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Orientation of the Putative Recognition Helix in the DNA-Binding Domain of Hin Recombinase Complexed with the Hix Site[†]

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ABSTRACT: On the basis of sequence similarity with other known DNA-binding proteins, the DNA-binding domain of Hin recombinase, residues 139–190, is thought to bind DNA by a helix–turn–helix motif. Two models can be considered that differ in the orientation of the recognition helix in the major groove of DNA. One is based on the orientation of the recognition helix found in the 434 repressor (1–69) and λ repressor–DNA cocrystals, and the other is based on the NMR studies of *lac* repressor headpiece. Cleavage by EDTA·Fe attached to a lysine side chain (Ser[183] → Lys[183]) near the COOH terminus of Hin(139–184) reveals that the putative recognition helix is oriented toward the center of the inverted repeats in a manner similar to that seen in the 434 and λ repressor–DNA cocrystals.

The structural class of DNA-binding proteins best characterized by crystallographic studies contains the helix–turn–helix motif. Comparison of the three dimensional structures of λ cro, λ repressor, and catabolite gene activator protein (CAP) led to the postulate that a conserved α-helix–turn–α-helix motif is involved in recognition of DNA in the major groove and may be a common structural motif for sequence-specific DNA affinity (Anderson et al., 1981; McKay & Steitz, 1981; Pabo & Lewis, 1982; McKay et al., 1982; Ohlendorf & Matthews, 1983; Pabo & Sauer, 1984; Schevitz et al., 1985). The X-ray structure determination of three proteins containing helix–turn–helix motifs bound to their DNA operator sites elucidates the DNA-binding domain of the 434 repressor (1–69) (Anderson et al., 1985, 1987; Aggarwal et al., 1988), the DNA-binding domain of λ repressor (1–92) (Jordan & Pabo, 1988), and the *trp* repressor (Otwinowski et al., 1988). These high-resolution views reveal the complexity of protein–DNA interfaces. The α helices, linked by turns of varying

length, contact the sugar–phosphate backbone as well as the base pairs in the major groove. The specificity depends on a set of correlated interactions, and changing any one may affect others (Jordan & Pabo, 1988; Aggarwal et al., 1988). The combination of direct protein–DNA contacts mediated by multiple hydrogen bonds and the sequence-dependent conformational effects in DNA limits our ability to make detailed structural predictions, even if a new protein can be assigned to a structural class such as the helix–turn–helix. Several more examples of protein-DNA complexes containing helix–turn–helix motifs will be needed to determine whether general principles emerge (Otwinowski et al., 1988; Aggerwal et al., 1988; Jordan & Pabo, 1988).

DNA-Binding Domain of Hin Recombinase. Hin recombinase is a 190-residue enzyme thought to recognize DNA by use of a helix–turn–helix motif on the basis of sequence similarities with repressor DNA-binding proteins (Pabo & Sauer, 1984). The 52 residues at the COOH terminus of Hin(139–190) have been shown to contain the DNA-binding domain (Bruist et al., 1987). Affinity cleaving studies using Hin(139–190) equipped with the DNA-cleaving moiety ethylenediaminetetraacetic acid (EDTA)·Fe at the NH₂ terminus have revealed that the NH₂ terminus of the DNA-binding domain of Hin lies proximal to the minor groove near the symmetry axis of Hin recombination sites (Sluka et al., 1987,
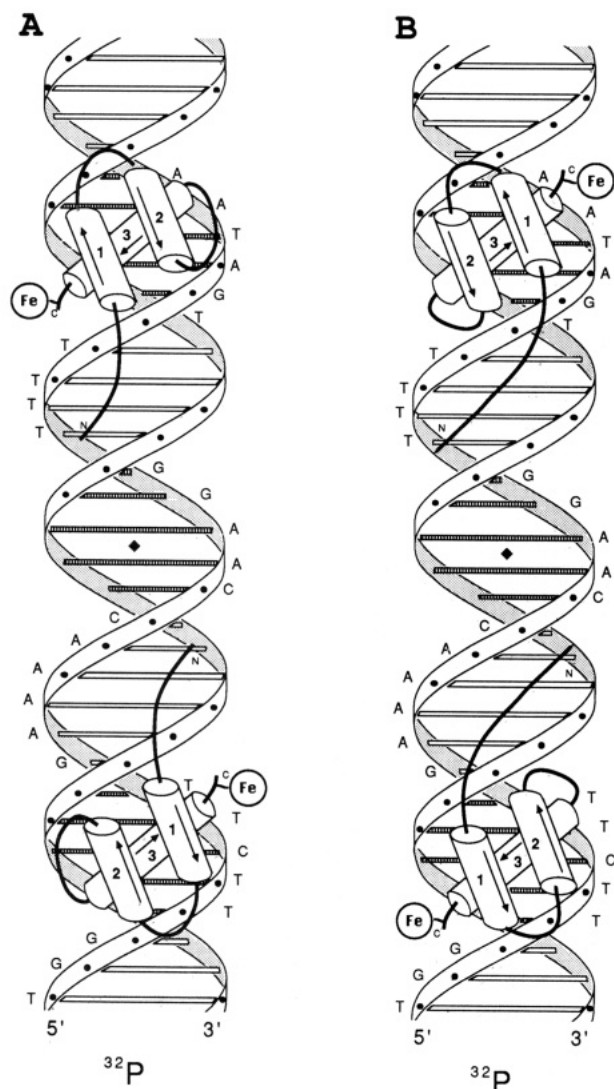
FIGURE 1: Two models for the DNA recognition domain of Hin-(139–190) binding to *hix*L. The sequence of *hix*L is shown along the 5′ strand. The pseudo-C_2 axis is indicated by a solid diamond. Putative α helices are shown as cylinders with an arrow pointing from the NH_2 to the COOH terminus. The model on the left is based on the 434 repressor–operator cocrystal with the following changes: (i) 434 repressor α_4 helix has been removed, and (ii) the nine-residue amino tail of Hin(139–184) is added as an extension to the 434 repressor α_1 helix amino terminus. The model on the right represents the recognition helix orientation of the *lac* repressor headpiece, which is rotated 180° from the 434–operator complex. This requires reorganization of the remaining helices in order to maintain the proper positioning of the amino terminus of the peptide. The α_3 helix of *lac* repressor has been removed. The α_2 helix of Hin(139–184) is reversed so that the amino terminus of the α_1 helix is toward the C_2 axis of *hix*L as required by the affinity cleaving data. In both models EDTA·Fe is shown attached to the side chain of a Lys[183], which replaced Ser[183] in the sequence.

