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Construction of Peptide β-Hairpins Recognizing DNA Sequences

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We synthesized fluorescently labeled 16-unit linear and cyclic peptides with sequences found in β-hairpins with four-residue loops; in the cyclic peptide the hairpin was stabilized by an S-S bond between two cysteines. The two peptides were shown to form two types of complex with DNA, with stoichiometry of 1:1 and 2:1 per binding site of four base pairs. For the linear peptide, the DNA titration curve depended on the solvent, and in 20% trifluoroethanol was S-shaped, suggesting cooperative binding. For the cyclic peptide the titration curve was S-shaped without TFE as well as in 20% TFE and 20% methanol. The thermodynamic parameters of peptide binding were determined without and with 20% TFE. The cyclic peptide preferred poly(dG)•poly(dC) to poly(dA)•poly(dT) and poly[d(GC)]•poly[d(GC)]; the former complex persisted to 0.06 M NaCl whereas the latter two dissociated at 0.04 M. The linear peptide formed a tighter complex with poly(dG)•poly(dC) than its cyclic counterpart. Both peptides were shown to compete for binding sites on poly(dA)•poly(dT) with distamycin A and its analog binding in the DNA minor groove.

Key words: DNA-binding peptides; two-strand peptide motif; β-hairpin construction; cooperative effects

Along with studies on the structure of DNA-protein complexes, of considerable interest are synthetic peptides identical or similar in structure to those found in the DNA-binding domains of regulatory proteins. The advantage of the model approach is that relatively small peptides are considered instead of quite complex macromolecules, and their primary sequences may often be significantly simplified in accordance with the *a priori* requirements of the model. Some model peptides are known [1-8] to be capable of binding to the same DNA regions as the natural proteins. The search for specific DNA-binding peptides may provide building blocks for designing ligands that would selectively bind to certain DNA regions [9, 10].

The goals of the present work were to synthesize 16-residue linear and cyclic peptides **I** and **II** (Fig. 1) and to study their binding with nucleic acids by fluorescence techniques and CD spectroscopy. The cyclic peptide differs from the linear one by the S-S bond between cysteines; such bonding is known to stabilize the β-structure in a short peptide *bis*(BocAlaCysOMe) [11]. We have earlier described the design of 26-unit linear and cyclic peptides modeling β-hairpins with four-residue loops [12], and have shown that they bind selectively with pseudosymmetrical nucleotide sequences at the periphery of phage 434 operators O_R1-3. The design involved the minimalistic approach, i.e., the peptides were composed of the minimal set of amino acids required to attain the goal. The same was applied to the 16-unit peptides in this work. This facilitates the structural analysis of their DNA complexes by NMR, IR and Raman spectroscopy.

The basic idea of the design was that the DNA-binding domains of some regulatory proteins contain two antiparallel chains in p-conformation. One can mention the *Escherichia coli* host integration factor and the eukaryotic transcription activator TBP recognizing the TATA box [13-15]; a similar motif is found in antibiotics of the triostin group [16]. In the crystalline complex of triostin A with self-complementary 5'-CGTACG-3', the depsipeptide ring of the antibiotic is inserted into the DNA minor groove [17, 18]. In

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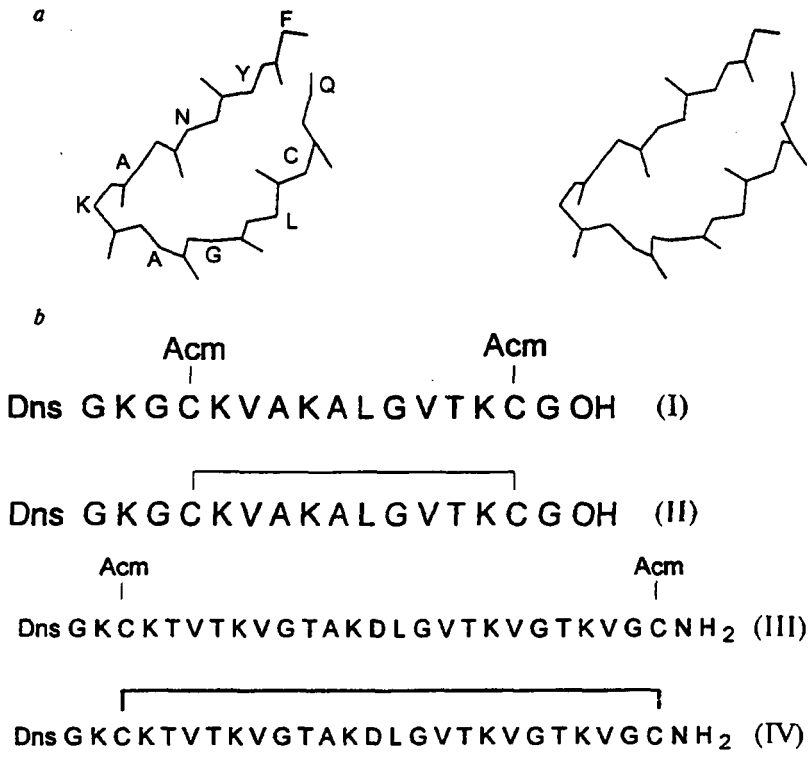

