Binding of Oligopeptides to d-AGATCTAGATCT and d-AAGCTTAAGCTT: Can Tryptophan Intercalate in DNA Hairpins?†

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Received September 12, 1991; Revised Manuscript Received December 18, 1991

ABSTRACT: The interactions of three tryptophan-containing peptides, KWK, KGWK *tert*-butyl ester, and KGWGK, with two self-complementary dodecamers of the same base composition but different sequence were studied by UV, CD, and fluorescence spectroscopy. The oligonucleotides, d-AGATCTAGATCT and d-AAGCTTAAGCTT, contain tandem repeats of the recognition site for the restriction enzyme *Bgl*II in the former and *Hin*dIII in the latter. Thermal transition data in dilute solutions and in 0.01 M NaCl indicate these dodecamers to be present in hairpin forms. Binding of peptides to these hairpins was followed by tryptophan fluorescence quenching titrations at 10 mM Na^+; the data suggest intercalation of the indole ring. The association constants for the peptide–oligonucleotide (PN) complexes are an order of magnitude higher (10^5 M) than those reported with polynucleotides [10^4 M; Rajeswari et al. (1987) *Biochemistry 26*, 6825]. The pentapeptide, KGWGK, discriminates between *Bgl*II and *Hin*dIII sequences with higher affinity for the *Hin*dIII dodecamer. The CD maximum of KGWGK, at 220 nm, is drastically diminished upon interaction with oligonucleotides. The ellipticity at 220 nm is halved at 10 times less P/N ratio with the *Hin*dIII dodecamer than the *Bgl*II dodecamer, suggesting stronger binding to the *Hin*dIII dodecamer. The results are discussed in terms of two different modes of binding of oligopeptides to the DNA hairpins.

Hairpin loops in DNA, though less common, are important structural signals that play an essential role in proper positioning and recognition of regulatory proteins (Weaver & De Pamphillis, 1984). Many replication origins contain unusual sequences such as direct repeats, palindromes, and true palindromes, which enable these regions to assume hairpin configurations recognized by specific initiation proteins (Marian, 1985). Studies with model DNA hairpins, using synthetic oligonucleotides constrained to form such structures, have been reported only recently (Blommers et al., 1989; Xodo et al., 1988), including structures with only two residues in the loop (Howard et al., 1991). Two-base-loop hairpins are stereochemically and energetically feasible in both DNA (Raghunathan et al., 1991) and RNA (Cheong et al., 1990). We present here a report of two deoxyoligonucleotides, d-AGATCTAGATCT and d-AAGCTTAAGCTT, which contain direct repeats of recognition and cleavage sites for the restriction endonucleases *Bgl*II and *Hin*dIII, respectively. These oligomers, depending upon conditions, will form duplexes as well as hairpin structures. A hairpin with a two-base loop in these sequences will constitute a single recognition site for the corresponding endonucleases. These enzymes achieve stringent discrimination of DNA sequences by both direct read-out (protein base contacts) and indirect read-out (features like bends, kinks, and sequence-dependent local distortions in DNA conformation) mechanisms (McLarin et al., 1986; Lesser et al., 1990). We have studied interaction of the two dodecamers with tri-, tetra-, and pentapeptides, each having a central tryptophan residue, by UV, CD, and fluorescence spectroscopy. The important questions we want to address through this model study are as follows: How different is the indirect read-out of the hairpins from that known in duplexes? Does tryptophan intercalate in hairpin structures? Do these peptides discriminate between *Bgl*II and *Hin*dIII sequences? We show here that these peptides bind to hairpins more strongly than to polynucleotide duplexes reported earlier [Rajeswari et al. (1987) and references cited therein] and that the pentapeptide discriminates between the two oligonucleotide hairpins.