1990a,b). Binding models for Hin(139–190) include a helix–turn–helix–turn–helix structure in the major groove with residues at the NH_2 terminus extending across the DNA phosphodiester backbone to the adjacent minor groove. However, the *orientation* of the putative recognition helix for Hin remains unknown.

λ cro, λ repressor, and 434 repressor bind to pseudo-C_2-symmetric DNA sequences as dimers (Pabo & Sauer, 1984). High-resolution X-ray crystal structures of λ repressor and 434 repressor (1–69) with their operators reveal that the COOH termini of the recognition helices are oriented toward the symmetry axis of the dimer-binding site (Anderson et al., 1987, Aggarwal et al., 1988; Pabo & Jordan, 1988). However,
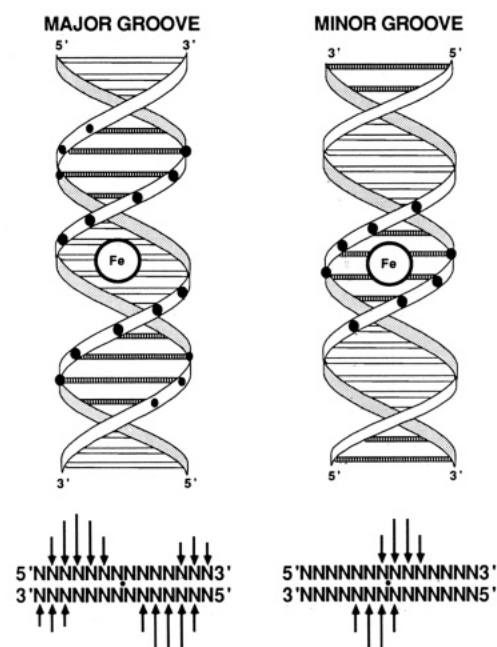

FIGURE 2: (Left) Cleavage patterns produced by a diffusible oxidant generated by EDTA·Fe localized in the major groove of right-handed DNA (Moser & Dervan, 1987; Griffin & Dervan, 1989). The cleavage patterns are consistent with the oxidant diffusing from the major groove to the adjacent minor grooves. (Right) Cleavage pattern produced by a diffusible oxidant generated by EDTA·Fe localized in the minor groove of right-handed DNA (Taylor et al., 1984; Dervan, 1986). The edges of the bases are shown as open bars for the major groove and crosshatched for the minor groove, respectively. The filled circles show the deoxyribose position along the DNA backbone where cleavage is observed. The sizes of the circles indicate relative cleavage intensities.

NMR data with the *lac* repressor headpiece bound to operator indicate an *opposite orientation* with the COOH terminus of the recognition helix facing away from the symmetry axis of the binding site (Boelens et al., 1987). Two models for the DNA-binding domain of Hin were put forward that differ in the orientation of the recognition helix (Sluka, et al., 1987). One is based on the orientation found in 434 repressor (1–69) and λ repressor (Figure 1A), and the other is based on *lac* repressor headpiece (1–56) (Figure 1B). In order to distinguish between these two models for Hin(139–190), we have carried out affinity cleaving experiments designed to determine the orientation of the putative recognition helix in the major groove of the Hin-binding site (Figure 1).

Affinity Cleaving. Incorporation of the DNA-cleaving moiety EDTA·Fe at discrete amino acid residues along a protein allows the positions of those residues relative to the DNA bases to be mapped to nucleotide resolution. This allows us to put limitations on the organization of the folded protein chain. EDTA·Fe localized at a specific DNA-binding site cleaves both DNA strands, typically covering 4–6 base pairs via a diffusible species (Schultz et al., 1982; Taylor et al., 1984; Dervan, 1986). Due to the right-handed nature of double-helix DNA, the groove in which the EDTA·Fe is located can be identified by complementary strand analysis of the cleavage pattern. An asymmetric cleavage pattern with maximal cleavage loci shifted to the 3′ side on opposite strands corresponds to an EDTA·Fe location in or above the minor groove (Figure 2). When the EDTA is located in the major groove, the maximal cleavage loci are 5′-shifted; in addition, there appears cleavage of lower efficiency on the distal strands of the adjacent minor grooves. This results in a *pair* of 3′-shifted asymmetric cleavage loci of unequal intensity on opposite strands (Figure 2). These patterns can be explained if the

