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Quantitative estimation of the contribution of pyrrolcarboxamide groups of the antibiotic distamycin A into specificity of its binding to DNA AT pairs

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ABSTRACT

Interaction of DNA with the analogs of the antibiotic distamycin A having different numbers of pyrrolcarboxamide groups and labeled with dansyl was studied. The binding isoterms of the analogs to synthetic polydeoxyribonucleotides were obtained. Analysis of the experimental data leads to the following conclusions: (1) the free energy of binding of the analogs to poly(dA)·poly(dT) depends linearly on the number of amide groups in the molecule of the analog whereas attachment of each pyrrolcarboxamide group produces changes of 2 kcal/mole in the free energy; (2) attachment of a pyrrolcarboxamide unit to the GC pair results in the free energy change of 0.95 kcal/mole; (3) the binding of analogs to poly(dA)·poly(dT) is a cooperative process, presumbly, dependent on conformational changes induced by the binding of analogs to DNA.

INTRODUCTION

What is the mechanism of the predominant binding of regulatory proteins to specific DNA regions?[1,2] This problem is one of the key tasks of the present-day molecular biology.

Distamycin A (Dst) and Netropsin (Nt) can serve as simple models for the regulatory proteins. Earlier Zasedatelev et al. have suggested a specific stereochemical model for the molecular mechanism of recognition of AT pairs upon binding of Dst and Nt to DNA.[4-6] According to this model, the antibiotic molecule incorporates into the minor groove of the DNA double helix while the molecule's oligopyrrolcarboxamide backbone forms a helix isogeometrical to the double helix of DNA in the B-form. The AT-specificity of binding is provided by hydrogen bond formation between the amide groups of antibiotic and the O2 thymine oxygens and N3 adenine nitrogens fasing

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into the minor groove of the DNA double helix (Fig. 1).

The present paper describes quantitative estimation of the contribution of the pyrrolcarboxamide groups of Dst into the specificity of its binding to AT pairs of DNA. For this purpose a number of Dst analogs have been synthetized (Fig.2). Every analog contains a fluorescent chromophore. The binding of analogs to DNA is registered by changes in the quantum yield of the fluorescent group. We have studied dependence of the free energy of binding on the number of the amide groups in Dst analogs[7].

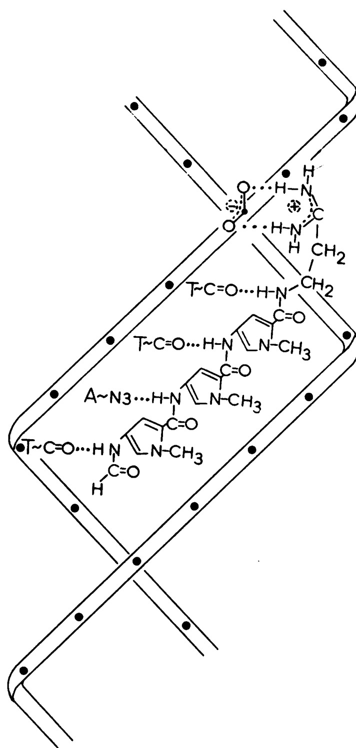
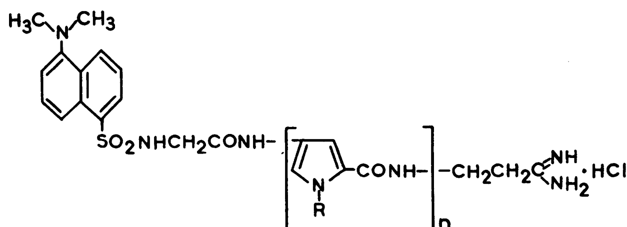


Fig.1. Schematic presentation of the Model for the complex of Dst and AT-rich region of DNA. The narrow groove side view. Bands symbolize two polynucleotide chains of the DNA double helix. The dotted lines show hydrogen bonds. For more details see the text.