Materials and Methods

The phosphoramidites were purchased from Applied Biosystems. The peptides Lys-Trp-Lys (KWK) was from Sigma, Lys-Gly-Trp-Lys *O-tert*-butyl ester (KGWK-O-tBu) was from Bachem (Switzerland). The pentapeptide Lys-Gly-Trp-Gly-Lys (KGWGK) was synthesized in solution phase by standard procedure. The two self-complementary oligonucleotides, d-5′AGATCTAGATCT (*Bgl*II) and d-5′AAGCTTAAGCTT (*Hin*dIII) were synthesized on a Cruachem PS100 semimanual DNA synthesizer using β-cyanoethyl phosphoramidite chemistry (Atkinson & Smith, 1984). Purification was done on a Q-Sepharose anion exchanger, details of which are reported elsewhere. The purity was checked on an FPLC analytical Mono-Q column as well as on urea PAGE after 5′-end labeling with ^{32}P. All chemicals used for making buffers were of analytical grade.

Concentrations were determined using E^M_{280} of 5700 M^{-1}, for the peptide, and E^M_{260} of 9000 M^{-1}, for the oligonucleotides, in dilute solution in 10 mM NaOH.

Fluorescence titrations of oligonucleotide solutions with peptides were performed on a Shimadzu RF-540 spectrofluorometer at room temperature (25 °C) in 10 mM cacodylate buffer (pH 7.0) containing 1 mM NaCl and 0.2 mM EDTA as described (Brun et al., 1975). The concentrations of oligonucleotides were 0.4×10^{-5} M to 2×10^{-5} M and the

† This work was accomplished with financial assistance from the Department of Science and Technology (Grant SP/SO/D43/86), Government of India. M.R.R. gratefully acknowledges the University Grant Comission for the Research Scientist Grant.

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0006-2960/92/0431-6237$03.00/0

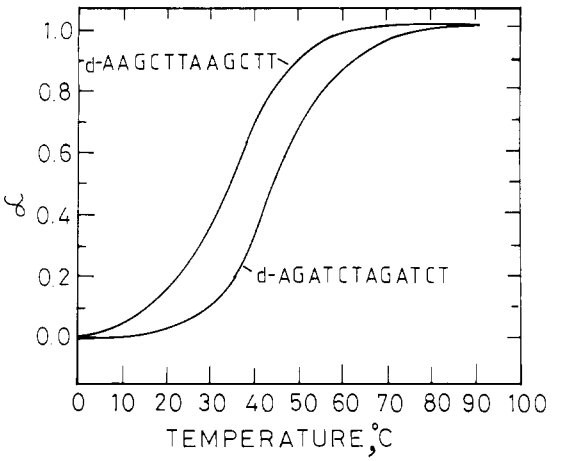

FIGURE 1: Ultraviolet melting curves of d-AAGCTTAAGCTT (*Hin*dIII) and d-AGATCTAGATCT (*Bgl*II) oligomers in 5 mM disodium phosphate buffer, pH 7.4, and 1 mM EDTA. α is the fraction of total change in absorbance upon thermal denaturation. T_m values are 35 and 44 °C for *Hin*dIII (2.0×10^{-5} M) and *Bgl*II (2.4×10^{-5} M) sequences, respectively.

peptide concentration varied from 1 to 6 μM. The isosbestic point in the difference absorption spectra (not shown) was 292 nm. However, the excitation wavelength chosen was 285 nm to ensure better quantum yield, since the absorption change due to binding at 285 nm was only about 1%. The emission maximum of the peptide was at 356 nm in both the presence and absence of the oligonucleotides.

The fluorescence quantum yield, Q, of peptide in the presence of oligonucleotide is expressed relative to that of free peptide, i.e., Q/Q_F. The observed difference in quantum yield ranged from 7% to 12%. The Q/Q_F values were plotted as a function of peptide concentration and extrapolated to zero peptide concentration to give the limiting value, Q_L. The screening effect due to light absorption by the oligonucleotides was taken care of by adding increasing amounts of NaCl up to 0.5 M (Brun et al., 1975). The fluorescence quantum yield of the dissociated complex in the presence of high salt was taken as Q_F.

UV absorption spectra and melting curves presented here were recorded on a Cecil 599S spectrophotometer with a water-jacketed cell holder. CD spectra were recorded on a Jasco J500A spectropolarimeter, which was routinely calibrated with D-camphorsulfonic acid.