α₁   α₂   α₃

HIN: 139-190;   G R P R A I N K H E Q E Q I S R L L E K G H P R Q Q L A I I E G I G V S T L Y R Y F P A S S I K K R M N

GIN: 138-193;   G R P P K L T K A E W E Q A G R L L A Q G I P R K Q V A L I Y D V A L S T L Y K K H P A K R A H I E N D D R I N

PIN: 138-184;   G R R P K L T P E Q W A Q A G R L I A A G T P R Q K V A I I Y D Y G V S T L Y K R F P A G D K

CIN: 138-186;   G R R P K Y Q E E T W Q Q M R R L L E K G I P R K Q V A I I Y D V A V S T L Y K K F P A S S F Q S

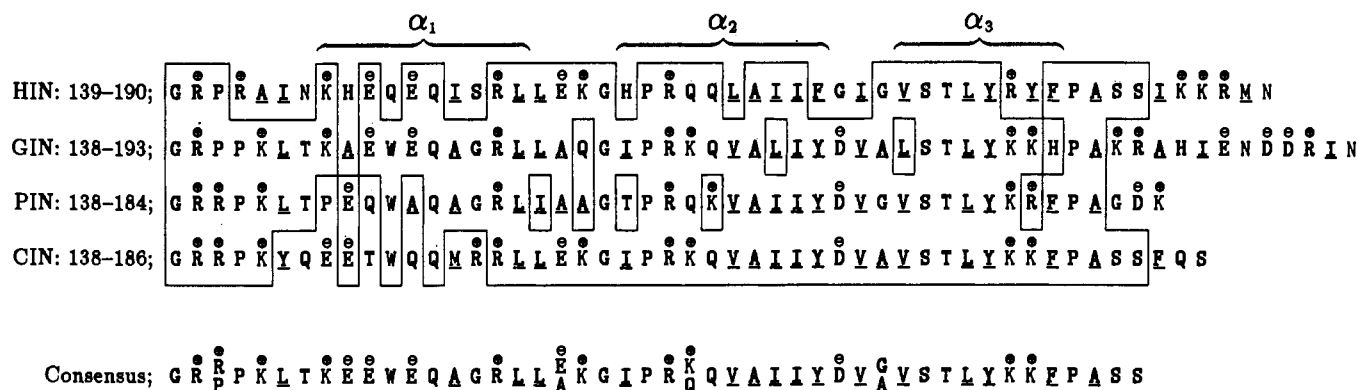Consensus;   G R P P K L T K E E W E Q A G R L L E K G I P R K Q V A I I Y D V G V S T L Y K K F P A S S

FIGURE 3: Protein sequences for the COOH-terminal domains of the Hin family of recombinases. Hydrophobic residues are underlined, and charged residues are marked with the appropriate sign. Net charges, excluding amino- and carboxy-terminal charges, for these domains are Hin +7, Gin +5, Pin +6, and Cin +6.

diffusible radical generated from the localized EDTA·Fe reacts with the major and minor grooves of DNA with unequal rates and preferentially (though not necessarily exclusively) in the minor groove. Several lines of evidence support this view. Tullius has shown that a sinusoidal cleavage pattern is obtained when a DNA is bound to a precipitate of calcium phosphate and allowed to react with EDTA·Fe, demonstrating that the two grooves have different reactivity toward EDTA·Fe (Tullius & Dombrowski, 1985). From our own work, we find that EDTA·Fe attached to minor-groove-binding molecules affords cleavage patterns with a single 3'-shifted asymmetric cleavage locus per EDTA·Fe position (Taylor et al., 1984; Dervan, 1986). However, for the case where EDTA·Fe is bound in the major groove of DNA by oligonucleotide triple helix formation, cleavage occurs along both strands of the adjacent minor grooves to afford a pair of cleavage loci of unequal intensity (Moser & Dervan, 1987; Griffin & Dervan, 1989). Cleavage is more intense along the strands proximal to the EDTA·Fe than along the distal strands of the adjacent minor grooves (Figure 2).

Hin(139–190) → Hin(139–184). To determine the orientation of the putative recognition helix of the Hin DNA-binding domain in the major groove of the DNA binding site, residues 139–190 were synthesized with EDTA near the COOH terminus. However, the cleavage pattern observed on the *hix*L binding site by using this synthetic protein was significantly more diffuse than the typical 4–6-base-pair cleavage pattern consistent with a highly localized EDTA·Fe (Sluka, 1988). The lack of a localized cleavage pattern suggests that the COOH terminus is conformationally flexible or extends some distance from the grooves of the *hix*L site. This lack of a discrete cleavage pattern does not allow an orientational assignment of the COOH terminus of Hin(139–190) (Sluka, 1988). One solution to this problem is to truncate the COOH terminus near the end of the postulated binding helix (Pro[181]), which presumably will have the COOH terminus more localized on the DNA. Before attaching EDTA to the COOH terminus of the truncated Hin DNA-binding domain, it was necessary to determine whether the shorter protein would fold and retain the same affinity and sequence specificity for the *hix*L site as Hin(139–190) (Sluka et al., 1987).