Dns-Gly-Dst-1	(n=1 , R=CH_3)
Dns-Gly-Dst-2	(n=2 , R=CH_3)
Dns-Gly-Dst-3	(n=3 , R=CH_3)
Dns-Gly-Dst(Pr)-2	(n=2 , R=$CH_2CH_2CH_3$)
Dns-Gly-Dst(Pr)-3	(n=3 , R=$CH_2CH_2CH_3$)

Fig.2. The structural formulae for the fluorescent analogs of Dst.

MATERIALS AND METHODS

The synthesis of Dst analogs has been previously described [8,9]. The concentration of analogs was measured spectrophotometrically while the following molar extinction coefficients were used: ε_{280} = 15000 for Dns-Gly-Dst-1, ε_{302} = 22500 for Dns-Gly-Dst-2, ε_{307} = 38000 for Dns-Gly-Dst-3. Extinction coefficients of the N-propyl analogs were taken equal to those of the corresponding N-methyl analogs. The absorption spectra of analogs are given in Fig.3.

All the experiments were run in the 0.06 M phosphate buffer, pH = 5.9, [EDTA] = $2.5\cdot10^{-4}$, t = 22°C. Earlier it has been shown that under such conditions Nt forms only AT-specific complex[4].

Synthetic polydeoxyribonucleotides were products of P-L Biochemicals, Inc. All the polymers with the exeption of poly(dG)•poly(dC) were dissolved in the above-described buffer and dialyzed against it. Poly(dG)•poly(dC) was dissolved at pH = 12.5 and dialyzed; similar method was described[10]. Concentration of synthetic polydeoxyribonucleotides in solution was determined spectrophotometrically while the following molar (per nucleotide pair) extinction coefficients were used:

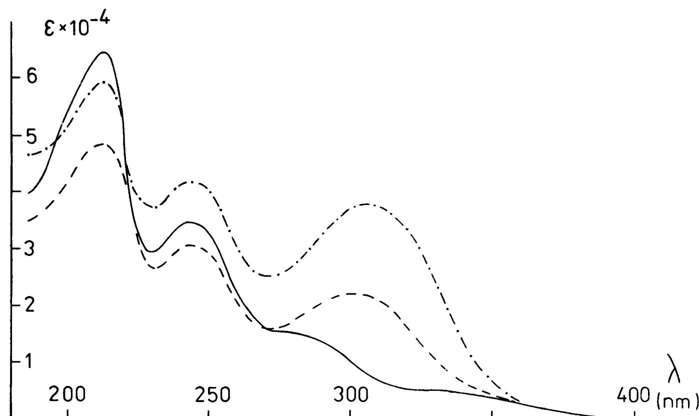


Fig.3. The absorption spectra of the fluorescent analogs of Dst in water. ——— Dns-Gly-Dst-1, - - - Dns-Gly-Dst-2, -·-·- Dns-Gly-Dst-3.

$\varepsilon_{259} = 1.2\cdot10^4$ for poly(dA)·poly(dT), $\varepsilon_{262} = 1.32\cdot10^4$ for poly(dA-dT)·poly(dA-dT), $\varepsilon_{253} = 1.48\cdot10^4$ for poly(dG)·poly(dC). The measured melting profiles of the synthetic polydeoxyribonucleotides were compared with the described data[11,12] and showed that all the polymers used were sufficiently long to be treated as infinite.

Binding isotherms were taken following the standard fluorescence technique. The Dst analogs were exited at $\lambda = 340$ nm, slit width s = 5 nm. The fluorescence measurements were made at $\lambda = 510$ nm, s = 20 nm. Under these conditions, the intensity of the fluorescence of the analogs bound to DNA increased 5- or 6-fold as compared to that of the free analogs in solution (Fig.4). The particular ratio of the intensities of fluorescence of the bound and the free analogs was found to be different for different analogs.