Fig. 1. *a)* Stereoscopic view of the β-hairpin in the bovine trypsin inhibitor (main chain), *b)* Linear and cyclic 16- and 26-residue peptides; amino acids in one-letter code, Acm is acetamidomethyl, Dns is dansyl.

accordance with our model of insertion of two antiparallel peptide chains into the minor groove [19], the NH and CO groups of the triostin A peptide ring act as donors and acceptors of H-bonds with DNA bases. Despite the similarity of TBP and triostin A complexes with DNA. there are important differences. In the TBP complex the DNA structure is deformed much more, and bases interact with the amino acid side chains rather than with the peptide backbone groups as in the triostin complex. Investigation of the structure of DNA complexes with β-peptides may shed light on the nature of these differences.

Figure 1 presents a stereoscopic view of the β-hairpin in the bovine trypsin inhibitor (residues 22-31); the atomic coordinates were taken from the Brookhaven Data Bank (protein code 5PTI). The hairpin motif is FYNAKAQLCQ, underlined are the four residues forming the loop, wherein glycine has dihedral angles ϕ and ψ corresponding to left-handed α-helix, which provides for an abrupt change in the polypeptide chain course needed to create a β-hairpin. Formation of the latter in a linear and cyclic peptide may also be promoted by salt bridging between the positive e-amino group of lysine at the N terminus and negative carboxyl at the C end.

MATERIALS AND METHODS

Peptides were synthesized by the solid-phase technique on a Merrifield resin (Aldrich, *1%* DVB, 200-400 mesh, 0.60 meq Cl/g) using N^{α}-Boc derivatives of amino acids. The side groups were protected with 2C1-Z (Lys), Bzl (Thr), and Acm (Cys); protected amino acids were introduced as activated 1-hydroxybenzotriazole esters, N-terminal glycine as Dns-GIy-OH. Synthesis was conducted in dimethylformamide, with condensation time of 1.5-24 h; in some cases 4-dimethylaminopyridine was used; condensation was checked as in [20]. Boc protection was removed with 50% TEA. in dichloromethane. All protective groups except Acm were removed with simultaneous detachment from the resin using a mixture of liquid HE, dimethylsulfide, *m*-cresol, and pentamethylbenzene (10:4:4:1). The reaction mix was subjected to evaporation, extracted with 30% aqueous acetic acid, lyophilized, and purified by gel filtration on a 2.5x60 cm column of Sephadex G-25 in 1 M acetic acid, and reversed-phase HPLC on a 10x250 mm Vydac C-18 (10 mkm) column with a 30-55% methanol gradient in water plus 0.1% TEA. (40 min, 3 ml/min, detection at 254 nm; retention time about 20 min). The structure of Acm-protected 16-unit linear peptide (mol. mass 1895.4 Da) was confirmed by SIMS: *m/z* 1896 $(M^{+}H)^{+}$. Cyclization was achieved by iodine treatment in 60% aq. acetic acid for 1.5 h at room temperature [21]. Excess iodine was eliminated with ascorbic acid. The cyclic peptide was purified as above except that the HPLC gradient was 20-55% methanol, detection at 220 nm, retention time about 46 min. The structure of cyclic 16-unit peptide (mol. mass 1751.2 Da) was