Hin is a member of a family of recombinases which includes Gin from phage Mu (Plasterk et al., 1983), Cin from phage P1 (Hiestand-Nauer & Iida, 1983), and Pin from the e14 element of *Escherichia coli* (Plasterk & van de Putte, 1985). Examination of these protein sequences indicates that, after the position corresponding to Ser[184] of Hin, there is variability present in the length and content of the remaining residues at the COOH termini of the proteins (Figure 3). We have

synthesized a protein that lacks six residues at the COOH terminus of Hin(139–190) to afford a 46-residue protein, Hin(139–184). EDTA has been attached to the NH₂ terminus of this protein to yield EDTA–Hin(139–184) for comparison with EDTA–Hin(139–190) (Sluka et al., 1987). We find that Fe·EDTA–Hin(139–184) cleaves DNA in a sequence-specific fashion similar to that of Fe·EDTA–Hin(139–190). It is therefore possible to attach EDTA near the COOH terminus of the putative recognition helix of Hin(139–184) to determine its orientation.

EXPERIMENTAL PROCEDURES

Materials. Manual peptide syntheses were carried out in 20-mL vessels fitted with coarse glass frits as described by Kent (1988). Automated syntheses were performed on an ABI 430A synthesizer (Kent et al., 1984, 1985), modified by the removal of in-line filters to the top and bottom of the reaction vessel, with a 20-mL Teflon/KelF reaction vessel. The synthetic protocols used were developed at the California Institute of Technology (Kent & Clark-Lewis, 1989; Clark-Lewis et al., 1986; Kent et al., 1988; Kent, 1988). Protected amino acid derivatives were purchased from Peninsula Laboratories. tBoc-L-His (DNP-protected) was obtained from Fluka and N^α-tBoc-N^ϵ-Fmoc-L-lysine from Chemical Dynamics Corp. 4-Methylbenzhydrylamine (BHA) resin was purchased from U.S. Biochemical Corp. Dimethylformamide (DMF), diisopropylethylamine (DIEA), dicyclohexylcarbodiimide (DCC) in dichloromethane, N-hydroxybenzotriazole (HOBt) in DMF, and trifluoroacetic acid (TFA) were obtained from Applied Biosystems. Dichloromethane (DCM) and methanol (HPLC grade) were purchased from Mallinckrodt, p-cresol and p-thiocresol were from Aldrich, and diethyl ether (low peroxide content) was from Baker.

Doubly distilled water was used for all aqueous reactions and dilutions. Plasmid pMFB36 DNA (Bruist et al., 1987) was isolated from *E. coli* and purified by CsCl centrifugation. Calf thymus (CT) DNA was purchased from Sigma and was sonicated, deproteinized, and dialyzed. Enzymes were purchased from Boehringer Mannheim or New England Biolabs.

Synthesis. N^α-tBoc-L-amino acids were used with the following side-chain protecting groups: Arg(Tos), Asp(OBzl), Glu(OBzl), His(DNP), Lys(Cl-Z), Ser(Bzl), Thr(Bzl), and Tyr(Br-Z). Manual assembly of the protected peptide on the solid support was carried out as previously described (Sluka et al., 1990a,b). Automated syntheses were carried out with modified cycles that are similar to the manual procedures (Kent, 1988). Double couplings were performed for every amino acid. tBoc protecting groups were removed from the α-amino group of the resin-bound amino acid by using 100%

TFA. The deprotected peptide resin was neutralized with 10% DIEA in DMF. Amino acids (except Asn, Gln, and Arg) were coupled to the free α-amino group as the symmetric anhydrides. In the first coupling, the symmetric anhydride was formed in an activating vessel with DCC in DCM. The dicyclohexylurea was removed by filtering the solution into a concentrating vessel, where the DCM was removed and replaced with DMF. The solution was then transferred to the reaction vessel, where the resin had previously been deprotected and neutralized. After the first coupling, the resin was neutralized with DIEA in DMF for the second coupling. The symmetric anhydride was formed in the activator in DCM, filtered, and transferred to the reaction vessel. DMF was added at the midpoint of the reaction cycle. Yields for double couplings were determined by quantitative ninhydrin monitoring (Sarin et al., 1981).

Asn, Gln, and Arg were coupled as the HOBt esters in DMF. For the first coupling the DCC in DCM was transfered to the concentrator and the solvent exchanged for DMF. HOBt and the amino acid in DMF were then added to the concentrator, and the active ester was allowed to form. The ester was transferred to the reaction vessel, where the amino acid was allowed to couple. After the first coupling the resin was neutralized with DIEA in DMF for the second coupling. The second coupling for Asn and Gln was identical with the first except that the DCM was not removed from the activating solution until after the ester formed. The second coupling for Arg was identical with the first symmetric anhydride coupling. Reaction times for HOBt esters were longer than for the symmetric anhydride due to the slower coupling reaction.

EDTA was attached to the NH₂ terminus of the protected peptide as the tribenzyl ester with a γ-aminobutyric acid linker (BEG) via an HOBt ester (Sluka et al., 1987, 1990b). EDTA was positioned at the COOH terminus by covalent attachment to a lysine side chain. N^α-tBoc-N^ϵ-Fmoc-L-lysine was coupled onto the growing chain as described above. Selective removal of the Fmoc protecting group was accomplished with 20% piperidine in DMF for 20 min. The tricyclohexyl ester of EDTA (Griffin, 1989; Sluka et al., 1990b) was then coupled to this amine as the HOBT ester in a reaction identical with the BEG coupling to the NH₂ terminus (Sluka et al., 1990a,b).