Three cells equally filled were used in every experiment. Equal amounts of the concentrated analog were simultaneously added to them. The first cell was filled with polydeoxyribonucleotide of high concentration that practically all the added analog was bound to it. This cell served as reference standard of fluorescence of absolutely bound analog. The second cell

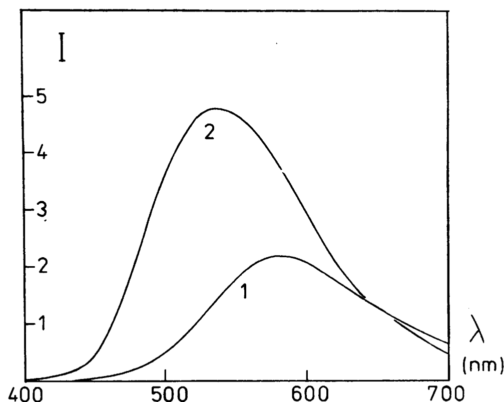


Fig.4. Fluorescence spectra of the free and the bound Dns-Gly-Dst-2. 1. - the free form; 2. - analog bound to the E.coli DNA. 0.06 M phosphate buffer, pH = 5.9 , $2.5 \cdot 10^{-4}$ EDTA, at a room temperature.

contained polydeoxyribonucleotide of such a concentration that could bind only a half of the analog added. This provided a reliable determination of the concentration of the bound and the free antibiotic in taking the binding isotherm. The third cell contained a buffer and was a reference standard for the fluorescence of the free analog. For each addition the concentration of the free analog "m" in the second cell was calculated using the following formula:

$$m = C \cdot (1 - F_2/F_1)/(1 - F_3/F_1) \qquad (1)$$

where "C" is the total concentration of the analog in the cells "F_1" is the fluorescence intensity of the first cell, "F_2" of the second cell, and "F_3" of the third. The concentration of the bound analog per base pair "r" was found fron the equation:

$$r = (C - m)/ [P/2]$$

where $[P/2]$ is the polydeoxyribonucleotide concentration expressed in base pairs.

All the measurements of fluorescence were made by spectrofluorometer AMINCO SPF-1000 CS. The absorption spectra of the

analogs were recorded with spectrophotometer CARY 118, circular dichroism spectra with dichrographe III, Jobin-Yvon.

RESULTS AND DISCUSSION

1. Binding isotherms. The dansyl group served as a fluorescence label to register the binding of the analogs to polydeoxyribonucleotides. Since the molecules of natural antibiotics Dst and Nt are not labeled with dansyl we made an additional experiment to investigate the influence of the fluorescence label on the binding of Dst analogs to DNA. We studied the circular dichroism of the analogs Dns-Gly-Dst-2 and Dns-Gly-Dst-3 complexed with poly(dA)•poly(dT). Fig. 5 shows that the adding of analogs to this polymer results in the increase of the circular dichro-