Results

Thermal Transitions. UV melting curves of the two oligomers in 5 mM disodium phosphate buffer (pH 7.4) and 1 mM EDTA are shown in Figure 1. The monophasic thermal transitions are broad but cooperative, indicating melting of ordered forms. The observed melting temperatures (T_m) were 44 °C for *Bgl*II and 35 °C for *Hin*dIII dodecamer, respectively. Under low salt conditions T_ms of both the oligonucleotides are practically independent of oligomer concentrations (Figure 2).

Fluorescence Measurements. The binding equilibria of oligopeptides and polynucleotides are treated in terms of formation of two types of complexes as per Schemes I and II (Brun et al., 1975), where P stands for peptide and N for polynucleotides. The complex $(PN)_1$ involves simple electrostatic binding to backbone phosphates without intercalation of the tryptophan side chain and hence does not contribute to fluorescence quenching, while in complex $(PN)_2$ the tryptophan moiety is intercalated between base pairs and is completely quenched (Montenay-Garestier et al., 1983). The existence of two types of complexes has been demonstrated by fluorescence lifetime measurements of KWK and KWGK-*O*-tBu in the presence and absence of polynucleotides (Montenay-Garestier et al., 1982; Rajeswari et al., 1987). We assume these results are of general validity and should hold well with oligonucleotides also.

Table I: Equilibrium Constants K_I, K_{II}, K_1, and K_2 for the Binding of Peptides to *Bgl*II Dodecamers[a]

peptide	K_I (=K_1) × 10^{-5} (M^{-1})	K_{II} (=K_1K_2) × 10^{-5} (M^{-1})	K_2
KWK	2.8 ± 0.2	0.20	0.07 ± 0.004
KWGK-*O*-tBu	1.5 ± 0.1	0.15	0.10 ± 0.005
KGWGK	3.3 ± 0.5	0.50	0.15 ± 0.010

[a] These binding constants were evaluated from Trp fluorescence quenching titrations using eqs 1 and 2 under the following experimental conditions: 10 mM cacodylate buffer containing 1 mM NaCl and 0.2 mM EDTA, pH 7.0 at 25 °C. The concentration range of peptides was 1–6 μM.

Table II: Equilibrium Constants K_I, K_{II}, K_1, and K_2 for the Binding of Peptides to *Hin*dIII Dodecamers[a]

peptide	K_I (=K_1) × 10^{-5} (M^{-1})	K_{II} (=K_1K_2) × 10^{-5} (M^{-1})	K_2
KWK	3.40 ± 0.4	0.31	0.09 ± 0.007
KGWK-*O*-tBu	0.70 ± 0.08	0.08	0.12 ± 0.01
KGWGK	36.00[b]	3.96	0.11 ± 0.01

[a] Experimental conditions were the same as in Table I. [b] Error is large due to the small slope in the graph shown in Figure 3, top panel.

Scheme I

$$P + N \rightleftharpoons (PN)_1 \quad (K_1)$$

$$P + N \rightleftharpoons (PN)_2 \quad (K_{II})$$

Scheme II

$$P + N \rightleftharpoons (PN)_1 \rightleftharpoons (PN)_2 \quad (K_1, K_2)$$

Schemes I and II are thermodynamically equivalent if $K_{II} = K_1K_2$, and the fluorescence titration data are interpreted according to Scheme II only, where K_1 and K_2 are evaluated from the following equation (Brun et al., 1975; Rajeswari et al., 1987):

$$Q_F/(Q_F - Q_L) = 1 + 1/K_2 + (1/K_1K_2)(1/[N]) \quad (1)$$

where Q_F is the fluorescence quantum yield of the free peptide and Q_L is the limiting value of the quantum yield of the complex (Q) extrapolated to zero peptide concentration. However, if the complexes $(PN)_1$ and $(PN)_2$ are two different species without isomerization between them, as in Scheme I, eq 1 can be written as

$$Q_F/(Q_F - Q_L) = 1 + K_I/K_{II} + (1/K_{II})(1/[N]) \quad (2)$$

In either equation, (1) or (2), a plot of $Q_F/(Q_F - Q_L)$ versus $1/[N]$ will give a straight line permitting evaluation of K_I and K_{II} (or K_1 and K_2). Such plots for binding of the pentapeptide, KGWGK, to the *Bgl*II and *Hin*dIII dodecamers are shown in Figure 3, and the values for association constants for all three peptides derived from the least-squares plots of Figure 3 are quoted in Tables I and II.

