washed with 50 mL of diethyl ether and was dissolved in 10 mL of methanol. The product was precipitated by addition of 50 mL of diethyl ether and was redissolved in 10 mL of methanol (five times). Yield: 1.68 g, 75.5%. TLC (silica gel; ammonium hydroxide:95% ethanol, 1:99 v/v): R_f = 0.8. NMR (CD_3OD; tetramethylsilane as reference): 3.15 (t, 2), 3.30 (t, 2), 7.32 (t, 1), 7.64 (d, 1), 7.80 (t, 1), 8.54 (d, 1).

EDTA Monoanhydride. In this and subsequent steps, glassware was acid-washed, and water and aqueous solutions were treated with Chelex-100 (Bio-Rad), in order to avoid trace metal contamination. EDTA monoanhydride was prepared by a modification of the procedure of Takeshita et al. (1982). EDTA dianhydride (Aldrich; 5.00 g, 19.5 mmol) was suspended in 32 mL of anhydrous dimethylformamide and was heated to 100 °C. The resulting solution was cooled to 75 °C. Into this solution was added dropwise over a period of 1 h deionized water (0.350 mL, 19.4 mmol). The reaction mixture was stirred for an additional 2 h at 75 °C, after which time the heat was removed, and the reaction mixture was stirred overnight to allow the product to precipitate. The product was collected by vacuum filtration and was dried under high vacuum. Yield: 4.09 g, 77%.

S-(2-Pyridylthio)cysteaminyl-EDTA. S-(2-Pyridylthio)-cysteamine hydrochloride (0.812 g, 3.65 mmol) and EDTA monoanhydride (1.00 g, 3.65 mmol) were suspended in 50 mL of anhydrous dimethylformamide. Into this mixture was added triethylamine (0.51 mL, 3.65 mmol). The reaction mixture was stirred for 17 h, after which time 10 mL of deionized water was added, and the reaction mixture was stirred for an additional 2 h. The reaction mixture was evaporated under high vacuum to a solid. The product was purified by flash chromatography (50 mL of dry silica gel, 230–400 mesh; ammonium hydroxide:95% ethanol, 1:9 v/v). Yield: 0.695 g, 37.3%. TLC (silica gel; ammonium hydroxide: 95% ethanol, 1:9 v/v): R_f = 0.2. NMR (D_2O): 2.79 (t, 2), 2.84 (t, 2), 3.05 (s, 2), 3.14 (s, 2), 3.18 (t, 2), 3.33 (t, 2), 3.63 (s, 4), 7.05 (t, 1), 7.60 (br m, 2), 8.15 (d, 1).

S-(2-Pyridylthio)cysteaminyl-EDTA-^{55}Fe(III). Reaction mixtures (100 μL) contained 10 mM *S*-(2-pyridylthio)-cysteaminyl-EDTA, 15 mM $^{55}Fe(III)NH_4(SO_4)_2$ (0.4 Bq/fmol), 50 mM potassium citrate (pH 5.6), and 200 mM KCl. Reactions were carried out in the dark and proceeded for 30 min at 23 °C.

Plasmids Encoding CAP and Cro Derivatives. Plasmids pXZCRP-178S and pXZCRP-170C;178S encode [Ser178]-CAP and [Cys170;Ser178]CAP, respectively, under control of the *crp* promoter (Zhang et al., 1991). Plasmids pAP119 (Pakula & Sauer, 1989; gift of Dr. Robert Sauer, Massachusetts Institute of Technology, Cambridge, MA) and pYCCRO-17C encode wild-type Cro and [Cys17]Cro, respectively, under control of the *tac* promoter. Plasmid pYCCRO-17C was constructed from plasmid pAP119 by use of site-directed mutagenesis [method of Kunkel et al. (1991), except that template DNA was prepared in *E. coli* strain XL1-Blue (Stratagene)].

[(Cysteaminyl-EDTA-^{55}Fe(III))-Cys170;Ser178]CAP. Reaction mixtures (500 μL) contained 0.006 mM [Cys170; Ser178]CAP [purified according to the procedure of Zhang et al. (1991)], 0.2 mM *S*-(2-pyridylthio)cysteaminyl-EDTA-^{55}Fe(III), 0.1 mM $^{55}Fe(III)NH_4(SO_4)_2$, 20 mM Tris-HCl (pH 8.0), 200 mM KCl, 1 mM potassium citrate, 0.1 mM EDTA, and 5% glycerol. Reactions were carried out in the dark and proceeded for 10 min at 23 °C. The product was purified by chromatography on Bio-Gel P-6DG (Bio-Rad), and the product was stored at −70 °C in a solution of 20 mM

Tris-HCl (pH 8.0), 200 mM KCl, and 5% glycerol.