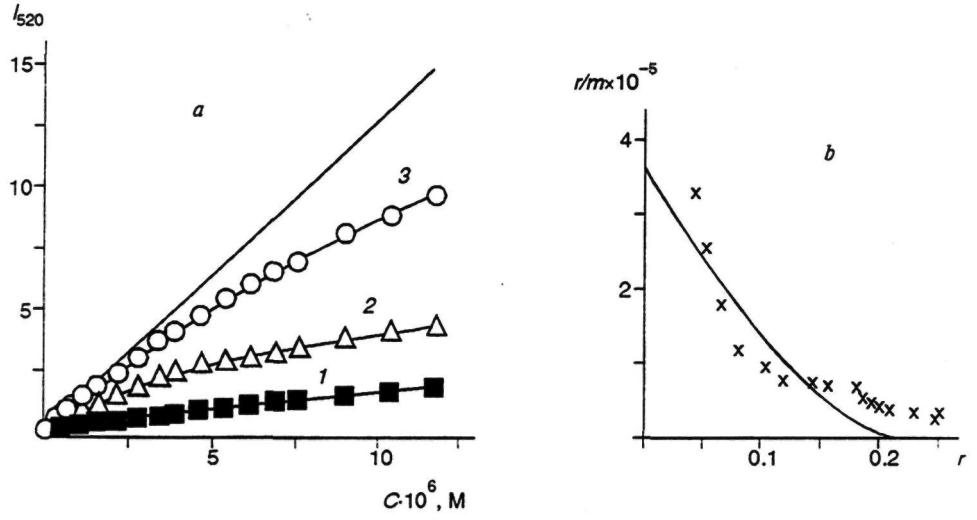

Fig. 2. Fluorimetric titration of DNA with peptide I *(a)* and Scatchard plot of the adsorption isotherm *(b). 1)* Peptide alone (control), with 2) 1.16 and *3)* 9.3 10^{-5}M (bp) DNA; straight line gives the initial slope of *3; C,* peptide concn.; *r.* bound peptide/basepair molar ratio; *m*. free peptide concn.

confirmed by FAB MS: *m/z* 1752 (M+H)+. Amino acid analysis yielded the following molar fractions (theoretical values parenthesized): Ala 2.11 (2), Gly 3.80 (4), Lys 3.82 (4), Leu 1.08 (1), Thr 1.04 (1), Val 2.15 (2); cysteine was not determined.

Synthetic peptides were stored lyophilized and were dissolved directly before experiments in 1 mM Na cacodylate (pH 7). Peptide concentration was determined spectrophotometrically, using an extinction coefficient e_{330} = 4300 $M^{-1}cm^{-1}$

Calf thvmus DNA (e_{259} = 13,300) was from Sigma; poly(dG)•poly(dC) (£253 = 14,800), poly(dA)•poly(dT) (e_{259} = 12,000), and poIy[d(GC)]•poly[d(GC] (e_{260} = 16,800) from PL Biochemicals (all extinctions given per mole base pairs). Poly(dG)•poly(dC) was dissolved in 0.1 M NaOH and dialyzed for 24 h against the same solution with 1 mM EDTA. Prior to use, all polynucleotides were dialyzed against two changes of the cacodylate buffer without EDTA. The CD spectra were taken and compared with the reference data. Slow changes were observed in the poly(dG)•poly(dC) spectrum during storage of solution at 4°C. When the spectrum was distorted, the pH was raised over 11 and dialysis was repeated. Fluorimetric measurements were made at 20°C with an Aminco SPF-lOOOSc instrument: excitation at 380 nm and detection at 520 nm (10 nm slit for excitation and emission). Absorption spectra were recorded with a Specord M40 spectrophotometer, CD spectra with a Jobin Yvon Mark III dichrograph.

RESULTS

Interaction of peptides with DNA in different solvents. Since peptides **I** and **II** carried a fluorescent label (dansyl) at their N termini, we used fluorimetric techniques to monitor their binding to DNA; peptide-DNA complexation is attended by a significant rise in fluorescence intensity. Figure 2 displays titration of different amounts of DNA with I in aqueous solution and the adsorption isotherm calculated from this data (Scatchard plot with r being the bound peptide/basepair molar ratio). The theoretical isotherm was generated with Eq. (16) in [22] derived for noncooperative binding of an extended ligand occupying *L* base pairs on DNA. The best fit was obtained with K_{ass} = 3.7•10^5 M^{-1}, X = 4 bp (Fig. 2b), whereby the mean square deviation (D2) of *r/m* was 2.3•10^4 M^{-2}.