Results with *Bgl*II show little change in K_I (or K_1) between the tri- and pentapeptide, while K_{II} values increased 2.5-fold with increased size of the peptide (Table I). With the *Hin*dIII dodecamer, the results are radically different; both K_I and K_{II} values are severalfold higher than KGWGK than with KWK. KGWK-*O*-tBu binds very weakly compared to KWK and

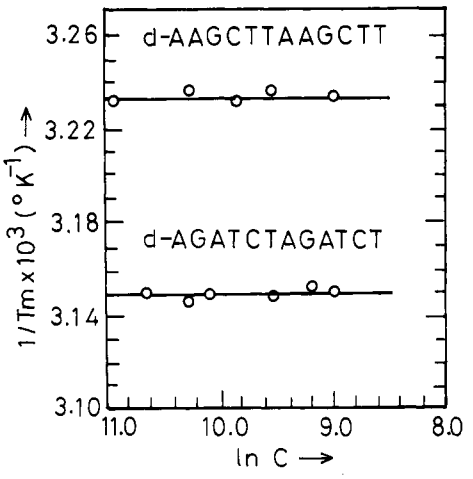

FIGURE 2: Dependence of $1/T_m$ vs ln C for the two deoxyoligonucleotides, where C is the oligomer concentration in moles of residues. Conditions: 2 mM sodium cacodylate, pH 7.0, 8 mM NaCl, and 1 mM EDTA.

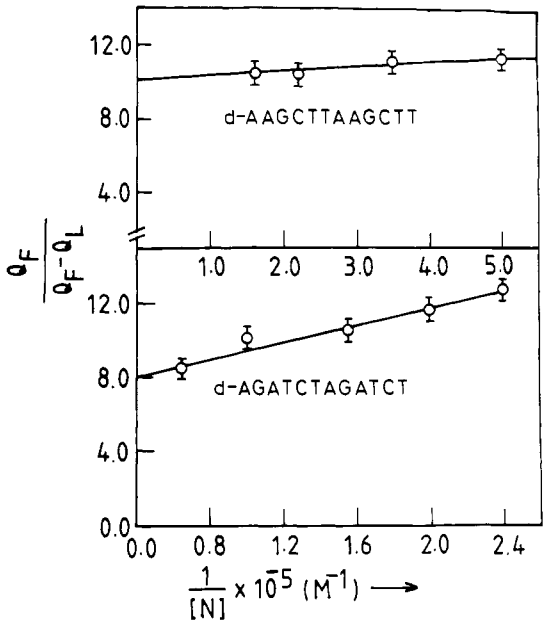

FIGURE 3: Analysis of fluorescence data for the binding of KGWGK to the two deoxyoligonucleotides, according to eq 2. Each point in the graph is from a complete fluorescence titration done in 10 mM sodium cacodylate, pH 7.0, containing 1 mM NaCl and 0.2 mM EDTA at 25 °C, using peptide concentrations ranging from 1 to 6 μM.

KGWGK (Tables I and II) despite the absence of negative charge at the carboxyl terminus. The variation of the isomerization constant, K_2, over the series of three peptides is very different from the trend shown in K_I or K_{II}.