Efficiencies of incorporation of EDTA–iron were determined by liquid scintillation counting in ScintiVerse II (Fisher).

[(Cysteaminyl-EDTA·^{55}Fe(III))-Cys17]Cro. Reactions were carried out as described in the preceding paragraph, using [Cys17]Cro [purified according to the procedure for purification of wild-type Cro of Leighton and Lu (1987)].

DNA Affinity Cleaving: [(Cysteaminyl-EDTA·^{55}Fe(III))-Cys170;Ser178]CAP. Reaction mixtures (100 μL) contained 0.1 nM ^{32}P-labeled DNA fragment ICAP [20 Bq/fmol (Ebright et al., 1989)], 100 nM [(cysteaminyl-EDTA·^{55}Fe-(III))-Cys170;Ser178]CAP, 1 mM ascorbic acid, 0.2 mM cAMP, 10 mM MOPS–NaOH (pH 7.3), 200 mM NaCl, and 50 μg/mL bovine serum albumin. Reactions proceeded for 3 h at 23 °C. Reactions were phenol extracted twice, were chloroform extracted, and were ethanol precipitated. Products were analyzed by denaturing gel electrophoresis through 20% polyacrylamide–8.3 M urea slab gels (Maxam & Gilbert, 1980). Following electrophoresis, gels were dried and were autoradiographed using Kodak X-Omat XAR5 film. Fragment lengths were determined by comparison to a Maxam–Gilbert G > A sequencing reaction (Maxam & Gilbert, 1980) of the same DNA fragment.

DNA Affinity Cleaving: [(Cysteaminyl-EDTA·^{55}Fe(III))-Cys17]Cro. Reactions were carried out as described in the preceding paragraph, using DNA fragment IλCRO [5′-G-C AACGCAATTCTATCACCGCCG GTGATAAATAG-GCACCC-3′/5′-GGGGTGCCTATTTATC AC C GGCG-GTGATAGAATTGCGTTG-3′; prepared according to the procedure of Ebright et al. (1989)] and [(cysteaminyl-EDTA·^{55}Fe(III))-Cys17]Cro, and omitting cAMP.

RESULTS AND DISCUSSION

S-(2-Pyridylthio)cysteaminyl-EDTA·^{55}Fe(III). *S*-(2-Pyridylthio)cysteaminyl-EDTA was prepared in two steps: (i) reaction of pyridyl disulfide with 2-aminoethanethiol hydrochloride, yielding *S*-(2-pyridylthio)cysteamine hydrochloride, and (ii) reaction of *S*-(2-pyridylthio)cysteamine hydrochloride with EDTA monoanhydride (Figure 1A). The overall yield was 28%. *S*-(2-Pyridylthio)cysteaminyl-EDTA·^{55}Fe(III) was prepared by incubation of *S*-(2-pyridylthio)cysteaminyl-EDTA with a 1.5-fold molar excess of $^{55}Fe(III)NH_4(SO_4)_2$ in citrate buffer at pH 5.6 (Figure 1B). *S*-(2-Pyridylthio)-cysteaminyl-EDTA·^{63}Ni(II) and *S*-(2-pyridylthio)cysteaminyl-EDTA·Eu(III) were prepared in an analogous fashion (Y. W. Ebright and R. H. Ebright, unpublished results).

CAP Derivative Having EDTA–Iron at Amino Acid 2 of the Helix–Turn–Helix Motif of CAP: [(Cysteaminyl-EDTA·^{55}Fe(III))-Cys170;Ser178]CAP. CAP is a helix–turn–helix motif sequence-specific DNA binding protein [review of CAP in de Crombrugghe et al. (1984); reviews of helix–turn–helix motif in Takeda et al. (1983) and Pabo and Sauer (1984)]. CAP binds to a 22-base-pair 2-fold-symmetric DNA site: 5′-AAATGTGATCTAGATCACATTT-3′ (Ebright et al., 1989). The crystallographic structure of CAP has been determined to 2.5-Å resolution (Weber & Steitz, 1987), and the crystallographic structure of the CAP–DNA complex has been determined to 3.0-Å resolution (Schultz et al., 1991). CAP is a dimer of two identical subunits, each of which is 209 amino acids in length and contains a helix–turn–helix motif. 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.