Figure *3a* presents analogous titration curves obtained in the presence of 20%(vol.) TFE. Curve *3* is S-shaped, testifying to cooperative binding. The peptide-DNA complex is saturated at two molecules of I per four base pairs. The thermodynamic model consistent with these data as well as with those obtained earlier for other peptides [10, 12, 23-25] can be briefly outlined as follows. Peptide binds to DNA as a monomer (β-hairpin) and as a dimer (β-sandwich) which occupy four base pairs. Monomers bind noncooperatively, while dimer binding is cooperative owing to direct contacts between sandwiches adsorbed on neighboring sites. For this model, the amount of bound peptide can be calculated with Eqs. (1)-(4) [10]:

$$r = \frac{K_1 m + 2K_2K_s m^2\left(\dfrac{aX_1 - a + 1}{X_1}\right)}{(L+1)X_1^L - LX_1^{L-1} - K_1 m\,(1 + aK_2K_s m)}, \quad (1)$$

$$r = \frac{\partial \ln X_1}{\partial \ln m}, \quad (2)$$

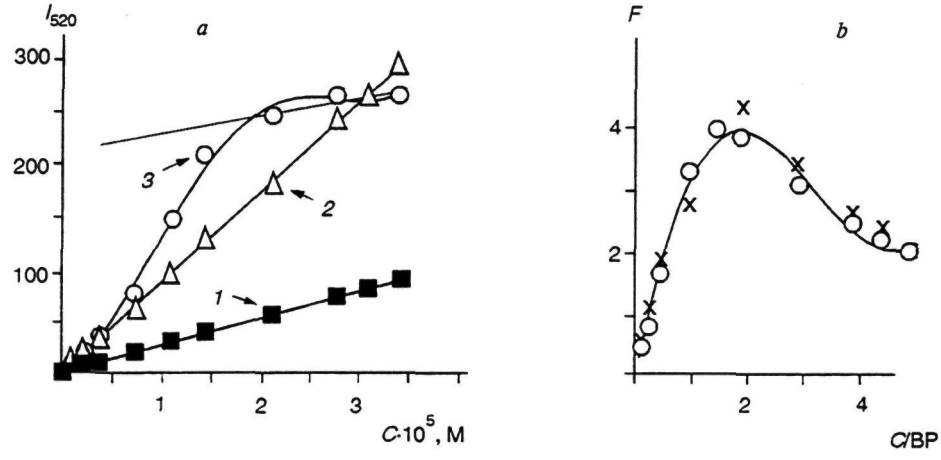

Fig. 3. Fluori metric titration of DNA with peptide I in the presence of 20% TFE. *Panel a: 1*) Peptide alone (control), with 2) $1.6 \cdot 10^{-4}$ and *3)* $7.7 \cdot 10^{-6}$ M (bp) DNA. *Panel b:* Experimental (crosses) dependence of *F* on C/BP (added peptide/basepair molar ratio) and theoretical best fit (circles and parabola) calculated with Eqs. (1-6) and parameters $L = 4$, $K_1 = 3 \cdot 10^4$ M^{-1}. $W = 2 \cdot 10^8$ M^{-2}. $a = 147$: $D2 = 0.9$.

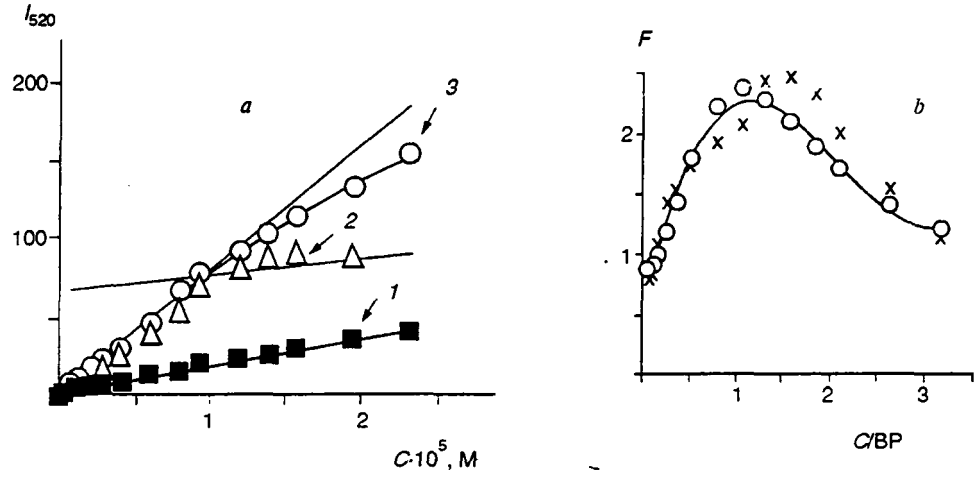

Fig. 4. Fluorimetric titration of DNA with peptide **II**: *1)* Peptide alone (control), with 2) $7.7 \cdot 10^{-6}$ and *3)* $9.6 \cdot 10^{-5}$ M (bp) DNA. Designations as in Fig. 3. parameters in text.

$$m = C - r\mathrm{BP}, \quad (3)$$

where X_1 is the largest root of the algebraic equation

$$X^{L+1} - X^L - K_1 mX - aWm^2(X - 1) - Wm^2 = 0. \quad (4)$$

Here $W = K_2K_s$, K_1 and K_2 are binding constants for monomer and dimer, K_s is sandwich formation constant, a is cooperativity parameter, C is peptide concentration and BP is DNA concentration (mole bp).