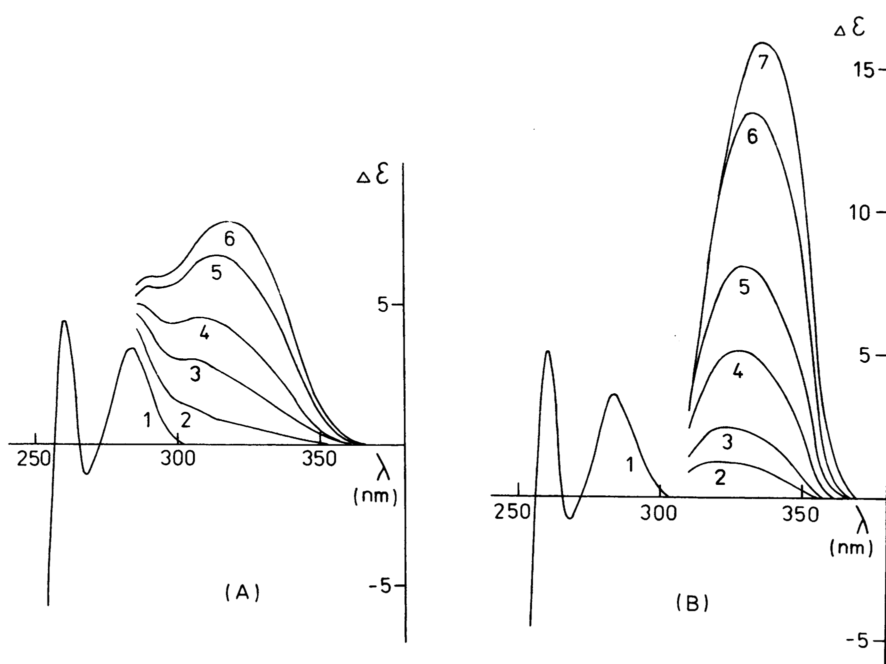


Fig.5. CD spectra of the complexes of Dns-Gly-Dst-2 and Dns-Gly-Dst-3 with poly(dA)•poly(dT). The spectra of complexes are shown for various number of the bound analog per base pair - "r". Δε represents the CD amplitude calculated per a base pair. (A) complexes of Dns-Gly-Dst-2 with poly(dA)•poly(dT), 1. r = 0, 2. r = 0.019, 3. r = 0.057, 4. r = 0.09, 5. r = 0.13, 6. r = 0.15. (B) complexes of Dns-Gly-Dst-3 with poly(dA)•poly(dT), 1. r = 0, 2. r = 0.012, 3. r = 0.024, 4. r = 0.048, 5. r = 0.072, 6. r = 0.12, 7. r = 0.135.

ism in the range of 310 - 330 nm. Similar effect may be observed upon binding of Dst and Nt to poly(dA)·poly(dT)[3,4,13]. This result supports our suggestion that the geometry of dansyl labeled analogs bound to DNA is similar to that of Dst and Nt.

Fig.6 represents binding isotherms of the analogs Dns-Gly-Dst-1, Dns-Gly-Dst-2 and Dns-Gly-Dst-3 with poly(dA)·.poly(dT) in Scatchard plots. Similar isotherms were obtained for the case of binding of those and N-propyl analogs to different polydeoxyribonucleotides. From the binding isotherms values of the binding constants K were obtained. The binding constants were found from the intersection of the isotherms with

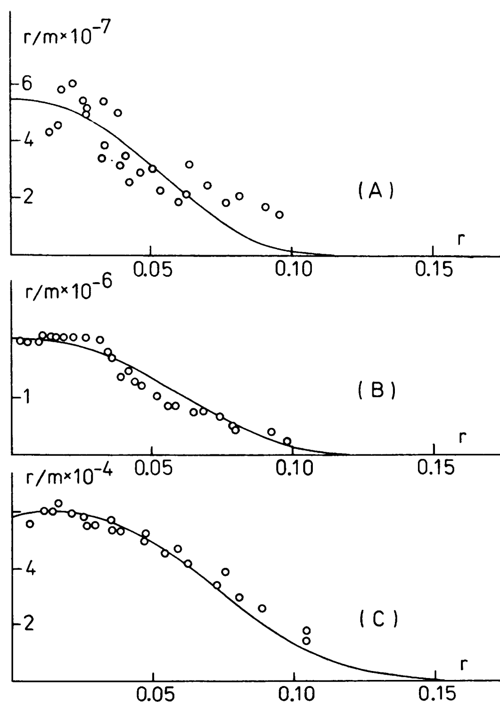


Fig.6. Binding isotherms of fluorescent analogs of Dst with poly(dA)·poly(dT) in Scatchard plots. The solid lines designate theoretical isotherms. Parameters of theoretical isotherms are given in parentheses. (A) - Dns-Gly-Dst-3 ($K = 5.5\cdot10^7$, L = 8, A = 1.6, D = 15); (B) - Dns-Gly-Dst-2 ($K = 2.0\cdot10^6$, L = 7, A = 1.6, D = 15); (C) - Dns-Gly-Dst-1 ($K = 5.8\cdot10^4$, L = 6, A = 1.6, D = 15).

the ordinate axis, i.e: $K = \lim(r/m)$, $r \to 0$.
All the K values obtained are summarized in Table.