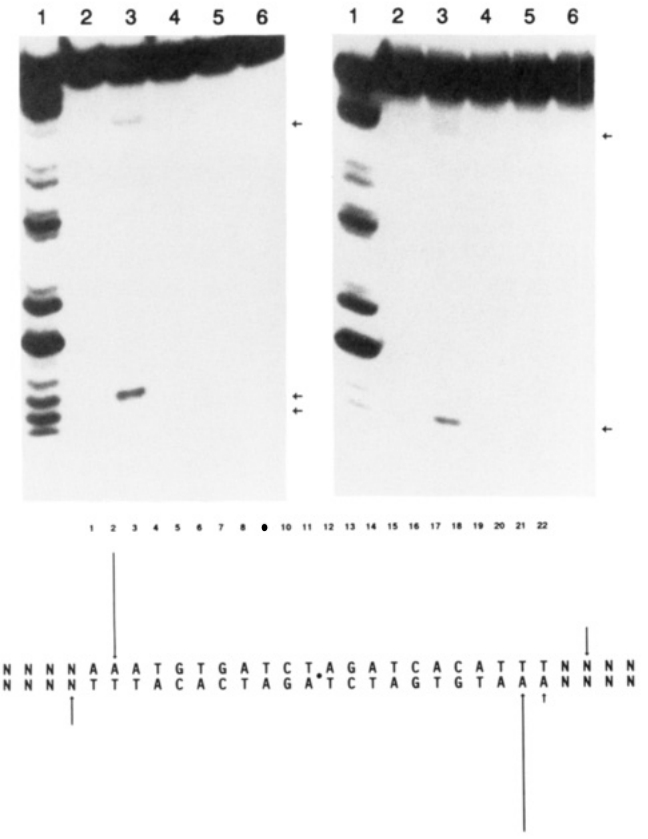

FIGURE 2: DNA affinity cleaving by the CAP derivative having EDTA–iron at amino acid 2 of the helix–turn–helix motif: [(cysteaminyl-EDTA–iron)-Cys170;Ser178]CAP. (A, top left) Data for DNA fragment ^{32}P-5′-end-labeled on the bottom DNA strand: lane 1, Maxam–Gilbert G > A sequencing reaction (Maxam & Gilbert, 1980); lane 2, uncleaved DNA fragment; lane 3, DNA affinity cleaving reaction; lane 4, control reaction omitting the CAP derivative; lane 5, control reaction omitting cAMP; lane 6, control reaction omitting ascorbic acid. Arrows indicate DNA cleavage products. (B, top right) Data for DNA fragment ^{32}P-5′-end-labeled on the top DNA strand. Lanes as in (A). (C, bottom) Summary of DNA affinity cleaving by the CAP derivative. The figure illustrates the 22-base-pair 2-fold-symmetric consensus DNA site for CAP (Ebright et al., 1989). Arrows indicate the nucleotides at which DNA cleavage occurs; the lengths of the arrows indicate the relative extents of cleavage. Note: Positions within the DNA site for CAP are numbered as in de Crombrugghe et al. (1984), Ebright et al. (1984a,b, 1987, 1989, 1990), Zhang et al. (1991), and Pendergrast et al. (1992). A different numbering convention is used in Schultz et al. (1991).

Inspection of the structure of the CAP–DNA complex suggested that covalent incorporation of EDTA–iron at amino acid 2 of the helix–turn–helix motif of CAP (i.e., at amino acid 170 of CAP) would place the EDTA–iron moiety in close proximity to DNA in the protein–DNA complex. Therefore, we have constructed and analyzed a CAP derivative having EDTA–iron at amino acid 2 of the helix–turn–helix motif: i.e., [(cysteaminyl-EDTA·^{55}Fe(III))-Cys170;Ser178]CAP.