To determine the thermodynamic parameters of binding, we compared the experimental and theoretical dependences $F = I/I_o - 1$ on C/BP (I and I_O are peptide fluorescence intensities with and without DNA). If the system under study contains only two forms of bound peptide, then

$$F = ((f_1/f_0 - 1)R_1 + 2(f_1/f_0 - 1)R_2)/(C/\mathrm{BP}), \quad (5)$$

$$r = R_1 + 2R_2. \quad (6)$$

where R_1 and K_2 are molar ratios of bound monomer and dimer to DNA base pairs; f_o, f_1 and f_2 are molar fluorescence intensities for free peptide and bound monomer and dimer, respectively. The f_1/f_o ratio can be found by measurements on the peptide alone and in the presence of a large excess of DNA; the titration curves *1* and *3* in Fig. *3a* show that $f_1/f_o = 3.5 \pm 0.5$. The f_2/f_o can be obtained form the data for nearly saturating binding, when the contribution of monomers can be neglected; in our estimates $f_2/f_o = 22 \pm 1$. As shown in Fig. *3b,* the best match of theoretical and experimental F is obtained at $L = 4$, $K_1 = 3 \cdot 10^4$ M^{-1}, $W = 2 \cdot 10^8$ M^{-2}, $a = 147$; therewith $D2 = 0.9$. We have found earlier that without TFE and with 20% methanol there is no β-sandwich binding, and the peptide binds to DNA noncooperatively as a monomer. In the presence of 20% TFE the monomer biding

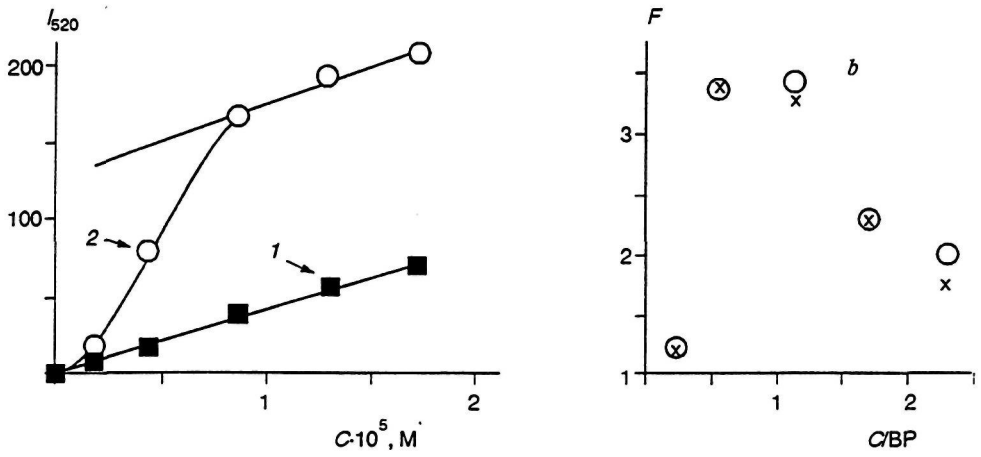

Fig. 5. Fluorimetric titration of DNA with peptide **II** in the presence of 20% TFE: 1) Peptide alone (control) and with 2) $7.7{\cdot}10^{-6}$ M (bp) DNA. Designations as in Fig. 3. parameters in text.