Deprotection and Purification. Because the histidine protecting group dinitrophenol (DNP) is stable to HF conditions, it was first necessary to remove this group from the resin-bound peptide. This was accomplished by thiolysis using 20% 2-mercaptoethanol and 10% DIEA in DMF. This treatment was repeated twice for 30 min each time. After removal of the N^α-tBoc group with TFA and drying of the resin, all other side-chain protecting groups as well as the peptide–resin bond were cleaved by using anhydrous HF in the presence of p-cresol and p-thiocresol as scavengers for 60 min at 0 °C (Stewart & Young, 1981). The HF was removed under vacuum. The crude protein was then precipitated with diethyl ether, collected on a fritted funnel, dissolved with water, and washed through, leaving the resin on the frit. A small sample was then removed, filtered, and subjected to analytical HPLC (25 cm × 4.6 mm C_4 column, 0–60% acetonitrile/0.1% TFA over 60 min). The remaining solution was frozen and lyophilized. Residual DNP groups were removed from the crude peptide by treatment in 4 M guanidine hydrochloride, 50 mM Tris, pH 8.5, and 20% 2-mercaptoethanol for 1 h at 50 °C (Kent, 1988). This solution was injected directly onto a semipreparative C_4 HPLC column (25 × 1 cm) and run in H_2O/0.1% TFA until the guanidine and 2-mercaptoethanol had eluted. A gradient of 0–60% acetonitrile/0.1% TFA was run over 240 min, and
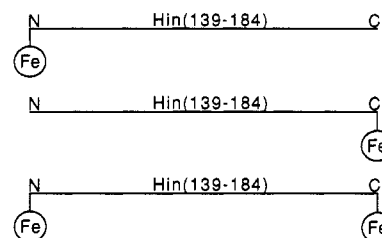

FIGURE 4: Synthetic proteins from the DNA-binding domain of Hin recombinase with EDTA·Fe positioned at the NH_2 terminus, the COOH terminus, and at both termini of residues 139-184.
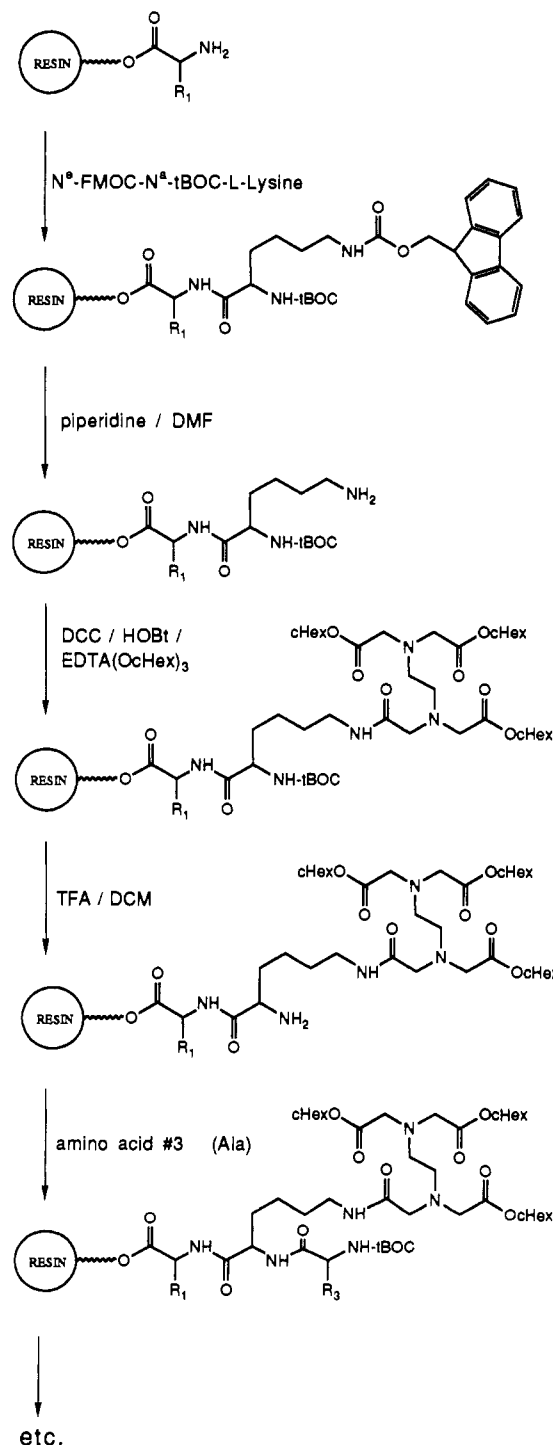

etc.