Wild-type CAP has a preexisting solvent-accessible cysteine residue at a position other than amino acid 2 of the helix–turn–helix motif and has no preexisting solvent-accessible cysteine residue at amino acid 2 of the helix–turn–helix motif (Ebright et al., 1985, 1990; Zhang et al., 1991; Pendergrast et al., 1992). To construct the CAP derivative of interest, we used two successive cycles of site-directed mutagenesis: one to eliminate the preexisting solvent-accessible cysteine residue at the position other than amino acid 2 of the helix–turn–helix motif [by replacement of Cys178 by serine, constructing [Ser178]CAP (Zhang et al., 1991)] and one to introduce a unique solvent-accessible cysteine residue at amino acid 2 of the helix–turn–helix motif [by replacement of Gln170 by

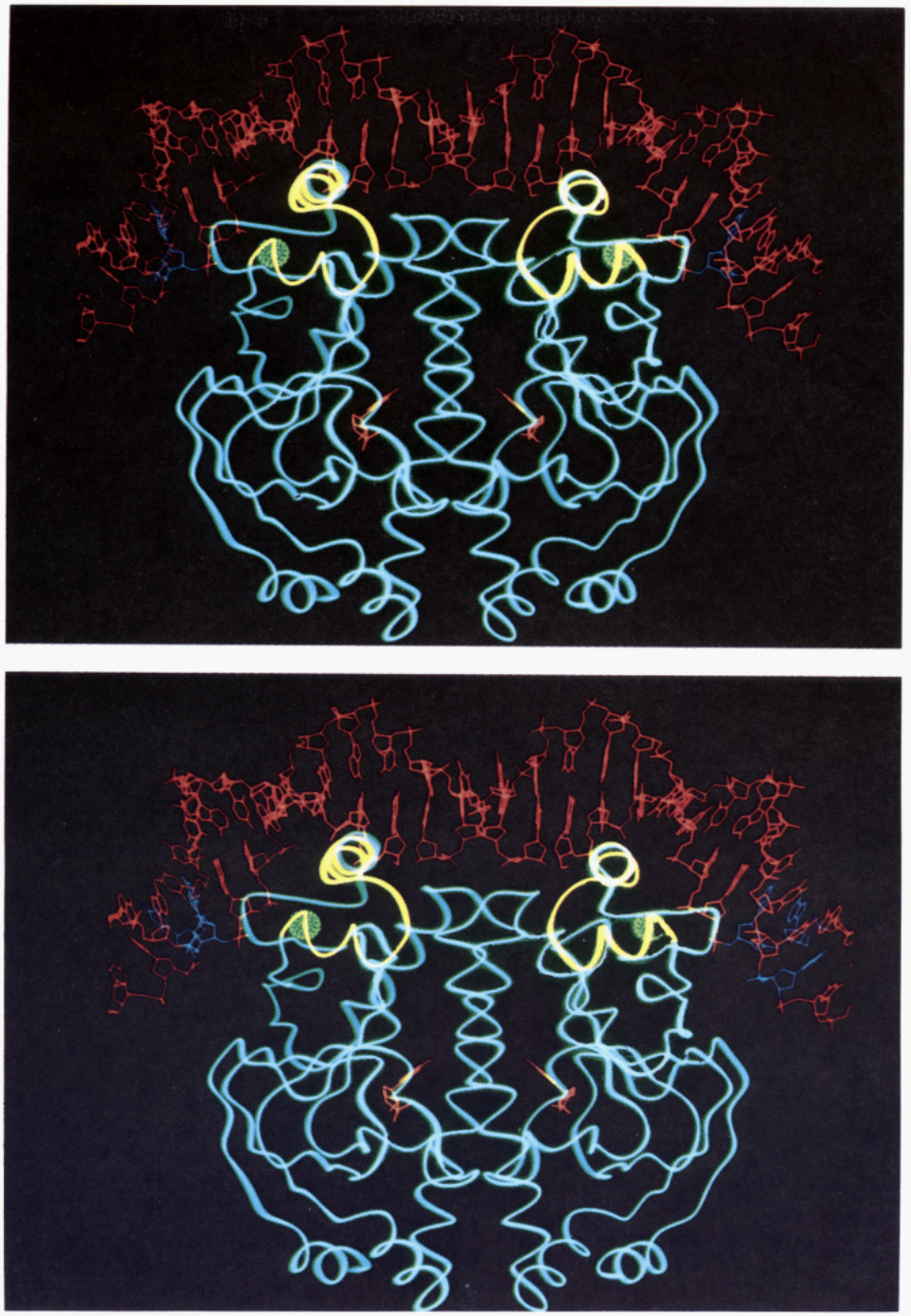

FIGURE 3: Structure of the CAP–DNA complex (Schultz et al., 1991). The helix–turn–helix motif of CAP is illustrated in yellow, and the α carbon of amino acid 2 of the helix–turn–helix motif of CAP is illustrated in green. In (A, top), the nucleotides at which highest DNA affinity cleaving occurs are illustrated in blue. The mean distance between the α carbon of amino acid 2 of the helix–turn–helix motif and the C1′ atoms of the nucleotides at which highest DNA affinity cleaving occurs is 11 ± 0 Å. In (B, bottom), all nucleotides at which DNA affinity cleaving occurs are illustrated in blue. The mean distance between the α carbon of amino acid 2 of the helix–turn–helix motif and the C1′ atoms of all nucleotides at which DNA affinity cleaving occurs is 14 ± 3 Å (11–16 Å). Method: Coordinates for the crystallographic structure of the CAP–DNA complex were obtained from the Brookhaven Protein Data Bank (accession code 1CGP).

cysteine, constructing [Cys170;Ser178]CAP (Zhang et al., 1991)]. We then reacted [Cys170;Ser178]CAP with *S*-(2-pyridylthio)cysteaminyl-EDTA·^{55}Fe(III). Quantitation of radioactivity indicated that 0.3 mol of EDTA–iron was incorporated per mole of [Cys170;Ser178]CAP. In control experiments, we reacted [Ser178]CAP with *S*-(2-pyridylthio)-cysteaminyl-EDTA·^{55}Fe(III). Quantitation of radioactivity indicated that <0.03 mol of EDTA–iron was incorporated per mole of [Ser178]CAP. We conclude that incorporation of EDTA–iron in the case of [Cys170;Ser178]CAP occurs exclusively, or nearly exclusively, at amino acid 2 of the helix–turn–helix motif.

