our procedure of correction for oligonucleotide contribution in the peptide region. In conclusion, we find that KGWGK can discriminate between *Bgl*II and *Hin*dIII sequences, possibly because of its flexibility and larger size, while KWK, a smaller rigid peptide, cannot do so.

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Hairpin and Duplex Forms of a Self-Complementary Dodecamer, d-AGATCTAGATCT, and Interaction of the Duplex Form with the Peptide KGWGK: Can a Pentapeptide Destabilize DNA?†

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ABSTRACT: Ordered forms of a synthetic dodecamer, d-AGATCTAGATCT, a direct repeat of the *Bgl*II recognition sequence, have been investigated using UV, CD, and fluorescence spectroscopy. Complex hairpin–duplex equilibria are manifest in UV thermal transitions, which are monophasic in the presence of very low or high NaCl concentrations but distinctly biphasic at intermediate ionic strengths. In 100 mM NaCl, the $1/T_m$ vs ln C curve has a reasonable positive slope, which yields ΔH and ΔS for duplex formation as −66.2 kcal/mol and −190 cal/mol, respectively. Interaction of the dodecamer in duplex form with a tryptophan-containing peptide, KGWGK, has also been investigated to test the "bookmark" hypothesis (Gabbay et al., 1976) under the uniform structural constraint of the oligonucleotide of defined sequence. CD spectra of the peptide (P), the oligonucleotide (N), and their mixtures at different P/N ratios show a dramatic change in peptide spectrum but little change in nucleic acid dichroism with peptide binding. The T_m of P–N complexes decreases with an increase in peptide binding and levels off at saturation binding of P/N = 2.0. The data are interpreted in terms of a groove-cum-intercalation mode of binding, where intercalation to the tryptophan side chain destabilizes the double helix. A Scatchard plot of the binding data is nonlinear, with best-fit values for an overall association constant $K = 4.33 \times 10^5$ M^{-1}, and the number of binding sites $n = 3.23$ when fitted to the site-exclusion model of binding.

The reported structure of *Eco*RI complexed with its cognate deoxyoligonucleotide substrate (Frederick et al., 1984; McLarin et al., 1986) and several other reports (Lesser et al., 1990; Newman et al., 1990; Mazarelli et al., 1989) put in evidence that these enzymes achieve stringent discrimination by both direct and indirect read-out mechanisms. The direct read-out involves protein base contacts through hydrogen bonding and van der Waals contacts with the functional groups on base edges, and the indirect read-out relates to the overall conformation of DNA. The reported cocrystal structure demonstrates the importance of sequence-dependent distortions, bends, and kinks in the DNA double helix in achieving tight complementarity between the DNA and protein surfaces.

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Various physicochemical studies suggest that partial insertion of a tryptophan residue between successive base pairs could induce a local bend in DNA structure (Gabbay et al., 1976). Such intercalation, though not demonstrated in crystals, has been studied in great detail by many investigators (Helene & Maurizot, 1981; Bresloff & Crothers, 1981; Chaires, 1986; Rajeswari et al., 1987) using polynucleotides of random or alternating sequences. This intuitively attractive bookmark hypothesis, however, should be tested with model peptides and oligonucleotides of defined sequences.

Here, we report a study on the sequence d-AGATCTAGATCT and its interaction with a synthetic pentapeptide, lysyl-glycyl-tryptophyl-glycyl-lysine (KGWGK), by UV, CD, and fluorescence spectroscopy. The deoxyoligonucleotide is designed to contain a tandem repeat of the recognition and cleavage site of the restriction endonuclease *Bgl*II. Such a dodecamer will form, depending upon the conditions, duplex or hairpin structures. We present results describing the complex hairpin–duplex equilibria and the binding of the pentapeptide to the duplex form. Interaction of KGWGK with the dodecamer assumes added significance in view of the presence of one or two restriction sites in the hairpin or duplex form, respectively. We show that the pentapeptide destabilizes the DNA helix upon binding, and to our knowledge, ours is the first report of this nature. Interaction of the hairpin form with several tryptophan-containing peptides is reported in the preceding paper in this issue (Rajeswari et al., 1992).