FIGURE 5: Synthetic scheme for the attachment of the tricyclohexyl (cHex) ester of EDTA to the ε-amino group of Lys[183] in the second amino acid position on the resin.

fractions were collected. Fractions were checked by analytical HPLC, and those containing the desired peak were pooled and
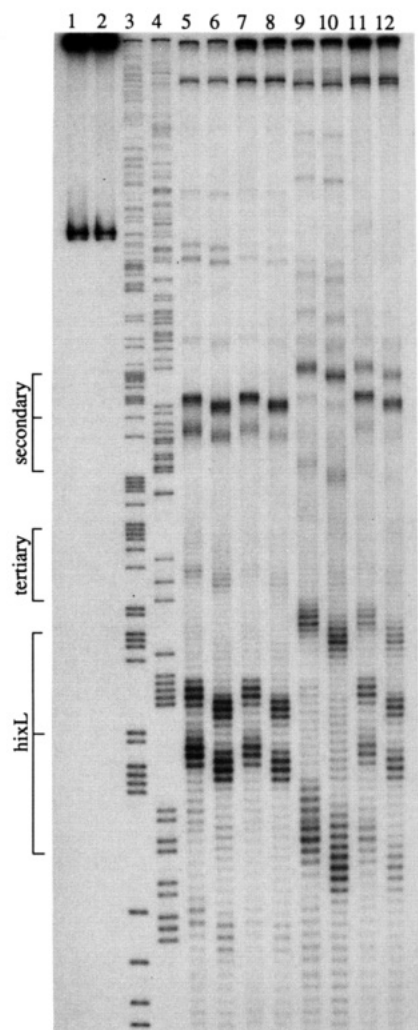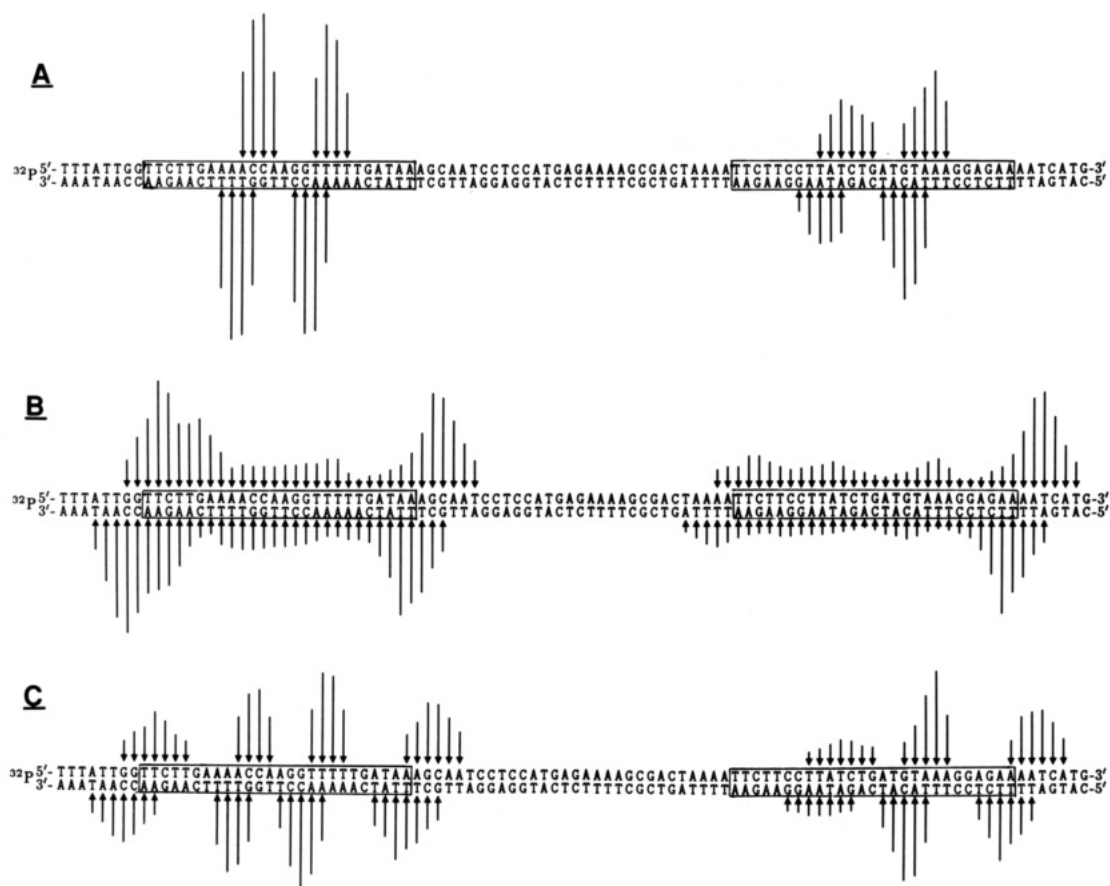
FIGURE 6: (Top) Autoradiogram of a high-resolution denaturing polyacrylamide gel of affinity cleaving reactions on a ^{32}P-end-labeled fragment (*Xba*I–*Eco*RI) from pMFB36 (Bruist et al., 1987). Reaction conditions were 20 mM NaCl, 20 mM phosphate, pH 7.5, 100 μM calf thymus DNA, 5 mM DTT, and $\approx$15000 cpm ($\simeq$1 ng) of end-labeled DNA in a total volume of 15 μL. Reactions were run for 60 min at 22 °C and terminated by ethanol precipitation. Cleavage products were analyzed on an 8%, 1:20 cross-linked, 50% urea denaturing polyacrylamide gel. Odd- and even-numbered lanes contain 5′- and 3′-end-labeled DNA, respectively. Lanes 1 and 2 are DNA controls, and lanes 3 and 4 are A-specific sequencing lanes (Iverson & Dervan, 1987). Lanes 5 and 6 contain 5 μM EDTA·Fe–Hin-(139–190), lanes 7 and 8 contain 10 μM EDTA·Fe–Hin(139–184), lanes 9 and 10 contain 5 μM Hin(139–184)–EDTA·Fe, and lanes 11 and 12 contain 10 μM EDTA·Fe–Hin(139–184)–EDTA·Fe. (Bottom) Histograms of the cleavage data. The sequence left to right is from the bottom to the middle of the gel. The left and right boxed sequences represent *hix*L and the secondary site, respectively. Arrow heights indicate the extent of cleavage at the indicated base. Panels A–C are cleavage patterns for EDTA·Fe–Hin(139–190), Hin(139–184)–EDTA·Fe, and EDTA·Fe–Hin(139–184)–EDTA·Fe.