To assay DNA affinity cleaving by the CAP derivative, we used a 40-base-pair DNA fragment containing the consensus DNA site for CAP [DNA fragment "ICAP" (Ebright et al., 1989)]. We reacted the ^{32}P-end-labeled DNA fragment with the CAP derivative, cAMP (the allosteric effector required for DNA binding by CAP), and ascorbic acid, and we analyzed the reaction products by denaturing polyacrylamide gel electrophoresis followed by autoradiography. The results in

Figure 2 indicate that DNA cleavage occurs primarily at one nucleotide on each DNA strand. The nucleotides at which DNA cleavage occurs occupy 2-fold-symmetry-related positions within the DNA site for CAP; i.e., DNA cleavage occurs primarily at the top-strand nucleotide of base pair 2 of each DNA half-site. Control experiments establish that DNA cleavage requires the CAP derivative, requires cAMP, and requires ascorbic acid. Additional control experiments establish that neither underivatized CAP nor underivatized CAP plus equimolar EDTA–iron is able to substitute for the CAP derivative. We conclude that amino acid 2 of the helix–turn–helix motif of CAP is close to the top-strand nucleotide of base pair 2 of the DNA half-site in the CAP–DNA complex in solution. We conclude further than the helix–turn–helix motif of CAP is oriented such that its N-terminus is far from, and its C-terminus is close to, the 2-fold axis of the DNA site in the CAP–DNA complex in solution. These conclusions are in excellent agreement with the crystallographic structure of the CAP–DNA complex (Figure 3; Schultz et al., 1991) and with the results of genetic (Ebright et al., 1984a,b, 1987; Zhang & Ebright, 1990) and photo-cross-linking (Pendergrast et al., 1992) analyses of the CAP–DNA complex. In the crystallographic structure of the CAP–DNA complex, the α carbon of amino acid 2 of the helix–turn–helix motif of CAP is 11 Å from the C1′ atom of the top-strand nucleotide of base pair 2 of the DNA half-site (Figure 3; Schultz et al., 1991).

Cro Derivative Having EDTA–Iron at Amino Acid 2 of the Helix–Turn–Helix Motif of Cro: [(*Cysteaminyl-EDTA·*^{55}*Fe(III))-Cys17*]*Cro*. To demonstrate that the procedure of this report is generalizable, we have performed analogous experiments with a second helix–turn–helix motif sequence-specific DNA binding protein: Cro (Brennan et al., 1990). Cro binds to a 17-base-pair 2-fold-symmetric DNA site 5′-TATCACCGCCGGTGATA-3′ (Takeda et al., 1989). The crystallographic structure of Cro has been determined to 2.8-Å resolution (Anderson et al., 1981), and the crystallographic structure of the Cro–DNA complex has been determined to 3.9-Å resolution (Brennan et al., 1990).