Materials and Methods

The deoxyoligonucleotide was synthesized on a PS 100 Cruachem DNA synthesizer using solid-phase synthesis and β-cyanoethyl phosphoramidite chemistry (Atkinson & Smith, 1984). The pentapeptide was synthesized in solution by standard methods. The purity of the peptide and oligonucleotide was checked on analytical FPLC using Mono-Q anion-exchange and RPC columns (Pharmacia, Sweden). Molar extinction coefficients used for determining the concentrations were E_{280} = 5700 for the peptide and, for the oligonucleotide, E_{260} = 9000 in 10 mM NaOH solution. UV spectra and melting curves were usually recorded on a Gilford 250 spectrophotometer fitted with a Model 2627 temperature programmer, except the melting curve at 10 mM NaCl, which was measured on a Cecil 599S fitted with a water-jacketed cell holder. All the chemicals and reagents were of analytical grade. Unless specifically mentioned solutions were made in cacodylate buffer, pH 7.0. CD spectra of the peptide, oligonucleotide solutions, and their mixtures at different peptide/oligonucleotide mole ratios (P/N) in 50 mM disodium phosphate buffer (pH 7.4) were recorded on a Jasco J500A spectropolarimeter. Fluorescence measurements were made on a Shimadzu RF 540 spectrofluorometer at 25 °C.

Results

Thermal Transitions. Representative UV melting curves of the deoxyoligonucleotide at different salt concentrations are shown in Figure 1. The cooperative thermal transitions are monophasic at very low and high salt concentrations but distinctly biphasic at intermediate ionic strengths, suggesting melting of two ordered forms, possibly hairpins and duplexes. Due to technical difficulties the bottom plateau of the melting curves recorded on the Gilford spectrophotometer could not be fully accessed and the curves are normalized with respect to the upper plateau ($A_{rel} = A/A_f$). The actual T_ms were determined from the first derivatives of the observed thermal transitions plotted manually. From such a plot it was not possible to extract the two T_ms for the two ordered forms, since

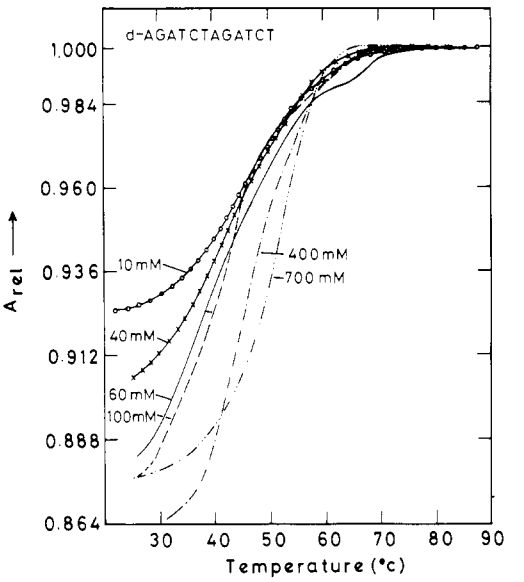

FIGURE 1: Ultraviolet melting profiles of the oligonucleotide d-AGATCTAGATCT in 10 mM (○), 40 mM (×), 60 mM (—), 100 mM (– – –), 400 mM (–·–), and 700 mM (–···–) NaCl with 1 mM EDTA and 2 mM sodium cacodylate buffer, pH 7.0. Oligomer concentration was 6.94 × 10^{-5}M. Ar_{el} is A/A_{final}.

