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BINDING OF *LAC* REPRESSOR HEADPIECE TO POLY[d(A-T)]. A THERMAL DENATURATION STUDY.

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The binding of the *lac* repressor headpiece to poly[d(A-T)] has been investigated using thermal denaturation experiments. The binding of the headpiece leads to a stabilization of the double-stranded structure. Competition experiments using circular dichroism measurements confirm that the headpiece binds stronger on the double-stranded poly[d(A-T)] than on a single-stranded nucleic acid like poly(A). Theoretical analyses of the melting curves allow the determination of the binding constant and of the site size on the poly[d(A-T)]. This latter value is found to be 4 base pairs, in good agreement with that determined by other experimental approaches, and is much smaller than the values previously found for the *lac* repressor.

The *lac* repressor regulates the expression of the lactose operon in *E.coli* (1,2). The protein binds tightly to the *lac* operator preventing thereby the synthesis of the mRNA corresponding to the *lac* enzymes. In the presence of sugar inducer molecules the affinity of the repressor for the operator is decreased sufficiently to allow the remainder of the genome to compete effectively for binding to repressor in a non-specific mode (3).

The *lac* repressor is a tetrameric protein composed of domains, which may be separated after limited proteolysis at high ionic strength (4-6). Among these domains the N-terminal headpieces (residues 1-51, 1-56 or 1-59 depending on the conditions of hydrolysis) have been shown to bind non-specifically on DNA (5,7,8) as well as specifically on the *lac* operator (9-12).

In this paper we report results of a study on the non-specific binding of the headpiece on poly[d(A-T)] using thermal denaturation. Calculations of theoretical melting profiles using the theory developed by McGhee (13) allow us to determine the parameters of the binding process and particularly the size of the binding site of the headpiece on the poly[d(A-T)]. Previous studies by

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Clement and Daune (14) and by Wang *et al.* (15) have shown that the binding of *lac* repressor on poly[d(A-T)] induces a strong stabilization of the double-stranded structure of the nucleic acid. They reported values of the binding site of 20 and 13 base pairs respectively.

To perform the analysis according to the theory of McGhee (13) it was necessary to compare the binding of the headpiece to double-stranded structure with that to single-stranded one. This was done using circular dichroism measurements on the binding of the headpiece on the neutral poly(A) and on poly [d(A-T)] in presence of poly(A).

Materials and Methods

The *lac* repressor from *E.coli* BMH 493 was purified as described by Rosenberg *et al.* (16). The phosphocellulose column step was followed by incubation of the *lac* repressor (approx. 7 mg/ml) in a buffer containing 1 M Tris-HCl, pH 7.5, 30 % glycerol and 0.01 M 2-mercaptoethanol with trypsin as described by Geisler and Weber (5). Proteolysis was stopped after 3 hours by the addition of soybean trypsin inhibitor, and the products were fractionated on a Sephadex G-150 column. Tryptic digestion for 3 hours produces pure short headpieces (1-51) as shown by Ogata and Gilbert (9). The headpiece fraction was identified by its characteristic tyrosine fluorescence, concentrated by lyophilisation and further purified on a Sephadex G-50 column. Its purity was assessed by SDS polyacrylamide gel electrophoresis. By circular dichroism it was shown that it could bind non-specifically to poly[d(A-T)] and specifically on the *lac* operator. Its concentration was determined from absorption measurements using a molecular extinction coefficient of 4800 $M^{-1}.cm^{-1}$ at 280 nm (7).

Poly[d(A-T)] (S = 14) was purchased from Boehringer Mannheim and used without further purification. Its concentration was determined using a molecular extinction coefficient of 6650 $M^{-1}.cm^{-1}$ at 260 nm. Poly(A) (S = 8) was purchased from P.L. Biochemical Inc., and used without further purification. Its concentration was determined using a molecular extinction coefficient of 10000 $M^{-1}.cm^{-1}$ at 260 nm. The headpiece-poly[d(A-T)] and headpiece-poly(A) complexes were prepared by direct mixing of the components in a buffer containing 1.3 mM potassium phosphate, pH 7.25, 1 mM DTE and 0.2 mM EDTA.

Poly[d(A-T)] and headpiece-poly[d(A-T)] complexes melting transitions were monitored using a Beckman DU8 automatic recording spectrophotometer. All melting profiles were determined at 260 nm. The temperature was increased at a constant rate of 0.25°C per minute. The total melting of poly[d(A-T)] produced a 40 % increase in the A_{260} with respect to that of intact double-stranded poly[d(A-T)]. The melting profiles are presented as the "fraction coil" as a function of temperature and were normalized to allow comparison with calculated melting curves using the ligand-perturbated helix $\rightleftarrows$ coil theory of McGhee (13).