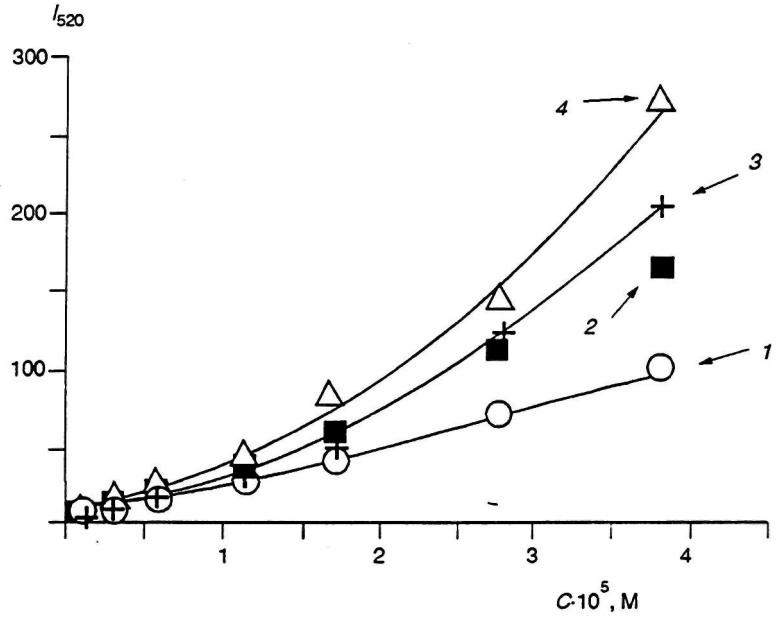

Fig. 6. Fluorimetric titration of polynucleotides G•C (2), A•T (3), and GC•GC *(4)* (all $1.6{\cdot}10^{-5}$ M) with peptide I *(I,* free peptide). Additions: 0.02 M NaCl. 20% TFE.

constant drops about 10-fold. This may reflect the binding-induced stabilization of β-conformation in the peptide and formation of a β-hairpin, which perhaps requires additional energy expenditures on altering the DNA conformation to enter the minor groove. However, with 20% TFE the peptide becomes capable of binding also as a dimer, so that its overall affinity for DNA increases. From the experimental data available, we can estimate only *W,* the product of the dimerization and dimer binding constants.

As to the cyclic peptide **II**, its titration curves are S-shaped without organic solvent, in 20%TFE, or 20% MeOH. The $f_2/f_0 = 9\pm1$**.** The best-fit parameters and mean square deviations obtained by matching the *F* dependences (Figs. 4 and 5) were $L = 4$, $K_1 = 4.5{\cdot}10^4$ M^{-1} $W = 4.9{\cdot}10^8$ M^{-2}, $a = 162$, $D2 = 0.8$ in aqueous solution and $L = 4$, $K_1 = 1.5{\cdot}10^4$ M^{-1}, $W = 5.2{\cdot}10^8$ M^{-2}, $a = 365$, $D2 = 0.09$ in 20% TFE. Interestingly, the binding constant for linear monomer in 20% TFE is close to that for cyclic peptide without TFE. In 20% TFE the K_1 for **II** is about half that for monomelic **I**, but *W* and *a* are about twice larger.

Binding with synthetic polynucleotides. Figures 6 and 7 display the titration of poly(dG)•poly(dC), poly(dA)•poly(dT), and poly[d(GC)]•poly[d(GC)] (prefixes "poly" and "d" are further omitted for simplicity) with peptides **I** and **II**, respectively. It has been shown that the quantum yield of dansyl fluorescence in complexes with DNA and synthetic polydeoxyribonucleotides does not depend on the GC content [23-26]. As evident from Fig. 6, initially there is no appreciable difference in peptide binding, whereas with increasing

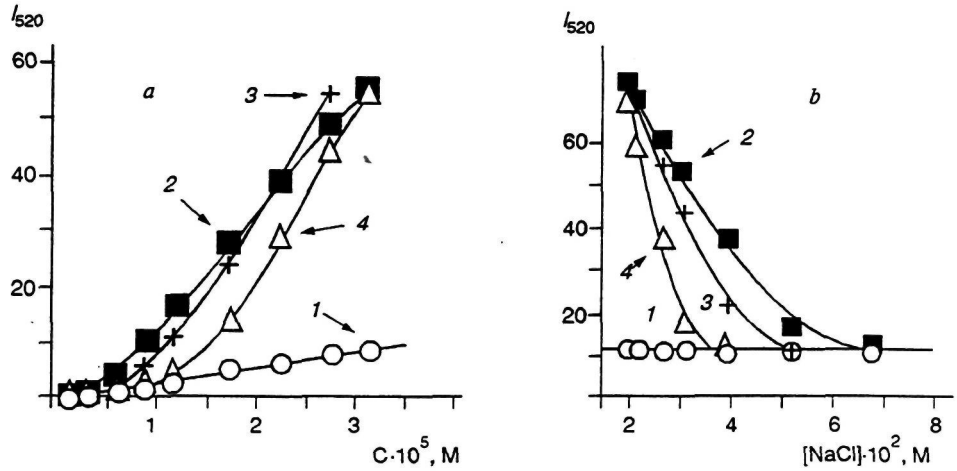

Fig. 7. *a)* Fluorimetric titration of polynucleotides G•C (2), AT (3), and GC•GC *(4)* (all 6.4•10^{-5} M) with peptide **II** (*1*. free peptide): conditions as in Fig. 6. *b)* Stability of respective complexes against NaCl.