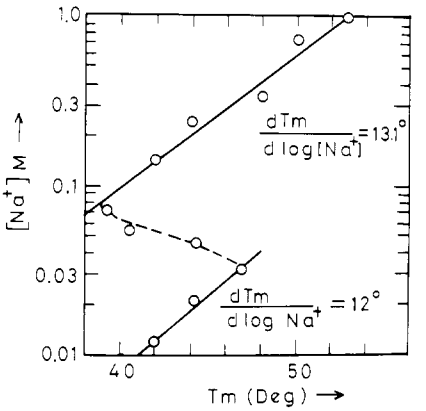

FIGURE 2: Salt dependence of the T_m of d-AGATCTAGATCT (6.94 × 10^{-5} M). Conditions: 2 mM sodium cacodylate buffer, pH 7.0, and 1 mM EDTA.

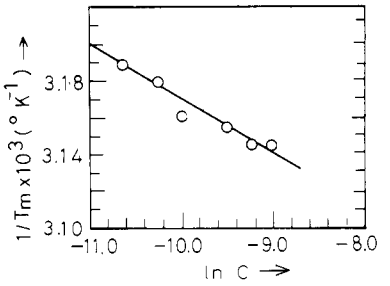

FIGURE 3: Plot of $1/T_m$ vs ln C. C is the residue molar concentration of *Bgl*II dodecamer in 100 mM NaCl, 2 mM cacodylate buffer, pH 7.0, and 1 mM EDTA.

the two phases of the biphasic curves were not sufficiently were separated. The reported T_ms in such cases therefore represent average values. The salt dependence of T_m (Figure 2) shows a distinct break around 40 mM Na^+; the T_m actually decreased progressively in the 40–75 mM salt concentration range and then increased in expected fashion. The dT_m/d log Na^+ values from the positive slopes of the two arms of Figure 2 are very similar, being 13 and 12 °C for the upper and lower arms, respectively. Figure 3 shows the dependence of T_m on oligomer

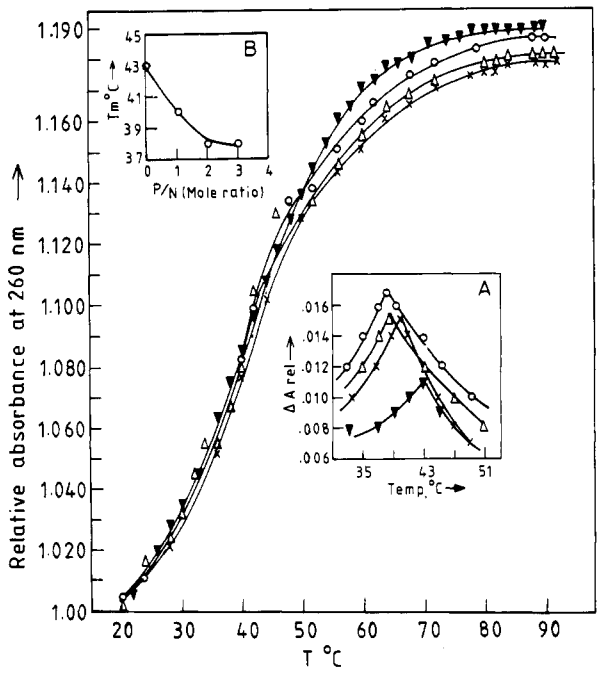

FIGURE 4: Melting curves of *Bgl*II dodecamer in the presence and absence of the peptide KGWGK at P/N ratios 0 (▼), 1 (×), 2 (△), and 3 (○) in 50 mM disodium phosphate and 0.2 mM EDTA at pH 7.4; the oligonucleotide concentration was 2.5×10^{-5}M. Inset A shows the ΔA between the successive points in the melting range vs mean temperature; inset B shows a plot of T_m vs P/N mole ratios. [N] is given in oligomer strand concentration.