Inspection of the structure of the Cro–DNA complex suggested that covalent incorporation of EDTA–iron at amino acid 2 of the helix–turn–helix motif of Cro (i.e., at amino acid 17 of Cro) would place the EDTA–iron moiety in close proximity to DNA in the protein–DNA complex—analogously to covalent incorporation of EDTA–iron at amino acid 2 of the helix–turn–helix motif of CAP. We have constructed and analyzed a Cro derivative having EDTA–iron at amino acid 2 of the helix–turn–helix motif of Cro: i.e., [(cysteaminyl-EDTA·^{55}Fe(III))-Cys17]Cro.

Wild-type Cro has no preexisting cysteine residues (Hsiang et al., 1977; Schwarz et al., 1978). To construct the Cro derivative of interest, we used site-directed mutagenesis to introduce a unique solvent-accessible cysteine residue at amino acid 2 of the helix–turn–helix motif (by replacement of Thr17 by cysteine, constructing [Cys17]Cro). We then reacted [Cys17]Cro with *S*-(2-pyridylthio)cysteaminyl-EDTA·^{55}Fe(III), using reaction conditions identical to those used for the CAP derivative. Quantitation of radioactivity indicated that 0.8 mol of EDTA–iron was incorporated per mole of [Cys17]Cro. In control experiments, we reacted wild-type Cro with *S*-(2-pyridylthio)cysteaminyl-EDTA·^{55}Fe(III). Quantitation of radioactivity indicated that 0 mol of EDTA–iron was incorporated per mole of wild-type Cro. We conclude that incorporation of EDTA–iron in the case of [Cys17]Cro occurs exclusively, or nearly exclusively, at amino acid 2 of the helix–turn–helix motif.

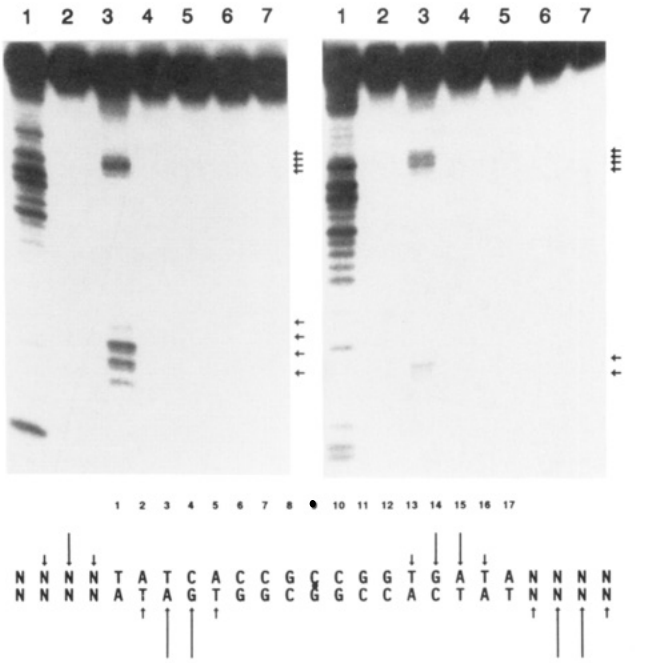

FIGURE 4: DNA affinity cleaving by the Cro derivative having EDTA–iron at amino acid 2 of the helix–turn–helix motif: [(cysteaminyl-EDTA–iron)-Cys17]Cro. (A, top left) Data for DNA fragment ^{32}P-5′-end-labeled on the bottom DNA strand: lane 1, Maxam–Gilbert G > A sequencing reaction (Maxam & Gilbert, 1980); lane 2, uncleaved DNA fragment; lane 3, DNA affinity cleaving reaction; lane 4, control reaction omitting the Cro derivative; lane 5, control reaction omitting ascorbic acid; lane 6, control reaction substituting underivatized Cro for the Cro derivative; lane 7, control reaction substituting underivatized Cro plus equimolar EDTA–iron for the Cro derivative. Arrows indicate DNA cleavage products. (B, top right) Data for DNA fragment ^{32}P-5′-end-labled on the top DNA strand. Lanes as in (A). (C, bottom) Summary of DNA affinity cleaving by the Cro derivative. The figure illustrates the 17-base-pair 2-fold-symmetric consensus DNA site for Cro (Takeda et al., 1989). Arrows indicate the nucleotides at which DNA cleavage occurs; the lengths of the arrows indicate the relative extents of cleavage.