Results and Discussion

The variation of hyperchromicity, if any, due to the binding of the *lac* repressor headpiece to poly[d(A-T)] is small enough, not to interfer with the large hyperchromicity due to the melting of the poly[d(A-T)]. Figure 1 shows UV melting profiles of *lac* repressor headpiece-poly[d(A-T)] complexes at various

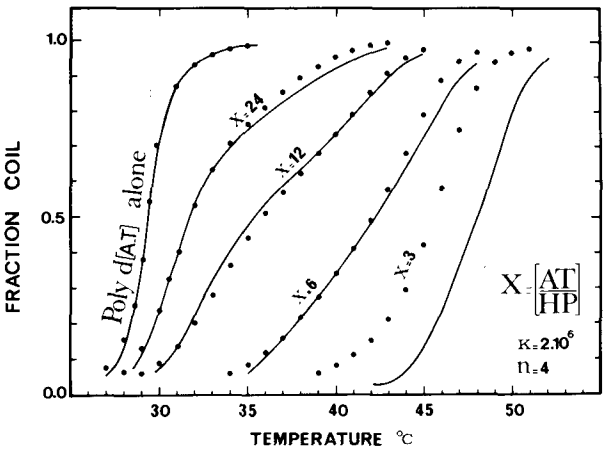

Figure 1 - Melting curves of poly[d(A-T)] in presence of various concentrations of *lac* repressor headpiece : (●●●) experimental curves ; (——) theoretical curves according to the theory (see text). X is the ratio of poly[d(A-T)] concentration (expressed as base pairs) to that of headpiece. The theoretical curves were calculated with a site size of 4 and a binding constant of 2 x 10^{-6} M^{-1}.

d(A-T) to headpiece ratios. Clearly headpiece binding increases the melting temperature of this double-stranded nucleic acid, indicating that the protein binds preferentially to this form of DNA. For low values of the d(A-T) to headpiece ratio the stabilization is accompanied by a broadening of the transition. For a value close to one headpiece per 12 base pairs the melting curve is obviously biphasic. This is seen more clearly on the derivative of the melting curve than on the melting curve itself. For higher concentrations of the headpiece the melting profile sharpens again.

The theory of the melting of DNA in presence of large binding ligands has been developed by McGhee (13) and can be used to extract some parameters of the binding process. Briefly the DNA is treated in the infinite homogenous Ising model approximation, and the calculations are done by the Lifson's method of sequence generating function. Several parameters are necessary to calculate a melting profile in the presence of a ligand : The association constants to the helix and the coil from of DNA, respectively K_h and K_c ; the ligand cooperativity parameters ω_h and ω_c ; the sizes of the binding sites on the helix and coil form n_h and n_c. Additionally it may be taken into account that the association constants might be temperature dependent. An important simplification in the calculation is obtained if one can assume that the binding to one form is ne-

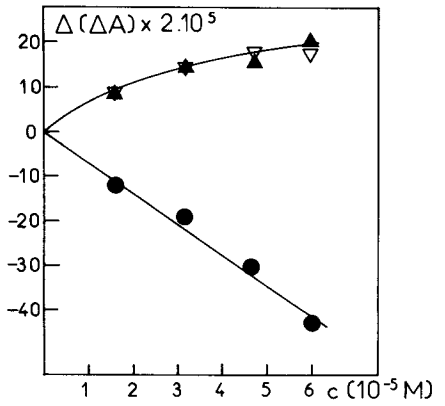

Figure 2 - Variation of the intensities of the CD signals at 265 nm of poly(A) (●●●), poly[d(A-T)] (▲▲▲), and an equimolar concentration of poly(A) and poly [d(A-T)] (▽▽▽), as a function of headpiece concentration. Concentration of both polynucleotide was 5 x 10^{-4} M in each experiment.

gligible when compared to that to the other. To test whether this is the case with the headpiece we have compared its binding on the double-stranded poly [d(A-T)] to that on the single-stranded poly(A). This was done using circular dichroism measurements.

When *lac* repressor headpiece is added to the single-stranded form of poly(A) one can observe a small decrease of the intensity of the large positive CD signal at 262.5 nm. This indicates that the headpiece binds on poly(A) and changes its conformation. The observed effect on the CD spectrum of poly(A) is qualitatively similar to that which has been previously reported when the *lac* repressor binds on the same polynucleotide (17). Even at the higher concentrations of headpiece added to the poly(A) the decrease of the CD signal does not reach a plateau (Figure 2) indicating that the binding is weak. This absence of plateau also does not allow calculation of the binding constant from the CD variation. To make a comparison with the binding process on poly[d(A-T)] we have performed competition experiments. We made use of the fact the CD signal of poly[d(A-T)] is increased when the headpiece binds on the double helix. The figure 2 shows that the increase of the CD signal of poly[d(A-T)] at 265 nm is very similar to that of a mixture containing the same amount of poly[d(A-T)] plus an equivalent concentration of poly(A). This indicates that in our experimental conditions all the headpiece binds on the double stranded structure of poly[d(A-T)].