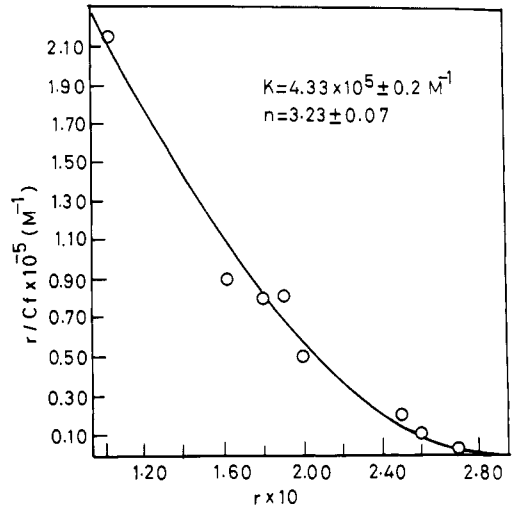

FIGURE 5: Scatchard plot for binding of KGWGK to d-AGATCT-AGATCT in 50 mM disodium phosphate (pH 7.4) and 0.2 mM EDTA at 25 °C. The line drawn is the best fit curve obtained by the Enzfitter program with $K = 4.3 \times 10^{-5}$ M and $n = 3.23$.

concentration in 100 mM NaCl; the slope of the $1/T_m$ vs ln C curve is comparable to those reported for other oligonucleotide duplexes (Breslauer et al., 1986; Aboul-ela et al., 1985). We estimate ΔH and ΔS for duplex formation from the slope of the least-squares plot at 100 mM NaCl as −66.2 kcal/mol and −190 cal/mol, respectively (Figure 3).

The UV melting curves of *Bgl*II complexed with the peptide at different P/N mole ratios in 50 mM disodium phosphate buffer were measured and the T_ms were plotted against respective P/N values (Figure 4, inset B). Surprisingly, the melting temperature actually decreased with an increase in peptide binding, leveling off at a P/N ratio around 2.0.

Binding Studies. The binding isotherm and the overall association constant for binding of KGWGK to the *Bgl*II

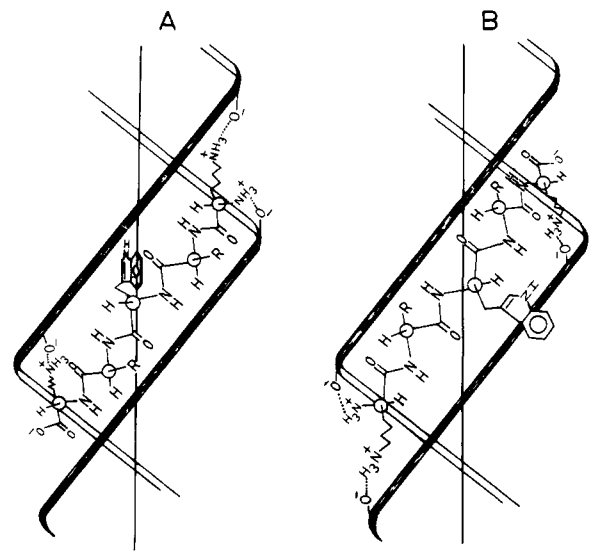

FIGURE 6: Schematic presentation of a model for KGWGK–duplex binding; interstrand binding (A) with the Trp side chain intercalated between base pairs and (B) with Trp facing out of the helix.