Table. The Binding Constants of the Dst Analogs to polydeoxyribonucleotides.

ANALOG	Binding Constants K [l/mole]		
	poly(dA)· ·poly(dT)	poly(dA-dT)· ·poly(dA-dT)	poly(dG)· ·poly(dC)
Dns-Gly-Dst-1	$5.8 \cdot 10^4$	$1.3 \cdot 10^4$	
Dns-Gly-Dst-2	$2.0 \cdot 10^6$	$1.6 \cdot 10^6$	$8.0 \cdot 10^3$
Dns-Gly-Dst-3	$5.5 \cdot 10^7$	$1.6 \cdot 10^7$	$5.0 \cdot 10^4$
Dns-Gly-Dst(Pr)-2	$1.1 \cdot 10^6$		
Dns-Gly-Dst(Pr)-3	$1.2 \cdot 10^7$		

Accuracy of the K values is at the order of 10%.

2. Free Energies of Binding of the Analogs to Different DNA Base Pairs. The values of the free energies of binding $\Delta G = -RT \ln K$ of all the analogs under study were deduced from the experimental values of the binding constants. In Fig.7 the RT lnK values are plotted depending on the number of the amide groups in the analog molecule. In the case of binding of the N-methyl analogs to the homopolymer poly(dA)·poly(dT) that dependence appeared to be strictly linear. This shows that each pyrrolcarboxamide unit of a Dst molecule contributes equally to the binding constant of the entire molecule to DNA. Therefore it is experimentally proved that Dst is a ligand having several equivalent reaction centers. Earlier a theoretical model has been suggested according to which the ligand recognizing definite base sequences of DNA ought to have several regularly disposed nucleotide-specific reaction centers[15].

As is seen in Fig.7 the energy contribution of a pyrrolcarboxamide unit equals to 2 kcal/mole comparable to the value of formation of one hydrogen bond. There is no doubt that other interactions preconditioned by the entire pyrrol nucleus may contribute to the value of 2 kcal/mole [10]. Anyhow the present result agrees well with the suggestion that hydrogen bond formation occurs between the nitrogens of the antibiotic amide

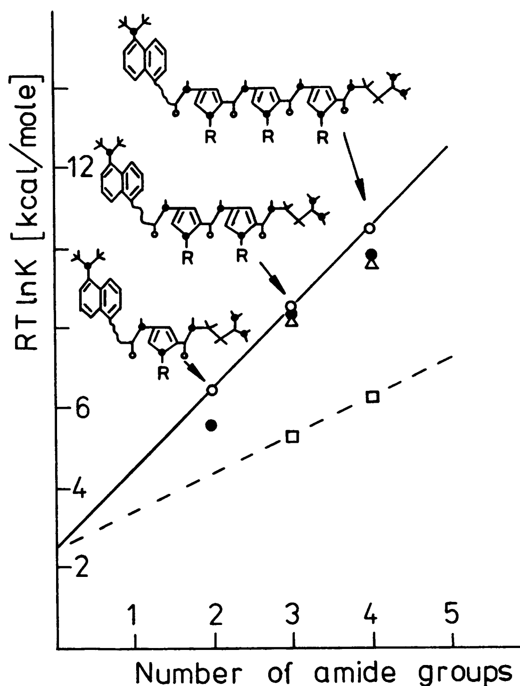


Fig.7. Dependence of the free energy of binding of Dst analogs on the number of amide groups in its molecule.○ - Dns-Gly-Dst-n and poly(dA)•poly(dT); ● - Dns-Gly-Dst-n and poly(dA-dT)•poly(dA-dT); △ - Dns-Gly-Dst(Pr)-n and poly(dA)•poly(dT); □ - Dns-Gly-Dst-n and poly(dG)•poly(dC); n - number of pyrrolcarboxamide units in analog's molecule.

groups and the O2 thymine oxygens and the N3 adenine nitrogens. This would also satisfy the Model of the Dst-DNA Complex[4-6].