A

^{32}P 5′- TTTATTGGTTCTTGAAAACCAAGGTTTTTGATAAAGCAATCCTCCATGAGAAAAGCGACTAAAATTCTTCCTTATCTGATGTAAAGGAGAAAATCATG-3′
3′- AAATAACCAAGAACTTTTGGTTCCAAAAACTATTTCGTTAGGAGGTACTCTTTTCGCTGATTTTAAGAAGGAATAGACTACATTTCCTCTTTTAGTAC-5′

B

^{32}P 5′- TTTATTGGTTCTTGAAAACCAAGGTTTTTGATAAAGCAATCCTCCATGAGAAAAGCGACTAAAATTCTTCCTTATCTGATGTAAAGGAGAAAATCATG-3′
3′- AAATAACCAAGAACTTTTGGTTCCAAAAACTATTTCGTTAGGAGGTACTCTTTTCGCTGATTTTAAGAAGGAATAGACTACATTTCCTCTTTTAGTAC-5′

C

^{32}P 5′- TTTATTGGTTCTTGAAAACCAAGGTTTTTGATAAAGCAATCCTCCATGAGAAAAGCGACTAAAATTCTTCCTTATCTGATGTAAAGGAGAAAATCATG-3′
3′- AAATAACCAAGAACTTTTGGTTCCAAAAACTATTTCGTTAGGAGGTACTCTTTTCGCTGATTTTAAGAAGGAATAGACTACATTTCCTCTTTTAGTAC-5′

lyophilized. The peptide was packaged into (5×10^{-9})-mol samples on the basis of OD_{275} (ϵ = 2800 for 2 Tyr).

DNA Cleavage Reaction. The plasmid pMFB36 (Bruist et al., 1987) was linearized by cleavage with restriction endonuclease *Xba*I. Labeling at the 3' end was accomplished with $[\alpha\text{-}^{32}P]dATP$ and the Klenow fragment of DNA polymerase I. The 5' end was labeled with ^{32}P by dephosphorylation with calf alkaline phosphatase (CAP) followed by treatment with $[\gamma\text{-}^{32}P]ATP$ and T4 polynucleotide kinase. Cleavage with restriction endonuclease *Eco*RI yielded 3'- and 5'-end-labeled fragments of 557 bp, which were isolated by nondenaturing polyacrylamide gel electrophoresis. Specific chemical cleavage reactions were then carried out.

DNA cleavage reactions were run in a total volume of 15 μL. Final concentrations were 20 mM phosphate, pH 7.5, 20 mM NaCl, 100 μM (in base pairs) CT DNA, ≈15 000 cpm of ^{32}P-end-labeled restriction fragment, 5 mM dithiothreitol (DTT), and 5 μM Fe·EDTA–protein. The proteins were allowed to equilibrate with the DNA for 10 min at 25 °C, and cleavage was then initiated by the addition of DTT and allowed to proceed for 60 min at 25 °C. The reactions were terminated by ethanol precipitation, dried, and resuspended in 5 mL of 100 mM Tris–borate–EDTA and 80% formamide solution. The ^{32}P-labeled DNA products were analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiography. Densitometric analysis of the gel autoradiogram and comparison of individual lanes with sequence marker lanes allowed assignment of DNA cleavage to nucleotide resolution.

RESULTS AND DISCUSSION

Three 46-residue proteins based on the DNA-forming domain of Hin recombinase were synthesized with EDTA at the NH_2 terminus, the COOH terminus, and both termini (Figure 4). Hin(139–184) was synthesized by automated solid-phase techniques on benzhydrylamine (BHA) resin using tBoc-protected amino acids (Sluka et al., 1990a,b). The average yield per cycle, as determined by quantitative ninhydrin analysis of the resin sample (Sarin et al., 1981), over 45 couplings was 99.4%. Tribenzyl-EDTA–GABA (BEG) was attached to the NH_2 terminus of the protected peptide–resin as previously described (Sluka et al., 1987, 1990a,b). Attachment of EDTA near the COOH terminus of the protein was accomplished by a combination of tBoc and Fmoc protection schemes (Figure 5). N^ϵ-Fmoc-N^α-tBoc-lysine was substituted for the Ser^{183} of the protein. The Fmoc protecting group was then selectively removed by using piperidine in DMF (Stewart & Young, 1981). Coupling of the tricyclohexyl ester of EDTA to the side-chain amine was accomplished by using DCC and HOBT in DMF (Sluka et al., 1990b). Ninhydrin analysis indicated at 99.9% yield in 1 h. The tBoc group was then removed by using TFA in DCM. The synthesis was continued manually for two residues and completed by using automated techniques with an average yield of approximately 99.1% per cycle. BEG was attached to the NH_2 terminus of a small portion of this protected peptide resin to afford EDTA–Hin(139–184)–EDTA (Figure 4). All proteins were deprotected with anhydrous HF and purified by reverse-phase HPLC.