Circular Dichroism. All the CD spectra were recorded in 50 mM disodium phosphate, pH 7.4, and 0.2 mM EDTA ([Na^+] = 101 mM). Figure 4 shows the CD spectra of the pentapeptide, KGWGK, in the presence and absence of oligonucleotides at different P/N ratios. The free peptide spectrum shows a single positive band centered around 220 nm but no dichroism above 240 nm. The spectra of the peptide in the presence of oligonucleotides were corrected for nucleic acid contribution calculated from the CD spectrum of free oligonucleotide, since the dichroic changes observed in the nucleic acid absorption region (240–300 nm) in the mixture were very small. About 50% reduction in ellipticity of the peptide band occurs at 10 times less P/N ratio for the *Hin*dIII dodecamer (P/N = 0.43) than for the *Bgl*II (P/N = 4.2)

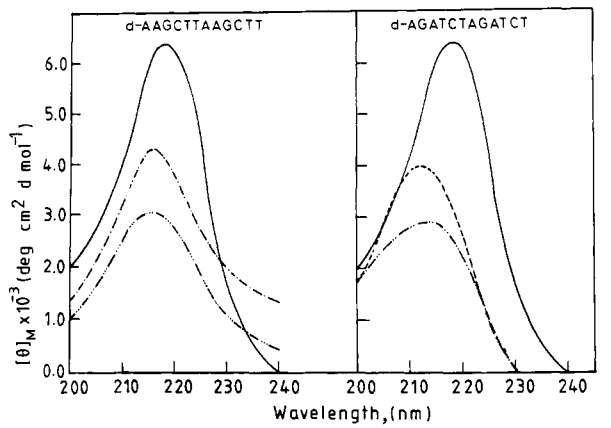

FIGURE 4: Circular dichroic spectra of peptide KGWGK in the absence and presence of the two oligonucleotides in 50 mM disodium phosphate buffer (pH 7.4) and 0.2 mM EDTA at 25 °C. The peptide (P) concentration was 5×10^{-5} M and P/N mole ratios were 0 (—), 0.14 (-·-), and 0.43 (-···-) for d-AAGCTTAAGCTT and 0 (—), 1.4 (---), and 4.2 (-···) for d-AGATCTAGATCT. [N] is given in oligomer strand concentration. The *Hin*dIII dodecamerr exists as a mixture of hairpins and duplexes, while the *Bgl*II dodecamer is almost completely in duplex form under these conditions (unpublished results).

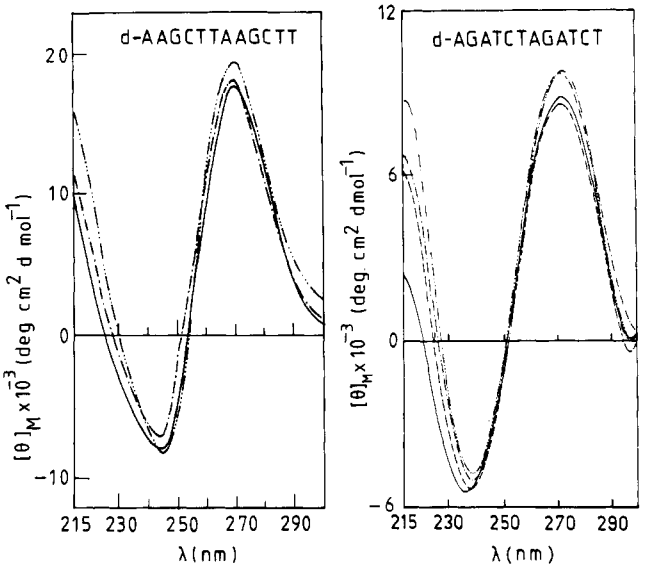

FIGURE 5: Circular dichroic spectra of the oligonucleotides, free (—) and in the presence of KGWGK at different P/N mole ratios ([N] is given in oligomer strand concentration): for d-AAGCTTAAGCTT, P/N = 0 (—), 0.14 (-·-), and 0.43 (-···-); for d-AGATCTAGATCT, P/N = 0 (—), 1.4 (---), 2.1 (-·-), and 4.2 (-···-). Conditions: 50 mM disodium phosphate, pH 7.4, and 0.2 mM EDTA at 25 °C.

dodecamer. The CD spectra of the oligonucleotides in the range 215–290 nm in the presence and in the absence of the peptide are also shown in Figure 5. The changes in the ellipticity are very small but distinct.