To assay DNA affinity cleaving by the Cro derivative, we used a 40-base-pair DNA fragment containing the consensus DNA site for Cro (Takeda et al., 1989). The results in Figure 4 indicate that DNA cleavage occurs at 7–8 nucleotides on each DNA strand. The nucleotides at which DNA cleavage occurs occupy 2-fold-symmetry-related positions within the DNA site for Cro; i.e., DNA cleavage occurs at the top-strand nucleotides of base pairs –3, –2, and –1 and the bottom-strand nucleotides of base pairs 2, 3, 4, and 5 of each DNA half-site. Control experiments establish that DNA cleavage requires the Cro derivative and requires ascorbic acid. Additional control experiments establish that neither underivatized Cro nor underivatized Cro plus equimolar EDTA–iron is able to substitute for the Cro derivative. We conclude that amino acid 2 of the helix–turn–helix motif of Cro is close to the top-strand nucleotides of base pairs –3 to –1 and the bottom-strand nucleotides of base pairs 2 to 5 of the DNA half-site in the Cro–DNA complex in solution. We conclude further that the helix–turn–helix motif of Cro is oriented such that its N-terminus is far from, and its C-terminus is close to, the 2-fold axis of the DNA site in the Cro–DNA complex in solution. These conclusions are in excellent agreement with the crystallographic structure of the Cro–DNA complex (Figure 5; Brennan et al., 1990; R. Brennan, S. Roderick, and B. Matthews, personal communication). In the crystallographic structure of the Cro–DNA complex, the α carbon of amino acid 2 of the helix–turn–helix motif of Cro is an average of 13 Å (7–16 Å) from the C1′ atoms of the top-strand