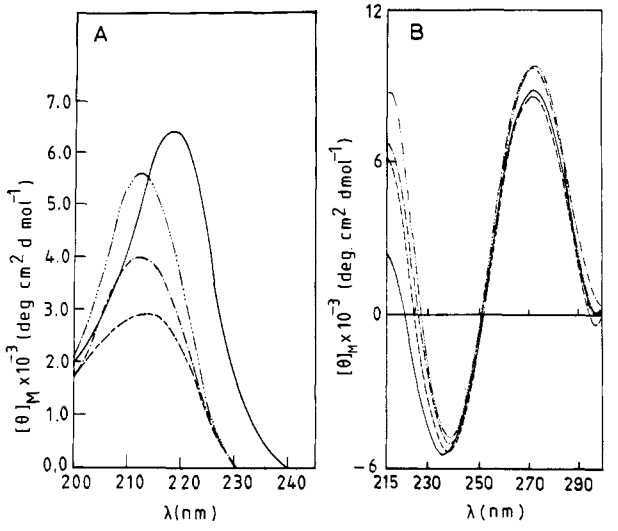

FIGURE 7: (A) Circular dichroic spectra of 5×10^{-5} M peptide KGWGK, free (—) and in the presence of *Bgl*II oligomer at different P/N mole ratios: 4.16 (– – –); 2.08 (– · –), and 1.4 (– ··· –) in 50 mM disodium phosphate buffer (pH = 7.4) and 0.2 mM EDTA. (B) CD spectra of the oligonucleotide free (—) and in the presence of KGWGK at the same condition and P/N mole ratios as given in (A). [N] is given in oligomer strand concentration.

dodecamer in 50 mM disodium phosphate buffer (pH 7.4) was determined from fluorescence quenching measurements. The results were analyzed using the following equation (McGhee and von Hippel, 1974):

$$r/C = K(1 - nr)[(1 - nr)/1 - (n - 1)r]^{n-1} \quad (1)$$

where r is the molar ratio of bound peptide per nucleotide and C is the concentration of free peptide given by $C = (C_t - C_B)$, where C_t is the total peptide concentration and C_B is the concentration of bound peptide, calculated from fluorescence quenching data as described (Brun et al., 1975). K and n are the overall binding constant and the number of nucleotide residues covered by each peptide molecule. The r/C and r values were fitted to the equation using the Enzfitter program, giving the best-fit values for $K = 4.33$ (±0.2) $\times 10^5$ M^{-1} and $n = 3.23$ (±0.07), respectively (Figure 5).

CD Measurements. Figure 7A shows the changes in the CD spectrum of the peptide upon addition of the oligo-

nucleotide. The spectrum of the free peptide shows a broad positive band centered around 220 nm and no dichroism above 240 nm. The CD spectra of peptide/oligonucleotide mixtures were corrected for oligonucleotide contributions in the peptide region (200–240 nm), calculated from the measured ellipticity of the free oligonucleotide. The intensity of the peptide CD band diminishes drastically upon interaction with oligomer, suggesting strong complexation. The CD spectra of the oligonucleotide at 230–300 nm in the presence and absence of the peptide are also shown in Figure 7, panel B. The change in ellipticity in this region is very small.

Discussion

We will first discuss the stability and the ordered forms of the oligonucleotide and subsequently its interaction with the pentapeptide. The UV thermal transition data clearly indicate the existence of two ordered forms. At room temperature, the oligomer possibly exists as hairpins below 40 mM Na^+ and as duplexes at 100 mM Na^+ and above, with a mixed population at intermediate salt concentration. This interpretation is borne out by the observed dependence of the melting temperature on salt and oligonucleotide concentrations (Figures 2 and 3). The graph of T_m vs log Na^+ shows a distinct break between 40 and 70 mM Na^+; we interpret the two straight lines below and above this range as representing the salt dependence of the hairpins (lower arm) and the duplexes (upper arm). The decrease in T_m observed between 40 and 70 mM NaCl concentration is possibly an artifact arising out of the presence of mixed species, duplexes and hairpins. Sheffler et. al (1968) have shown that at moderate and high counterion concentrations hairpin helices of $d(TA)_i$ oligomers slowly convert to duplex structures at low temperature. From theoretical arguments they calculate that the population of dimeric helices increases at the expense of the monomeric hairpins at temperatures much below the T_m of the hairpins, because the nonbonded loop in hairpins become bonded in the duplex structure and helix stability increases with chain length [see discussion in Scheffler et al. (1968)]. In Figure 2, at 42 °C and 10 mM salt, the oligomer exists almost completely in hairpin form, but in 60 mM salt, at the same temperature, the population of hairpin is ca. 40% and duplex ca. 60%. It may be noted that in 60 mM Na^+, T_m of the hairpins is pushed up to ca. 49 °C, and hence at 42 °C (lower than T_m), conversion of hairpins to duplexes is expected. A similar decrease in T_m with an increase in oligonucleotide concentration in two specific instances of hairpin forms has also been reported [see Figure 2, bottom panel, in Howard et al. (1991)], which, we suspect, could be due to formation of mixed species. The observed dependence of T_m on oligomer concentration further confirms our interpretations (Figure 3). At 10 mM NaCl, T_m is independent of oligomer concentration (Rajeswari et. al., 1992), as would be expected for hairpin forms. In 100 mM NaCl, the slope of the $1/T_m$ vs ln C curve is as expected for the duplex forms, and the calculated ΔH and ΔS values are in excellent agreement with the reported values of −68,0, −64.5, and −62.8 kcal/mol for ΔH and −196, −183, and −179 cal/mol for ΔS of other oligonucleotide duplexes (Aboul-ela et al., 1985).