Discussion

Melting curves of the two deoxyoligonucleotides are monophasic at low salt concentration but are distinctly biphasic at higher ionic strengths (results not shown), suggesting the presence of hairpin structures. The monophasic thermal transition presented in Figure 1 must represent melting of hairpin forms since the T_ms of these oligomers in dilute salt solutions are independent of oligomer concentrations (Figure 2). The self-complementary d-GGTACGCGTACC and its derivatives with GA and IA mispairs at positions 6 and 7 or 5 and 8 in dilute salt solutions show monophasic melting curves and also exhibit negligible slope in $1/T_m$ vs ln C curves

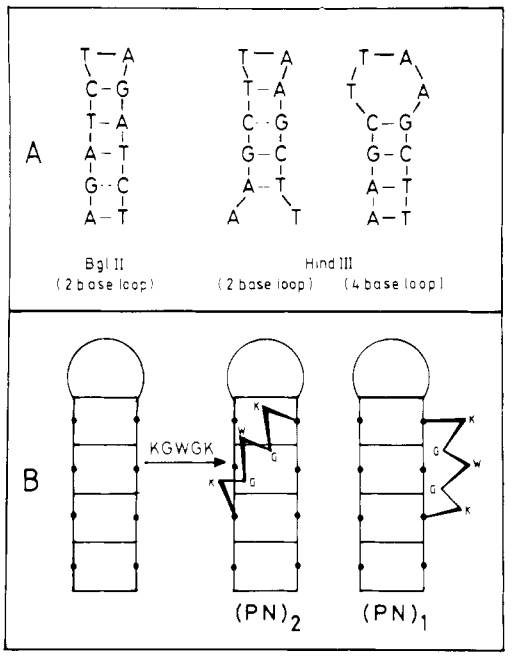

FIGURE 6: Schematic representations of (A) possible hairpin structures for the two dodecamers presented in the study and (B) binding of KGWGK to the deoxyoligonucleotide hairpins.

(Howard et al., 1991). The dependence of thermal stability on the position of the mispair is interpreted in terms of hairpin structures with a two-base loop and a five-base-pair stem of three GC and two AT pairs for those melting above 50 °C; the less stable 5,8 derivatives having a four-base loop and four-base-pair stem of two GC and two AT pairs (see Figure 5 in Howard et al., 1991). With our oligomers, a five-base-pair stem would have two GC and three AT pairs, whereas a four-base-pair stem would have two GC and two AT pairs. Although we cannot conclude about the number of residues in the loop, the observed T_ms of our dodecamers suggest that in low salt solutions the *Bgl*II dodecamer (T_m = 44 °C) exists as a hairpin with a two-base loop and a five-base-pair stem, and the *Hin*dIII hairpin (T_m = 35 °C) has a four-base-pair stem with either two- or four-base loops as shown in Figure 6 (panel A). We therefore choose to discuss the binding of peptides to our oligomers in terms of two separate $(PN)_1$ and $(PN)_2$ complexes with DNA hairpins, as per Scheme I, as schematically shown in Figure 6 (panel B). We also assume that intercalated complexes, $(PN)_2$, are formed at the stem region of hairpins, as a two-base loop will be two small to accommodate any of the peptides as an intercalated complex.

Interesting results of the present study are that the oligopeptides bind more strongly to these DNA hairpins than to polynucleotide duplexes; K_1 values ($\approx 10^5$ M) for $(PN)_1$ complexes are 1 order of magnitude higher than those reported for polynucleotides (Rajeswari et al., 1987; Brun et al., 1975). We think K_I involves not only coulombic forces but also van der Waals contacts and hydrogen bonding with the DNA backbone. In that case bends, kinks, and sequence-dependent distortions in DNA structure will be an important factor. It is possible that geometries of these hairpins are stereochemically more favorable for interactions with small peptides. Moreover, in such a scenario with the hairpins, all binding lattices are uniform, whereas with polynucleotides binding sites comprise a heterogeneous population involving those with very low affinities leading to a low average value. We may mention that K_1 with poly(rA)·poly(rU) is 12 times higher than that with poly(rA)·poly(dT) (Rajeswari et al., 1987) though they both have basically A-type structures. Surprisingly, KGWK-*O*-tBu binds very poorly compared to KWK or KGWGK. We do not have any explanation at present except for possible steric interference by the bulky ester group. K_I values are similar for KWK binding to the two dodecamers but are radically different for KGWGK binding.