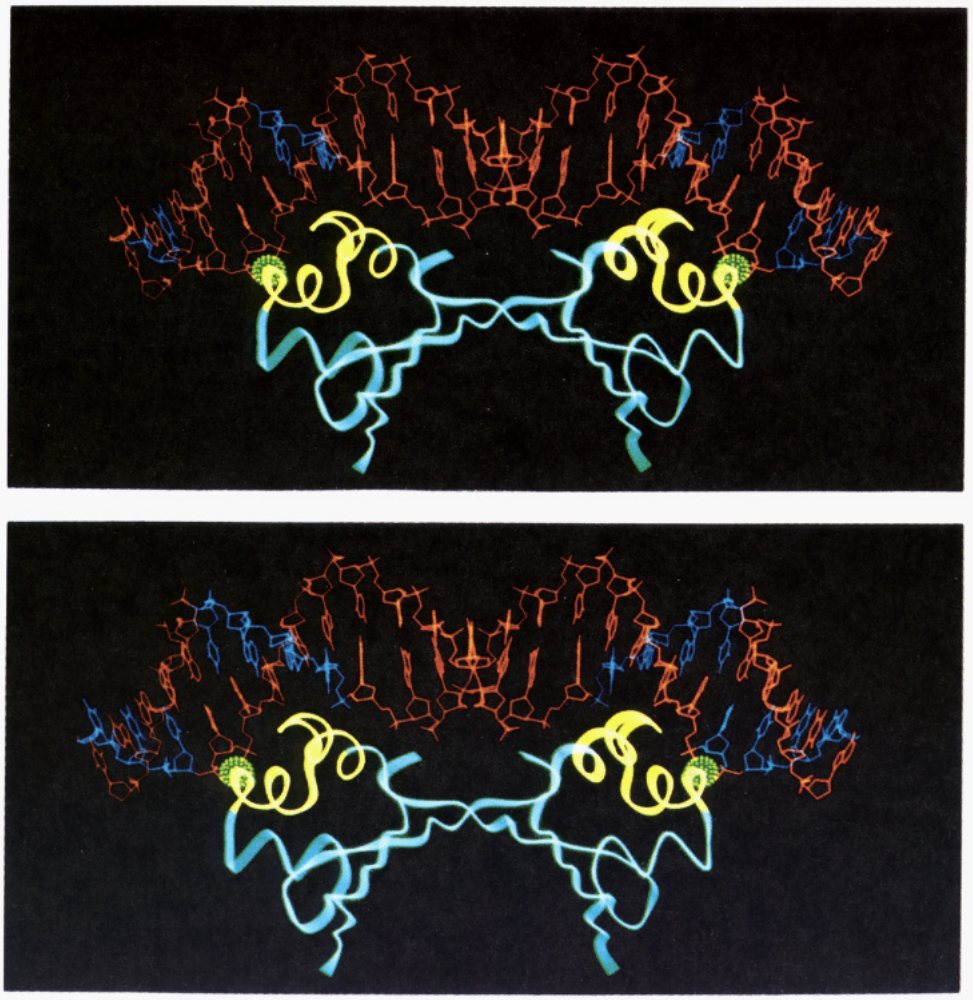

FIGURE 5: Structure of the Cro–DNA complex (Brennan et al., 1990; R. Brennan, S. Roderick, and B. Matthews, personal communication). The helix–turn–helix motif of Cro is illustrated in yellow, and the α carbon of amino acid 2 of the helix–turn–helix motif of Cro is illustrated in green. In (A, top), the nucleotides at which highest DNA affinity cleaving occurs are illustrated in blue. The mean distance between the α carbon of amino acid 2 of the helix–turn–helix motif and the C1′ atoms of the nucleotides at which highest DNA affinity cleaving occurs is 13 ± 3 Å (7–15 Å). In (B, bottom), all nucleotides at which DNA affinity cleaving occurs are illustrated in blue. The mean distance between the α carbon of amino acid 2 of the helix–turn–helix motif and the C1′ atoms of all nucleotides at which DNA affinity cleaving occurs is 13 ± 3 Å (7–16 Å). Method: Coordinates for the crystallographic structure of the Cro–DNA complex were obtained from R. Brennan, S. Roderick, and B. Matthews, University of Oregon, Eugene, OR. The positions of Cro backbone atoms and the positions of DNA backbone atoms have estimated uncertainties of ≈1 Å. The crystallographic structure of the Cro–DNA complex does not include base pairs –4 to –1 of the DNA half-site. Model coordinates for base pairs –4 to –1 of the DNA half-site were generated assuming that base pairs –4 to –1 of the DNA half-site exhibit canonical B-DNA conformation, and were generated using the program DNA FIT MAN (Warwicker et al., 1987).

nucleotides of base pairs –3 to –1 and the bottom-strand nucleotides of base pairs 2 to 5 of the DNA half-site (Figure 5; Brennan et al., 1990; R. Brennan, S. Roderick, and B. Matthews, personal communication).

Prospect. The procedure of this report permits covalent incorporation of an EDTA–metal complex at a rationally selected site within a full-length protein. In the work in this report, we have incorporated a radioactive and reactive EDTA–metal complex, EDTA·^{55}Fe(III), at a rationally selected site in CAP (a protein of 209 amino acids) and at a rationally selected site in Cro (a protein of 66 amino acids). The work in this report represents the first instance of covalent incorporation of an EDTA–metal complex at a rationally selected site in a protein of more than 56 amino acids and the first instance of covalent incorporation of an EDTA–metal complex at a rationally selected site in a protein produced from an expression system.

We anticipate that the procedure of this report will be generalizable to other EDTA–metal complexes. In unpublished work, we have used the procedure of this report to incorporate a second radioactive EDTA–metal complex, EDTA·^{63}Ni(II), at a rationally selected site within CAP and to incorporate a fluorescent and heavy-atom EDTA–metal complex, EDTA·Eu(III), at a rationally selected site within CAP (Y. W. Ebright and R H. Ebright, unpublished results).

In addition to EDTA–iron DNA affinity cleaving with helix–turn–helix motif sequence-specific DNA binding proteins and with other sequence-specific DNA binding proteins, the procedure of this report has potential applications in site-specific radioactive labeling of protein, site-specific fluorescent labeling of protein, and site-specific heavy-atom labeling of protein.

Note: While this report was under review for publication, Ermácora et al. (1992) described the synthesis of *S*-(2-pyridylthio)cysteaminyl-EDTA (by a different synthetic route) and application of *S*-(2-pyridylthio)cysteaminyl-EDTA in EDTA–iron protein cleaving.

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