Binding of lactose repressor to poly d(A-T) : OD and CD melting of the complex

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ABSTRACT

The binding of lactose repressor to poly d(A-T) at low ionic strength has been investigated by heat denaturation. The poly d(A-T) melting is monitored by optical density and the protein melting by circular dichroism. From the modification of the poly d(A-T) melting curve we estimate a maximum binding ratio of about one tetrameric repressor to about 20 bases pairs. The repressor melting can be interpreted as a global shift from α to β structure of about 25 residues per subunit. The melting curves of poly d(A-T) and repressor have not a shape easy to interpret; nevertheless both show a cooperative transition in the same temperature range where we can evaluate that about 3.8 aminoacid residues shift from α to β structure when 1 basespair melt.

INTRODUCTION

Since the operator region cannot be isolated in large amounts, the binding of lactose repressor to operator cannot be studied directly by the physicochemical methods and one must study non-specific binding to DNA. The study of the DNA perturbation may be correlated with the study of protein perturbation. Here we use the hyperchromic effect of poly d(A-T) and the ellipticity of the protein to investigate the thermal behavior of repressor-poly d(A-T) complex.

MATERIALS AND METHODS

Growth of bacteria and purification of repressor from the strains BMH 461 or 593 (the generous gift of B. Müller-Hill)

followed the method described by Müller-Hill et al. (1).

The buffers were the same as described by Müller-Hill (1), except that DTE ($2 \cdot 10^{-4}$ M) replaced β -mercaptoethanol in the gradient elution of the phosphocellulose column. Binding of IPTG to repressor was measured by the CD at $\lambda = 225$ nm with added IPTG or by equilibrium dialysis against ^{14}C -IPTG (from CEA - France). Purified repressor was stored at -20° in the elution buffer (around 0.2 M phosphate) after addition of glycerol to 30% by volume.

Poly(~~AT~~) was purchased from P.L. Biochemicals. Complexes were obtained by direct mixing at 4° C of convenient volumes of stock repressor, buffer and poly(~~AT~~) dissolved in the buffer; the mixture was then dialysed against the buffer. Before mixing, the stock repressor was clarified by centrifugation (around 10000 g for 15 min.); after dialysis, the mixture was also clarified. The ratio of protein to DNA was obtained from the spectrum of the complexes assuming a ratio OD(230)/OD(280) of 7 for repressor; r is expressed in subunit per 100 phosphates.

The refractive index increment was measured with a Zeiss interferometer with a two-compartment cell in a stirred water bath at room temperature. Circular dichroism measurements were made with the Roussel Jouan dichrograph II in a thermostated cell. OD melting profiles were obtained from a DB-G Beckman spectrophotometer. The shape of the melting curves both by CD and OD was not affected when varying the rate of heating from 0.5 to 2° C per min.

EXPERIMENTS AND RESULTS

Estimation of molar absorption coefficient.

The usefulness of the molar absorption coefficient is limited by the variety of values cited in the literature. For example values of E (0.1%; 280 nm) from 1.38 (1) to 0.69 (2) have been used; thus we were obliged to determine our own value for E .

A stock solution of lactose repressor was extensively dialysed. After dialysis we centrifuged to remove aggregated material and measured the optical density. Then the stock solution was diluted with the dialysis buffer to perform the index increment measurement at various concentrations as quickly as possible to avoid further aggregation. We plotted the fringe shift versus the OD (280) to get the slope from which we derived the absorption coefficient. Assuming a reference value of $0,190 \text{ cm}^3/\text{g}$ for the index increment, we get $0.66 < E(0.1\%; 280 \text{ nm}) < 0.72$.

Using the absorption coefficients for the individual amino-acids (3) and the amino-acid composition (4), we estimated $E(280) = 0.55$ for a neutral aqueous medium and $E(280) = 0.71$ for an ethanolic medium in good agreement with our measurements. We shall use henceforth $E(280) = 0.69$.

Conformation and stability of the protein.

We obtained CD spectra approximately similar in shape to those reported (5,6), but a net discrepancy existed for the ellipticity scale. No drastic changes could be seen for various aqueous solvents at neutral pH, nor for concentrations lowered to about $15 \text{ } \mu\text{g/ml}$. We observed a net decrease of molar ellipti-

city at $\lambda = 220$ nm and of molar rotation, when a fresh thawed solution was kept a few days at 4° or heated from 20 to 40° C; in each case the shift of θ preceded a net aggregation of the protein as seen by increase in turbidity at $\lambda = 320$ nm. Saturating levels of the effectors, IPTG or pAPTG, had no effect on this instability, nor did excess levels of various DNA (in the range from 100 phosphates to 500 phosphates per subunit) at ionic strength around 0.2.