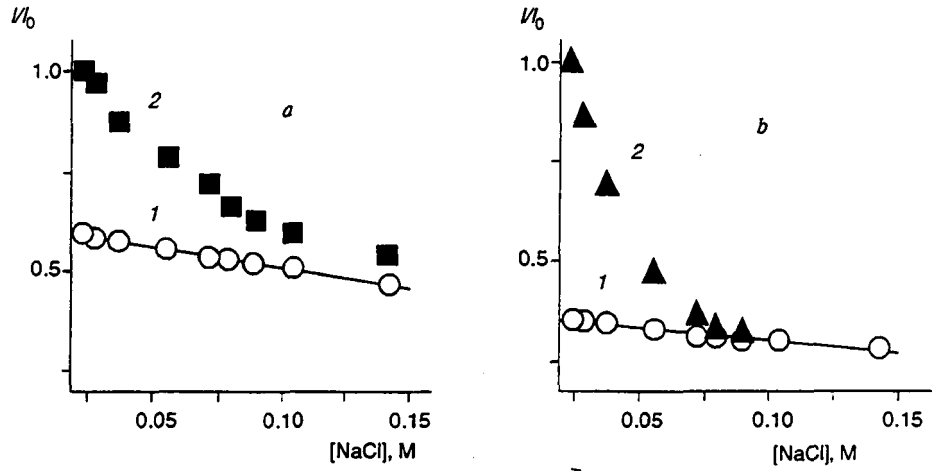

Fig. 8. Stability of peptide I complexes (2) with GC *(a)* and GC-GC *(b)* (all 1.6•10^{-5} M) against NaCI *(I,* free peptide), I_o is fluorescence intensity for the mixture in 0.02 M NaCl. *I* is that at current salt concentration.

peptide/polynucleotide ratio the interaction becomes cooperative.

For peptide **II** (Fig. 7), the fluorescence intensity with GC exceed that with the other two. Note that at low peptide concentrations with GC-GC the response virtually coincides with that of free peptide, indicating that the affinity of **II** to GC-GC is lower than for GC or AT. The differences in binding of **I** and **II** with GC and GC-GC are perhaps due to the substantial restrictions imposed on the peptide backbone conformation by the S-S bond. The latter stabilizes the β-structure even in very short peptides [11, 16, 17]. The linear peptide is much more flexible than the cyclic one, and probably one of its conformers possesses high affinity for GC-GC. The specificity of cyclic peptide binding appears to be determined by specific van der Waals contacts and H-bonding between the peptide NH and CO groups and the basepair groups exposed in the minor groove. In particular, peptide carbonyls may form H-bonds with 2-amino groups of guanine in one DNA strand. Thus, a part of GC-specific sites of **II** engaged in GC binding do not work with GC-GC.

The stability of GC and GC-GC complexes with **I** as a function of NaCl concentration is shown in Fig. 8: the complex with GC is still observed at 0.1 M, and the half-dissociation point is 0.06 M *(a),* whereat the complexes with GC-GC *(b)* and AT (not shown) are completely broken down. Analogous dependences for **II** are displayed in Fig. 7*b:* the stability decreases as GC > AT > GC•GC. Thus, the GC complexes are the most stable with both peptides, but the linear one forms a tighter complex.

Competition for poly(dA) •poly(dT) between peptides and distamycin A analog. Distamycin A is known to bind in the DNA minor groove, occupying five base pairs and forming H-bonds with adenine N3 and pyrimidine O2 [28, 29]. To ascertain in which groove