The proteins were allowed to react at micromolar concentrations (22 °C, pH 7.5, 20 mM NaCl) for 1 h in the presence of DTT with a ^{32}P-end-labeled restriction fragment containing the Hin-binding sites *hix*L and the secondary site (Sluka et al., 1987, 1990a,b). The DNA cleavage products were separated by high-resolution polyacrylamide gel electrophoresis and visualized by autoradiography (Figure 6, top panel). By comparison of Hin(139–184) and Hin(139–190), both with
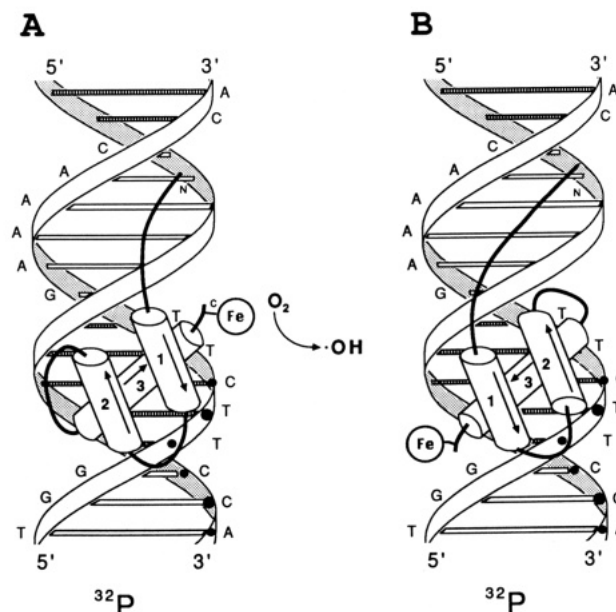

FIGURE 7: Schematic representation of two models of Hin(139–184)–EDTA·Fe bound to the bottom half of *hix*L, with filled circles indicating the sites of maximal cleavage produced by Hin(139–184)–EDTA·Fe. The model on the left (A) seems to fit the experimental cleavage data best.

EDTA at the NH_2 terminus, we find that Fe·EDTA–Hin-(139–184) cleaves DNA with the same sequence specificity as Fe·EDTA–Hin(139–190) but with reduced efficiency (Figure 6, compare lanes 7 and 8 with lanes 5 and 6). When EDTA·Fe is moved from the NH_2 to the COOH terminus of Hin(139–184), we find that the DNA cleavage locus for Hin(139–184)–EDTA·Fe is shifted further from the symmetry axis at the binding sites compared to Fe·EDTA–Hin(139–184) (Figure 6, lanes 9 and 10). Remarkably, the maximal cleavage patterns observed on opposite stands of the DNA for Hin-(139–184) with EDTA·Fe at the COOH terminus are shifted to the 3' direction. A protein equipped with EDTA at both the NH_2 and COOH termini, Fe·EDTA–Hin(139–184)–EDTA·Fe, affords a pair of cleavage patterns consistent with the combination of patterns from EDTA–Hin(139–190) and Hin(139–190)–EDTA (Figure 6, lanes 11 and 12 and panel B). The fact that the cleavage pattern for EDTA·Fe at Gly^{139} (NH_2 terminus) appears unchanged when EDTA·Fe is present or absent at the COOH terminus suggests that Hin(139–184) maintains the same structure independent of which terminus is modified with EDTA·Fe.

Histograms of cleavage at the *hix*L and secondary sites, which consist of imperfectly conserved 12-bp inverted repeats, on a DNA restriction fragment are shown in Figure 6, panels A–C. Although the binding-site specificity for Hin(139–184) and Hin(139–190) are identical, the binding affinities are different, suggesting that nonspecific affinity has been lost in the shorter protein. Three of the six residues removed contain positive charges, and it is possible that these residues, Lys^{186}-Lys^{187}-Arg^{188}, make important electrostatic contributions (at 20 mM NaCl). Thus, the removal of six residues (185–190) from the COOH terminus of the protein apparently leaves the tertiary structure and the sequence-specific binding elements of the protein intact (Figure 3).

The specific cleavage pattern observed for Hin(139–184)–EDTA·Fe is shifted to the 3' side and indicates that the Fe·EDTA attached near the COOH terminus of the recognition helix is positioned within the Hin-binding site above the minor groove of the sequence 5'-GGTT-3'. Due to the opposite

orientations of the recognition helix in each model, the EDTA·Fe should be located on opposite faces of the DNA (Figure 1). Therefore, the location of maximal cleavage expected for the two orientations should be different; the λ and 434 repressor orientation is closer to the symmetry axis of the inverted repeats. Two models for the DNA-binding domain of Hin, one based on λ and 434 repressor–DNA cocrystals and the other on *lac* repressor headpiece–DNA NMR studies, were superimposed on the experimentally observed cleavage data (Figure 7).

Comparison of the two models and the cleavage data obtained with an EDTA·Fe attached near the COOH terminus of Hin(139–184) supports the model where the recognition helix of Hin is oriented *toward the center of the hixL binding site* as in λ and 434 repressors (Figure 7A) and not as assigned for the *lac* repressor headpiece (Figure 7B).

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