The free energy of binding of the N-propyl analogs to poly(dA)•poly(dT) does not differ much from that of the N-methyl analogs, see Fig.7. This result may be interpreted within our model[4-6] according to which the N-methyl groups of a Dst molecule are situated in the external position with respect to the DNA double helix.

Of specific relevance is the closeness of the values of the free energies of binding of the N-methyl analogs to poly(dA)•poly(dT) and to poly(dA-dT)•poly(dA-dT). This points to a similar character of binding of the Dst analogs to the ho-

mopolymer and to the alternating polymer which has - unlike the homopolymer - not a canonical B-form in solution[16,17]. Anyhow the values of the free energies of binding of the analogs to poly(dA-dT)•poly(dA-dT) are somewhat less than those obtained from the binding to poly(dA)•poly(dT). This result implies that adenine and thymine are - though close - not equivalent to the amide groups of Dst by the energies of hydrogen bond formation.

Fig.7 demonstrates also the points corresponding to the binding of Dns-Gly-Dst-2 and Dns-Gly-Dst-3 to poly(dG)•poly(dC). The intersection of the dotted line is close to the similar intersection for the case of binding of the analogs to poly(dA)•poly(dT). The slope of the dotted line shows that the interaction of a pyrrocarboxámide unit with a GC pair corresponds to a free energy change of 0.95 kcal/mole. The result obtained characterizes the extent of nonspecific interaction of pyrrolcarboxamide unit with the DNA double helix and, presumbly discloses the presence of a weak hydrogen bond between the amide groups of Dst analogs and the cytosine O2 atoms. The bond is weakened because the cytosine O2 atom is involved into hydrogen bond formation with the guanine 2-amino group. The difference of 1.05 kcal/mole between the free energies of interaction of of the amide group with an AT or a GC pair is associated with a 6-fold change of the binding constant per each amide group. This fact does explain the high specificity of binding of Dst whose molecule possesses four amide groups.

3. Cooperative Binding of the Dst Analogs to poly(dA)•poly(dT).
The shape of the binding isotherms presented in Fig.6 demonstrates cooperative binding of the Dst analogs to poly(dA)•poly(dT). However a correction for the calculation of free energies of binding does not need to be applied since all the binding constants have been found from the ordinate intersections of isotherms (see Appendix). A possible explanation of the cooperativity observed is presented below.

A simple equation for the binding of the extended ligands to DNA was obtained by Crothers[18]. Curve (a) on Fig.8 shows his isotherm. To a first approximation that isotherm can be associated with our experimental curves, but it does not describe the plateau in the initial part of our isotherms.

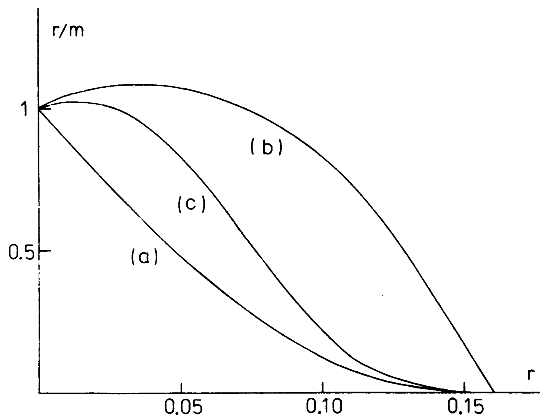


Fig.8. Theoretical curves for the binding of extended ligands to homopolymer. All the calculations are made by the formulae given in the Appendix. (a) - noncooperative binding, parameters L = 6, K = 1; (b) - the binding with the contact cooperativity, parameters L = 6, K = 1, A = 10; (c) - the binding with the distance cooperativity, parameters: L = 6, K = 1, A = 1.6, D = 15.