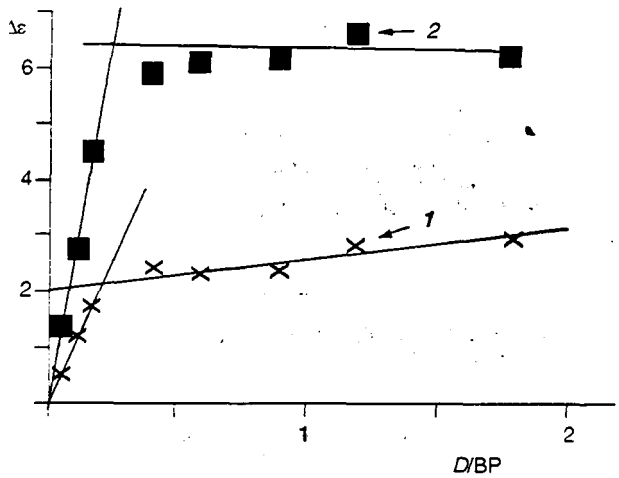

Fig. 9. Competition between peptide **I** and distamycin A analog for binding on AT: *1)* AT alone ($1.2 \cdot 10^{-5}$ M). 2) AT plus peptide ($3 \cdot 10^{-5}$ M): *D* is amount of distamycin analog added: conditions as in Fig. 6. ^e is CD at 315 nm per 1 cm light path per 1 mole base pairs.

peptides I and II are adsorbed, we assayed the binding of a distamycin A analog to AT in the absence and in the presence of the peptides. The analog contained two N-propylpyrrolecarboxamide fragments and two glycine residues at the N end; its thermodynamic parameters in DNA binding are given elsewhere [30]. In the presence of 0.02 M NaCl, its binding constant with AT is $3.2 \cdot 10^5$ M^{-1}, and the binding site is 3-4 bp. Complexation with DNA gives rise to CD at 300-350 nm, whereas the free antibiotic is optically inactive.

Figure 9 shows titration of the A•T - **I** complex and free AT with the distamycin analog. As can be seen, substantially less analog is bound in the presence of **I**. Analogous results were obtained when the analog was used as a ligand competing with **I** and **II** for the binding sites on AT (not shown). These results are most simply explained by assuming that the peptides, like distamycin A, are bound in the DNA minor groove.

DISCUSSION

Assessment of the effect of solvent on the interaction of synthetic peptides with DNA demonstrated that for the linear peptide I in 20% TFE the titration curve is S-shaped, testifying to formation of two types of complex. Such profiles are observed in cooperative binding. With the cyclic peptide II, titration curves are S-shaped regardless of solvent. In our previous works with *bis*(Lys-Gly-Val-Cys-Val-NH-NH-Dns), the peptide was shown to bind to DNA as a monomer (β-strand) and a dimer (β-sandwich) [10, 23]; both occupy 4-5 bp. Monomeric binding is noncooperative, while dimer binding is cooperative owing to interaction between sandwiches adsorbed on neighboring sites. Binding of **I** and **II** has much in common with the above case, and the thermodynamic model outlined agrees nicely with the experimental data in this work. For the linear peptide, the binding constant in 20% TFE decreases by an order of magnitude, but at the same time it acquires the ability of dimeric binding. The cyclic peptide forms both complexes regardless of TFE. The two peptides have close binding constants in 20% TFE. Both exhibit some selectivity in binding, the cyclic II preferring the G•C homopolymer and the linear **I** more affine to GC•GC. However, at elevated ionic strength in both cases the G•C complexes prove the most stable. It is noteworthy that **I** forms a tighter G•C complex than **II**. A possible explanation is that the S-S bridge in a short β-hairpin creates a structure with a somewhat larger interchain distance, so that insertion of the cyclic peptide in the minor groove requires greater DNA deformation. The issue widely discussed at present is that optimal binding of regulatory proteins with DNA requires their mutual adaptation, i.e., conformational changes in both the protein recognition site and DNA. Earlier we have found a transition from random to p conformation upon binding of a 26-unit cyclic peptide (**IV** in Fig. 1) with DNA [12].

We have found competition for A•T between linear and cyclic peptides and the distamycin analog binding in the minor groove. This fact as such is not unambiguous evidence for that the peptides also occupy the minor groove. The reduction of the amount of antibiotic bound in the presence of peptide may be due to conformational alterations in DNA induced by peptide binding. Nonetheless, the possibility of insertion of an antiparallel β-strand in the DNA minor groove during binding of some regulatory proteins [13-15] and triostin antibiotics [17, 18] was demonstrated by X-ray analysis and NMR. Insertion of the β-strand is accompanied by significant changes in the DNA structure: expansion of the minor groove and impairment of basepair stacking. It should be admitted that the specificity toward GC pairs is not so pronounced in the peptides studied here as in the cystine peptide dimer considered earlier [10, 23]. However, the 26-unit cyclic analog of **II** exhibited appreciable selectivity. DNase footprinting patterns testified to specific peptide protection of sequences in the regulatory region of phage 434 gene *cro* [12]. All these data permit a conclusion that bipartite peptides and β-hairpins using a two-strand motif in binding with DNA may serve as building blocks for constructing novel specific DNA-binding ligands.

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