The overall association constant K_{II} for $(PN)_2$ intercalated complexes is only slightly higher for binding of KWK to the *Hin*dIII sequence than to the *Bgl*II sequence (0.31×10^5 and 0.2×10^5 M), but for KGWGK binding K_{II} is 8-fold higher than *Hin*dIII than with *Bgl*II (3.96×10^5 and 0.5×10^5 M). In $(PN)_2$ complexes, if the binding is of the polylysine type (Feughelman et al., 1955) the amino acid side chain will have direct read-out through the major or minor groove, facilitating intercalation of the indole ring of tryptophan between base pairs. The stability of such $(PN)_2$ complexes will be influenced by the size and flexibility of the peptide as well as by the conformation of the hairpins (indirect read-out). An optimum size, perhaps, is necessary for efficient binding in the stem region, and KGWGK is much more flexible than KWK because of the presence of two glycine residues on either side of tryptophan. The large difference in slopes (K_{II} values) may be related also to the different structures of *Bgl*II and *Hin*dIII hairpins.

The ellipticity of the positive band at 220 nm in the peptide CD spectrum is drastically reduced on addition of either oligonucleotide (Figure 4), suggesting strong complexation with a definite change in peptide conformation on binding to the oligonucleotides. The 220-nm band is possibly a composite band with contributions from the peptide n–Π* transition and the B_b band of the indole chromophore. Thus in $(PN)_2$ complexes, where Trp is intercalated between base pairs and the peptide backbone is stretched out along the groove, both base stacking and the peptide backbone are perturbed, causing a dramatic change in CD maxima. The ellipticity is halved at 10 times less P/N ratio with *Hin*dIII than with *Bgl*II, which also supports stronger complexation with *Hin*dIII hairpins as concluded from the fluorescence data. We realize that in 50 mM phosphate (100 mM Na^+) buffer the oligomers possibly exist as a mixture of hairpins and duplexes; *Bgl*II is almost completely in duplex form (Roy et al., 1992) but not *Hin*dIII. The CD data therefore do not really relate to the fluorescence titration conditions, where the oligomers are in hairpin forms. Nevertheless, we think that with the mode of binding as we have proposed, the peptides bound to hairpin stems and duplexes would have similar geometries since hairpin stems are basically duplex structures. The perturbations in the peptide conformation upon complexation and the consequent changes in peptide CD spectrum would then be similar for *Bgl*II and *Hin*dIII solutions irrespective of the composition of helical species. The binding affinities of the hairpin and duplex forms of any one dodecamer would also be similar. Indeed, the overall association constant of *Bgl*II duplex with KGWGK is 4.33×10^5 M^{-1} (Roy et al., 1992), while for hairpin form it is 3.8×10^5 M^{-1} (Table I). Under such circumstances, if KGWGK has stronger affinity for the *Hin*dIII sequence, much less peptide would be required in the case of *Hin*dIII to reduce the peptide CD band by the same extent as observed with the *Bgl*II dodecamer at a 10 times higher P/N ratio. This is what is shown in Figure 4 and is supportive of the conclusion arrived from fluorescence data. This implies, of course, that, like *Bgl*II, the hairpin and duplex forms of the *Hin*dIII dodecamer also do not differ much in their affinities for the peptide. Figure 5 shows very little change in the oligonucleotide CD spectrum in the 240–300-nm region, apparently suggesting negligible perturbation in oligomer structure and validating

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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Received September 12, 1991; Revised Manuscript Received April 13, 1992

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.

† This work was carried out with financial assistance from the Department of Science and Technology (Grant SP/SO/D43/86) and the University Grants Commission (MRR/8-55/SC/88(SAI)), Government of India.

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0006-2960/92/0431-6241$03.00/0