Using a method of analysis of CD spectra (7) with our own data (8), we got α and β estimations around $\alpha = 30\%$ and $\beta = 15\%$. These results have to be used with caution from the quantitative point of view, since in practice the calculation depends largely on the concentration and on reference spectra for α , β and disordered form, which are not yet completely specific in the literature.

Stability of repressor-polyd(AT) complexes.

Because of the general instability of the protein in solution we checked qualitatively the melting behavior of lactose repressor mixtures with various DNA by OD at $\lambda = 260$ nm varying the ionic strength at a ratio of protein to DNA up to 2 subunits per hundred phosphorus.

For mixtures containing DNA from Hemophilus influenzae or calf thymus at high ionic strength (0.11 Na-Phosphate pH 7.3; 0.1 mM EDTA; 0.1 mM DTE), only a decrease of $|\theta|$ was seen preceding aggregation around 50° C as in the case of the protein alone. For Hemophilus influenzae, calf thymus or λ DNAs in less concentrated buffers, we obtained thermal OD curves indicating some interaction, but the situation was not clear since several events are known to occur in the same temperature range : DNA

melting, protein melting and the consequences of these on DNA protein binding. We conclude that at low ionic strength we obtain a more stable protein in solution after some days of storage or after heating to a temperature where one would expect it to aggregate (as seen by optical density or circular dichroism), when mixed with DNA. Since one observes the same behavior when repressor was mixed with polyd(A-T), we used polyd(A-T) as a tool since it permits sharp thermal profiles at temperatures below the melting of the protein alone. For polyd(A-T) complexes, instability to storage or heating appears between 10^{-2} and 5×10^{-2} M NaCl.

Thermal OD transition curve.

No significative change of the total hyperchromic effect was seen but the shape of the melting curve of polyd(A-T) is largely affected by increasing amounts of lactose repressor; much heterogeneity indicates repressor binding (fig. 1); at first

Figure 1. OD (260) melting curves of poly d(A-T) with increasing levels of repressor at low ionic strength. The ordinate is expressed as a fraction (H) of the total hyperchromicity. The buffer is 1 mM Tris-HCl pH 7.3, 0.2 mM EDTA, 0.2 mM DTT, 2 mM NaCl. Curves correspond successively to : $r = 0 - 2.8 - 4.8 - 5.2$ and 11.

the curve seems biphasic and we can define two melting temperatures ($T_1 < T_2$), as reported for histone-DNA complexes. However, since the intermediate range produces a large part of the hyperchromic effect, we must describe the curve in three parts, quantified in an empirical way by their relative contribution to the hyperchromic effects : h_1 , h_1 and h_2 which are obtained from the intercepts of tangents at the inflexion points. We interpret the first part (h_1 , T_1) as the melting of polyd(A-T) parts without bound repressor ; this is supported by the facts that a) T_1 varies normally with ionic strength when adding NaCl up to $5 \cdot 10^{-2}$, where aggregation clearly occurs (fig. 2) and

Figure 2. Variation of OD melting curves with ionic strength. The buffer (see figure 2) and the concentrations of repressor and poly d(A-T) are the same for the three curves with $r = 4.8$, but the concentration of NaCl is 2 mM for curve A, 10 mM for curve B and 50 mM for curve C. In the case of curve C, the hyperchromicity seems larger due to aggregation, but true OD melting occurs also as indicated by the cooperative decrease in the cooling curve.

b) T_1 is close to the melting temperature expected for the free poly d(A-T) at the corresponding ionic strength (fig. 3). The

Figure 3. Plot of OD melting temperatures T_1 and T_2 versus protein to poly d(A-T) ratio r (for the composition of buffer, see figure 2). The various symbols correspond to separate series of melting experiments performed with different preparations of the complex but according to the same procedure.

slight increase of T_1 with r corresponds to the stabilization of free poly d(A-T) segments by adjacent repressor covered regions. We admit that all remaining hyperchromicity ($h_1 + h_2$) represents parts of poly d(A-T) bound to protein in one way or another. So we plot the ratio r of protein to poly d(A-T) versus ($h_1 + h_2$) (fig. 4) ; r is calculated from the ratio OD(230)/

Figure 4. Plot of protein to poly d(A-T) ratio r versus the fraction of hyperchromicity ($h_1 + h_2$) attributed to complexed part of poly d(A-T). The various symbols have the same meaning as in figure 3.

OD(260) in the complex before melting. In such a plot it is not easy to get values near $h = 0$ or $h = 1$, but, assuming a linear relation between r and $(h_1 + h_2)$, we can get a value for the maximum of r by extrapolating to $(h_1 + h_2) = 1$; we get so a stoichiometric ratio of 10 subunits of repressor per 100 phosphates of poly d(A-T). Such a value should be compared with that obtained when we try to prepare complexes of higher r values: in this case, we observe a large aggregation during dialysis and the 230/260 OD ratio after clarifying the mixture leads to similar values.