The binding isotherms with the positive and the zero initial slope and describing the cooperative binding of the ligands are to be found in the literature[19,20]. It is suggested that if the binding constant of the isolated ligand equals to K the ligand having only one contact with its neighbour will have the binding constant $K \cdot A$, and the ligand having two contacts $K \cdot A^2$, where A is the cooperativity factor. We shall term such cooperativity the contact cooperativity. Curve (b) on Fig.8 demonstrates an adequate isotherm. It however appears that such isotherm fail to describe the experimental curves presented in Fig.6 at any values of parameters.

We suggested that the shape of the experimental isotherms obtained by us is preconditioned by the distance cooperativity. This term means that the Dst molecule bound to DNA promotes the boarding on DNA not only for its closest neighbours but also for the ligand binding 1 - 2 twists of the double helix to its left and to its right. Physically speaking the ligand modifies the structure of 1 - 2 twists of the DNA double helix when bound to DNA. The hydrodynamic experiments showed that upon

binding to DNA the Dst molecule alters locally certain parameters of the double helix[13].

The distance cooperativity is described by the method of recurrent formulae[21]. The Appendix provides the formulae used in this work. We based our assumptions on the simple physical model according to which the binding constant of the ligand is A times larger as compared to the case of isolated binding if the ligand binds to DNA at a distance lesser or equal to "D" base pairs from the bound ligand. Within this model one can deduce the partition function "S_N" describing the ligand binding to a polymer of N units using the following recurrent formula:

$$S_N = S_{N-1} + K\cdot m\cdot S_{N-L-D} + A\cdot K\cdot m\cdot(S_{N-L} - S_{N-L-D}) \qquad (2)$$

where "m" is the concentration of the free ligand, "L" is the ligand length expressed in base pairs, "D" the range of cooperativity expressed in base pairs, "K" the binding constant of the isolated ligand and "A" the cooperativity factor. S_N was calculated for $N \gg L + D$ and the value "r" was obtained from the formula:

$$r = \frac{d(\ln S_N)}{N\ d(\ln m)} \qquad (3)$$

In such a way the points with the coordinates "r", "r/m" were obtained. A typical isotherm for the distance cooperativity binding is shown by curve (c) in Fig.8. Formulae (2) and (3) allowed to plot theoretical isotherms giving a proper description of the experimental isotherms. The solid lines in Fig.6 designate theoretical curves. Values of parameters K, L, A and D used to calculate the theoretical isotherms are given in the legend to Fig.6.

From the legend to Fig.6 one can see that parameters A and D are the same for all theoretical curves. Therefore it is assumed that all the analogs similarily alters the structure of the DNA double helix around the binding site, and the areas of cooperativity may be assumed to be equal for all the analogs.

Parameter L corresponding to the number of base pairs covered on DNA by the analog increases together with the increase of the number of pyrrolcarboxamide units in the analog molecule. This result is in good agreement with the Model for the

Dst-DNA Complex[6]. Besides, the above-mentioned data lead to the conclusion that Dns-Gly and the propioamidine residue occupy together four base pairs upon binding to DNA. Our estimation of the DNA areas covered by the analogs correlates with the van-der-Waals sizes of the molecules.

The data obtained support the Model for Dst-DNA Complex[6] proposed by us and estimate the role of the peptide groups of the regulatory proteins during formation of specific complexes with DNA. The approach described in this work may be applied to other ligands recognizing definite sequences of the DNA double helix.

APPENDIX

Strict and complete description of the recurrent formulae method for binding isotherms is given by Gursky and Zasedatelev [21]. The Appendix provides a basic deduction of the formulae used in this work.

For theoretical description of the binding isotherms the grand partition function should be calculated:

$$S = \sum_q Z_q \cdot m^q$$

where "m" is the concentration of the free ligand, "Z_q" the canonical partition function for the polymer with "q" ligands bound. The number of the bound ligands per a base pair "r" was found from the formula (3), where "S_N" is the grand partition function for the polymer of N units.

The recurrent formulae method allows to calculate a grand partition function "S_N". Below we demonstrate the method on the examples used in this work.