Before we discuss the interaction of the peptide with oligonucleotide, let us first consider the possible types of interaction. In 50 mM disodium phosphate buffer (100 mM Na^+) at room temperature, the dodecamer is in the duplex form. The peptide may bind to the duplex in two different ways, forming either intrastrand or interstrand complexes. An intrastrand complex may form by simple electrostatic binding of the two lysyl NH_3^+ groups to the phosphates on the same strand. In the interstrand complex, the lysyl NH_3^+ groups bind to two phosphates across the double helix on opposite strands with the peptide spanning along the major or minor groove. In either complex and tryptophan side chain may partially intercalate between base pairs, though for reasons discussed below we think with the oligonucleotide duplex intercalation in an interstrand complex is more likely.

The most interesting result of this study, which supports tryptophan intercalation in the *Bgl*II duplex, is the melting curves of P–N complexes shown in Figure 4. T_m of the *Bgl*II dodecamer decreases progressively upon peptide binding. This result is in contrast to earlier reports (Gabbay et al., 1976) but perhaps not unexpected. Usually the DNA double helix is stabilized by binding of a ligand in the major or minor groove through a number of hydrogen bonds and van der Waals contacts with the functional groups on base edges. In KGWGK, as the neighboring residues of tryptophan are glycines, which do not possess side chains either to contact or form hydrogen bonds with nucleobases, stabilization of DNA by ligand binding would be very weak even if the peptide binds in the groove. On the other hand, even a partial insertion of the indole ring between base pairs would cause unstacking of bases, causing destabilization. The T_m decrease of the complex levels off at saturation binding of 2 peptides per oligomer strand, i.e., 4 peptides per dodecamer duplex, which would mean each peptide molecule covering about three base pairs or three base residues per strand. This value is in excellent agreement with the value (3.2) obtained from the Scatchard plot shown in Figure 5. The overall association constant is 4.3×10^5 M^{-1}, a value which agrees well with the value reported for KGWGK binding to *Bgl*II hairpin stems (Rajeswari et al., 1992). This agreement is probably due to the fact that hairpin stems are duplex structures, and the binding mode will therefore be similar. The proposed mode of binding to the hairpin stems leading to intercalation and fluorescence quenching is similar to the interstrand complexes.