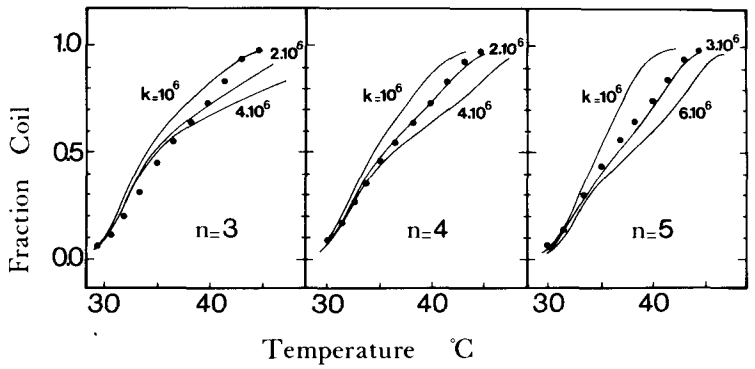

Figure 3 - Comparison between the experimental melting curve (●●●) corresponding to one headpiece per 12 base pairs and theoretical curves calculated with different values of n and K.

Therefore to perform the analysis of the melting profiles we have neglected the binding on the coil form and taken the values of K_c and n_c equal to zero. The value of ω_h, the cooperativity parameter was set to one, that is to say we have considered that there is no cooperativity in the binding process. In a study using fluorescence quenching we never needed cooperativity to analyse the binding of the headpiece on various DNAs (Schnarr, Durand and Maurizot, in preparation). Our ability to fit the melting data without introducing this factor indicates us that it is justified to take $\omega_h \simeq 1$.

In all our calculations a value of 8.0 Kcal/mole was used for the enthalpy change corresponding to the melting of one base pair of poly[d(A-T)] (13). The nucleation parameter σ, the statistical weight of a boundary between a helix and a coil region, was determined by the fit of the melting profile of poly[d(A-T)] in the absence of headpiece. The value of $5\ 10^{-4}$ found for σ is similar to that found by Jensen and von Hippel (18) on the same polymer.

In a first series of calculations we assumed that K_h is independent of temperature ($\Delta H_h = 0$) and adjusted by computer simulation the values of the size of the site and of the binding constant to fit the observed melting curves. Several values of n_h and K_h were tried and the experiments were all together best described using values of $n_h = 4$ base pairs and $K_h = 2\ 10^6\ M^{-1}$. The sensitivity of the calculation to these parameters is shown in figure 3 in which the experimental denaturation curve corresponding to one headpiece per 12 base pairs is compared with theoretical curves calculated with site sizes of 3, 4, 5 base

pairs (for n_h = 6 bp the fit was very bad whatever the binding constant). For each site size the curves calculated for several values of the binding constant are shown. The fit corresponding to n_h = 3 is worse than those obtained with 4 or 5 base pairs. One can see that values of n_h = 4 b.p. with $K_h = 2\ 10^6\ M^{-1}$ or values of n_h = 5 b.p. with $K_n = 3\ 10^6\ M^{-1}$ best fit this curve. If one considers all the experimental curves the best fit is obtained for n_h = 4 and $K = 2\ 10^6\ M^{-1}$. This shows that there is little choice for the values of the adjustable parameters.

Similar calculations were made using other values of ΔH_h (-8, -4, +4, +8 Kcal/mole). We did not notice large differences with the curve calculated using $\Delta H_h = 0$ and this did not improve the quality of the general fit. This result could be expected since the transition even when biphasic occurs within a few degrees and the binding constant does not vary to much in such a restricted temperature range. Using fluorescence quenching measurements (allowing the determination of isothermal binding curves) we found under buffer conditions similar to those used in this study a value of the site size of 3 base pairs and $K = 3\ 10^5\ M^{-1}$ for the interaction with a natural DNA (M.S., M.D. and J.C.M., to be published). Taking into account that McGhee (13) predicts a possible error of a factor 2 for the site size and of an order of magnitude for the association constant the two sets of data agree rather well. Furthermore the heat denaturation method determines the number of base pairs protected against melting, a number which may be slightly different from that really occupied by the protein as determined for example by fluorescence.

It is of interest to compare the results obtained on the headpiece (this study) with those obtained on the *lac* repressor (14,15). As the *lac* repressor the headpiece stabilizes the double-stranded structure of the nucleic acid. It is now commonly admitted that the *lac* repressor interacts with the DNA mainly if not exclusively *via* two headpieces (9,10,19-23). Values of 13 and 20 base pairs were reported for the size of the segment of DNA protected against thermal denaturation by the *lac* repressor (14,15). These values are by far larger than twice the value obtained with the headpiece. This probably reflects the

fact that in the *lac* repressor the core imposes constraints on the geometrical positions of the headpieces despite their large mobility.

Conclusions

The work presented in this paper shows an example of application of the theory of McGhee (13) on the melting of DNA in the presence of large binding ligands. Until now very few studies have used this method for the determination of binding parameters. It has been used for the binding of netropsin (13), an helix stabilizer, ribonuclease (18) and bacteriophage T4 gene 32-protein (24), two DNA melting proteins. The agreement between the parameter determined by this method and those determined by other methods, like fluorescence or circular dichroism can be regarded as encouraging.

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