1. Let us concider the noncooperative binding of ligands to the polymer of N units. Suppose the ligand length equals to "L" base pairs. Then the recurrent formula would be like this:

$$S_N = S_{N-1} + K \cdot m \cdot S_{N-L} \qquad \text{(A1)}$$

Here $K = \exp(-\Delta G/RT)$, ΔG is the free energy change upon binding of the ligand to DNA. The physical meaning of the formula

(A1) is simple. If the polymer of N-1 units is added with one more unit this N^{th} unit may be either free or occupied with the ligand. Therefore partition function S_N consists of two terms. The first S_{N-1} describes the case when the Nth unit is free, and the other $K \cdot m \cdot S_{N-L}$ the case when the Nth unit is covered by the ligand. Fig.9(A) illustrates the formula (A1).

2. Let us consider the ligands binding to DNA in the case of the contact cooperativity. The interaction is suggested to occur only under direct contact of the ligands, and the cooperativity factor equals to A. The recurrent formula is as follows:

$$S_N = S_{N-1} + K \cdot m \cdot S_{N-L-1} + A \cdot K \cdot m \cdot (S_{N-L} - S_{N-L-1}) \qquad (A2)$$

The first term S_{N-1} describes the case when the Nth unit is free, the second term $K \cdot m \cdot S_{N-L-1}$ corresponds to the case when the Nth unit is covered with the ligand, but this ligand does not contact the adjacent ligand, for this L + 1 last units should be free. The term $A \cdot K \cdot m \cdot (S_{N-L} - S_{N-L-1})$ is associated with the case when the Nth unit is covered with the ligand

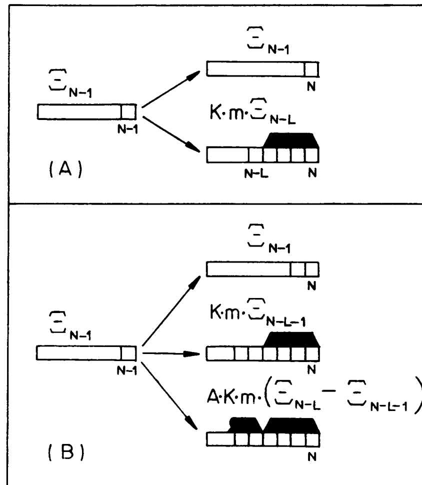


Fig.9. Illustrations to the Appendix. The figure demonstrates how by means of recurrent formulae one can express the partition function "S_N" describing the ligand binding to the polymer of N units via the partition functions for the polymer of a lesser length. Detailed explanation is given in the Appendix.

contacting its neighbour. The difference ($S_{N-L} - S_{N-L-1}$) describes the case when $N-L-1^{th}$ unit is covered obligatorily with the right end of the other ligand. Fig.9(B) illustrates the formula (A2). If in formula (A2) the cooperativity factor is suggested to be A=1 we obtain formula (A1). And A=1 means that between the ligands there is no interaction.

3. The distance cooperativity. The ligands bound to the polymer are suggested to interact not only directly but also at a distance of one, two, three, ... D-1 base pairs. For each distance between the ligands a cooperativity factor is introduced: A_0 for direct contact, A_1 for the distance of one base pair, A_2 for the distance of two base pairs, etc. The recurrent formula is as follows:

$$S_N = S_{N-1} + K\cdot m\cdot S_{N-L-D} + A_0 K\cdot m\cdot(S_{N-L} - S_{N-L-1}) +$$
$$+ A_1 K\cdot m\cdot(S_{N-L-1} - S_{N-L-2}) + A_2 K\cdot m\cdot(S_{N-L-2} - S_{N-L-3}) +$$
$$+ \dots A_{D-1} K\cdot m\cdot(S_{N-L-D+1} - S_{N-L-D}) \qquad (A3)$$

In our work we concider the most simple case of the distance cooperativity when $A_0 = A_1 = A_2 = \dots = A$. The physical meaning of such a model is that the ligand bound to DNA equally alternates the DNA structure of D-1 base pairs to the left and to the right from its binding site. In that case formula (A3) is simplified and rearrenged into formula (2) which was used in the given study.

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