The interstrand complex we propose is basically the Wilkins (1956) model, which has received experimental support from several studies [Gabbay et al. (1976) and reference cited therein]. In this model the polypeptide chain assumes single-stranded helical β-sheet structure, which can wrap around the DNA double helix, with the amino acid side chain alternately pointing "into" and "out of" the helix [see Figure 5 in Gabbay et al. (1976)]. With KGWGK, under the constraint of two terminal lysines being anchored to the opposite strands of DNA, the indole ring of tryptophan would either point into or out of the helix depending upon the orientation of the peptide, as shown in Figure 6. We want to point out that such binding is consistent with formation of two types of complex, one with complete quenching of tryptophan fluorescence and the other without quenching (Rajeswari et al., 1987). This model would also account for the much lower quenching observed in case of peptides bound to usual B-DNA structure (only 50% of bound molecule will contribute to quenching) compared to other type of structures of single-stranded nucleic acids (Rajeswari et al., 1987).

Our CD data are consistent with this model of intercalation in the interstrand complex. The broad positive band at 220 nm of the pentapeptide diminished drastically upon addition of oligonucleotide. Disordered oligopeptides usually show a weak band, either positive or negative depending upon solvent condition and peptide sequence, which is assigned to the peptide $n-\pi^*$ transition (Woody, 1985). The indole chromophore of tryptophan has a B_b transition at 225 nm and the corresponding CD band is usually positive in disordered peptides (Auer et al., 1973). Thus the broad positive band ob-

served for KGWGK (Figure 7) may be a composite band with contribution from both the peptide n–π^* and indole B_b transitions. The decrease in this band therefore indicates changes in the peptide backbone conformation as well as orientation of Trp side chain upon binding to the oligonucleotide duplex. The extended chain β-structure of polylysine in aqueous solution shows a negative CD band at $\simeq$220 nm assigned to the peptide n–π^* transition (Yang et al., 1976). If the peptide KGWGK binds to the *Bgl*II duplex in the fashion described above, as an interstrand complex, a drastic reduction in the 220-nm band is expected. In contrast, only negligible changes are seen in oligonucleotide CD bands (Figure 4B). As free peptide does not exhibit detectable CD above 240 nm, the spectrum in the 240–300-nm region is presumably devoid of interference from peptide CD. The possibility of induced dichroism in the peptide transitions in this region due to intercalation is ruled out, because CD difference spectra between poly(A) alone and its mixture with any of Lys-Trp-Lys, Lys-Tyr-Lys, and Lys-Phe-Lys are very similar (Durand et al., 1975). Gabbay et al. (1976) investigated binding of a series of oligopeptides with N-terminal Lys-Phe sequence to salmon sperm DNA by NMR, CD, and other techniques. Their NMR data clearly demonstrate the partial intercalation of the aromatic ring of phenylalanine between base pairs; viscometric and flow dichroism results support the partial insertion and also suggest consequent bending of DNA. Nevertheless, the DNA CD spectrum shows only a small decrease in the long-wavelength band at 275 nm and it has been concluded that DNA structure is not grossly affected (Gabbay et al., 1976). The small decrease is attributed to electrostatic binding of Lys NH_3^+ charges to phosphates (Durand et al., 1975; Gabbay et al., 1976). In view of these results, and the small changes observed in Figure 4B, we suggest that no major perturbation occurs in the duplex structure of the oligonucleotide despite intercalation of the indole ring.

Intercalation in an intrastrand complex, in our opinion, would involve major distortions in both peptide and DNA structures and should also be reflected in the DNA CD spectrum, which is not observed. In an interstrand complex, intercalation may only be nominal and consequently perturbation in the DNA helix is minimal. In view of above discussions, we think that the interstrand complex model agrees well with our data and possibly is the preferred mode of groove-cum-intercalation binding of KGWGK to the *Bgl*II duplex.

Added in Proof

We draw attention to the crystallographic study of MetJ repressor complexed with DNA, which has revealed the antiparallel β-ribbon as a DNA recognition motif. It has been suggested that the β-ribbon can be a popular motif for recognition through the major groove (Kim, 1992). In fact, a revised structure of an *Eco*RI–DNA cocrystal shows an extended chain motif (an isolated β-strand) that runs through the major groove (Kim et al., 1990). This adds support to our model proposed here.

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