Finally, the insensitivity of T_2 to r (fig. 3) is a local property of repressor bound poly d(A-T).

Thermal CD transition curves

The CD melting curves were obtained for the same solutions as studied by OD melting. If we assume that the spectrum and melting of poly d(A-T) are not drastically changed in the λ range studied by the binding of repressor, we can follow the behavior of the protein with temperature, in complexes with high r values. We obtained CD melting curves at $\lambda = 230$ nm without correction, where no significant signal was observed for poly d(A-T) melting alone. The melting curve did not change when r was varied up to saturation or when the curve was registered at $\lambda = 220$ nm and corrected slightly for poly d(A-T) contribution. The melting curve is not subject to a simple analysis, since it appears visually to be composed of two transitions: the first and major one with its maximum slope around 60°C and the second and minor one with its maximum slope around 70°C (fig. 5). By comparing the OD melting (poly d(A-T)) and

Figure 5. CD (230) melting curve of repressor in repressor-poly d(A-T) complex ($r = 4.8$).

the CD melting (protein) we can say that :

a) The complexed poly d(A-T) and the complexed protein melt in the same temperature range in the conditions of the experiments, except that the second transition in the protein melting (around 70°) does not correspond to any further poly d(A-T) melting.

b) the (h_1, T_1) poly d(A-T) melting occurs at a temperature when the CD melting is practically zero ; therefore during the first poly d(A-T) melting no modification of the complex occurs due to repressor melting. From this point of view, CD melting experiments support our interpretation of the OD melting, the stoichiometry proposed being that of the complex of poly d(A-T) with a "native" form of lactose repressor.

Another feature of the protein melting is that the total change of ellipticity corresponds only to a small part of

the initial structure : this increase of ellipticity was around 30% of the initial ellipticity for $\lambda = 220$ nm (corrected for poly d(A-T) contribution when necessary), and around 40% for $\lambda = 230$ nm. Actually these relative values correspond to absolute values approximately equal. No significant change was seen when varying r or the ionic strength. By examining the reference spectra (7, 8) for α , β and disordered form, we see that the ellipticities at these wavelengths are very different except for the ($\beta - \alpha$) combination which corresponds to the replacement of an α -residue by a β -residue (not necessarily at the same place) ; this is especially evident in difference spectra (fig. 6). By comparing the intensities of the difference spectra for melting of lactose repressor and for an α to

Figure 6. Part A : CD difference spectra for an α to β structure change obtained from reference spectra of Lemieux et al. (▲) or Chen et al. (●).

Part B : CD difference spectra for the melting of repressor in repressor-poly d(A-T) complex (the various symbols correspond to different r values between 2.8 and 10). In view of the experimental uncertainty no systematic variation of the difference spectra with r can be inferred from the different curves.

β shift using reference data (8), we estimate that around 25 aminoacid residues, or 7% of the total chain length of a sub-unit, are shifted from α to β form.

DISCUSSION

Since our experiments convinced of the difficulties in preparing the solutions and completing the experiments fast enough for study of the protein alone, we concentrated our attention on complexes with DNA, or particularly with poly d(A-T). In this case, we observed that the time-dependent irreproducibility disappears with solutions at low ionic strength. This observation is fundamental from the practical point of view and we have to take it into account in any further experiments.

Nevertheless the question of the instability of the protein in solution is not yet resolved ; perhaps it must be approached again in view of the more precise amino acid composition now available (4). This composition permits us to evaluate the net charge of the protein, if we assume that no other ions than H^+ are bound and that no extraordinary pK values exist in this protein. Thus, in the neutral media generally used, we may reasonably assume at least two positive charges, but we cannot explain the measured pH_i (1), unless we assume that the degree of amidation is not very stable, as outlined by Beyreuther (4). The main conclusion from these considerations is that we cannot be certain of working at a pH other than pH_i without titrating the degree of amidation of our solutions. Such a conclusion could explain at least partly the aggregation of the protein, since around pH_i the electrostatic part of

repulsion between oligomers is small at every ionic strength. It may be possible to get experimental values for the protein alone in the future by looking more deeply into this particular point.

Dimensional aspect

Although many remaining sources of uncertainty limit us to using it as a first approximation, we obtained a value for the stoichiometric ratio of the complex. We find that a tetrameric repressor protects about 20 pairs from thermal melting. Gilbert found that the specific binding protects 27 pairs from DNase digestion and that the symmetric sequence is contained in 21 pairs (9). Furthermore, from measurements of microcrystals (10), Steitz proposes a model in which a tetramer covers $135 \text{ \AA}$ of DNA length ; in other words 40 base pairs are sterically forbidden for further binding, unless lactose repressor tetramers can bind non-specifically at the same time on the two sides of the poly d(A-T) helix, in which case the region apparently covered would be 20 base pairs. Evidently the stoichiometry found may be discussed on a structural basis.

Electrostatic aspect

The protonation state of H is unknown ; if we assume that the pK of H is shifted to basic values due to the high polyelectrolyte field in the complex, we estimate that a tetramer bearing 36 net positive charges protects 40 phosphates.

Such a consistency between our two estimations of the size of covered region can be compared with that obtained with denatured histones ; A. Garel et al. (11) report that histones F_{2a2} and F_V protect from melting a part of DNA which corresponds

both to their dimensions and to their net positive charges.

Dissymmetric melting of complexed poly d(A-T)

In the case of repressor poly d(A-T) complexes, we do not obtain biphasic melting profiles with two different cooperative meltings separated by an intermediary temperature range with almost no melting ; we observe an intermediary non cooperative melting. We have attributed the first cooperative melting (h_1 , T_1) to free poly d(A-T) so we have now to consider possible explanations for the intermediary non cooperative melting (h_1) and the second cooperative melting (h_2 , T_2). If electrostatic binding of positive charges of protein and of consecutive phosphates is a valid explanation for the cooperative melting (h_2 , T_2), we can evaluate the number of positive charges involved in the binding. Since 10 phosphates are protected by one repressor subunit, and since the experimental values of h_2 and h_1 are roughly equal, we can estimate that 5 positive charges per repressor subunit should be electrostatically bound to a corresponding number of phosphate groups. In this hypothesis, the intermediate melting (h_1) remains to be explained. Nevertheless the heterogeneous protection of complexed poly d(A-T) could be related to the fact that the operator sequence is only partly symmetric (9) although the repressor is a tetrameric protein.

Possible relation between poly d(A-T) and protein meltings.

We have not yet considered the possible influence of the protein melting as observed from C.D. measurements. By comparing the OD and CD melting curves, we can see that the melting range of the complexed poly d(A-T) corresponds to the

first transition of the protein. If we plot the OD change at 260 nm versus the CD change at 230 nm, we get a combination of linear segments (fig. 7). We cannot say whether such a phenomenon is trivial or whether it represents the coupled melting of poly d(A-T) and bound fractions of the protein. From the slopes in such a plot we get an estimate of the number v of amino acids, shifting from α to β structure when a poly d(A-T) pair melts according to a simple relation :

Figure 7. Plot of the fraction (H) of total hyperchromicity of poly d(A-T) versus the relative change in ellipticity of repressor ($r = 4.8$).

$$v = \frac{\Delta\theta}{\Delta OD} \times \frac{\epsilon_H}{\theta_{\alpha\beta}}$$

where $\Delta\theta$ and ΔOD are the measured changes of OD and ellipticity at the same concentration ;

ϵ_H is the molar hyperchromicity in the melting of 2 phosphates ;

$\theta_{\alpha\beta}$ is the molar ellipticity in the shift of a residue from α

to β structure.

We find, as expected, that v is about zero at the first poly d(A-T) cooperative melting and very high at the last part of the protein melting at around 70°C. For the intermediary range we calculate v to be 1.5 and for the melting around 60°C $v = 3.8$; using the results of stoichiometry and the fact that the total hyperchromicity for each segment is about the same, the v values correspond respectively to 5 and 12 amino acids per subunit. We cannot say how these values can be related (or even are related) to the structure of amino acids bound respectively to native and melted poly d(A-T).

CONCLUSION

We tried to explain the OD and CD melting curve of repressor-poly d(A-T) complexes. Since the operator-binding property is rather unstable compared to inducer-binding, we are not sure that the studied complex is the only one we can obtain by varying the conditions of complex formation. The shape of the OD melting of protected poly d(A-T) and the possible relation with melting of the protein are not unambiguously explained.

Nevertheless, according to the dissymmetry of the stabilization of poly d(A-T) and the intensity of the second cooperative OD melting we are tempted to conclude that we observe in the studied complex the binding of the specific site of the protein.

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Abbreviations used

Poly d(A-T) : double stranded copolymer of deoxyadenylic and deoxythymidylic acids in alternating sequence.
DTE, DTT : respectively dithioerythritol and dithiothreitol.
IPTG, pAPTG : respectively isopropyl and para-amino-phenyl-thio-galacto-pyranoside.
CD : circular dichroism.
OD : optical density
E(0.1% ; 280) : absorption coefficient at $\lambda = 280$ nm based on a concentration of 0.1% weight by weight.
 θ : molar ellipticity on the amino acid residue concentration scale.
r : ratio of protein to DNA expressed as subunit of repressor per hundred DNA phosphorus.
H : one letter code for histidine.