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

R. Clement and M. P. Daune,

Institut de Biologie Moléculaire et Cellulaire du C. N. R. S., Groupe de Biophysique, 15, rue Descartes, 67000 Strasbourg, France

ASTRACT

      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 bases pair 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 (2.10^{-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.

Polyd(A-T) was purchased from P.L. Biochemicals. Complexes
were obtained by direct mixing at 4° C of convenient volumes of
stock repressor, buffer and polyd(A-T) 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 r 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 dichroïsm measurements were
made with the Roussel Jouan dichrograph II in a thermostated cell.
OD melting profiles were obtained from a DB-G Beckman spectro-
photometer. 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 cm^3/g for the index increment, we get 0.66 < E(0.1%; 280 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 µg/ml. We observed a net decrease of molar ellipti-

city at λ = 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 λ = 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 α = 30% and β = 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 λ = 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 <u>Hemophilus influenzae</u> 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 <u>Hemophilus influenzae</u>, 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 dichroïsm),

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, instabi-

lity 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;

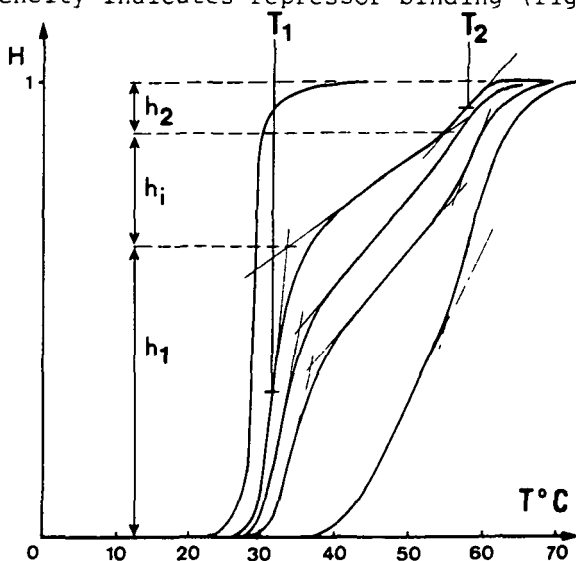much heterogeneity indicates repressor binding (fig. 1); at first

Figure 1. OD (260) melting curves of poly d(A-T) with increasing
levels of repressor at low ionic strength. The ordinate is ex-
pressed 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 tempera-
tures $(T_1 < T_2)$, 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_i and h_2 which are obtained
from the intercepts of tangents at the inflexion points. We inter-
pret 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
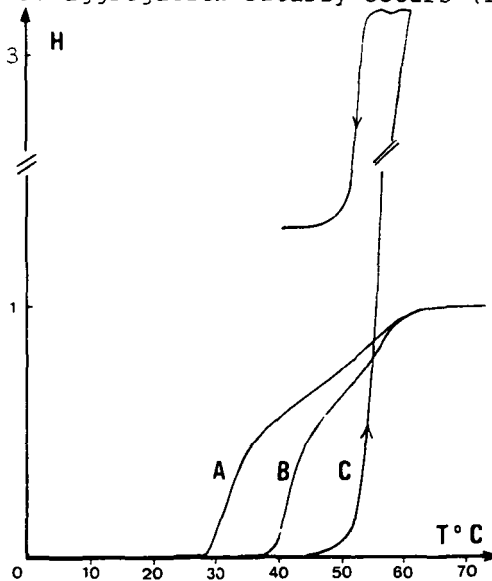to 5.10^{-2}, where aggregation clearly occurs (fig. 2) and

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 hyper-
chromicity 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

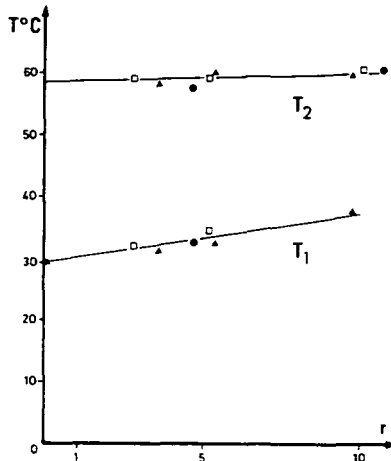poly d(A-T) at the corresponding ionic strength (fig. 3). The

Figure 3. Plot of OD melting temperatures T_1 and T_2 versus pro-
tein 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 re-

gions. We admit that all remaining hyperchromicity $(h_i + h_2)$

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

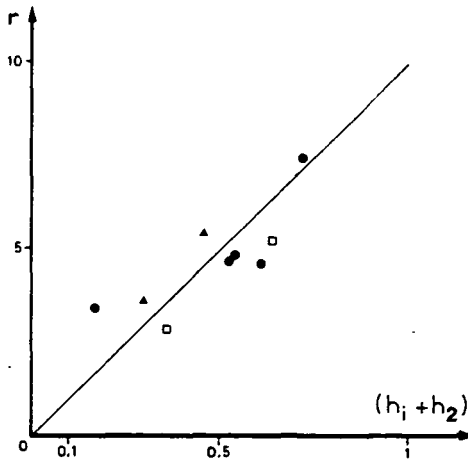$(h_i + h_2)$ (fig. 4) ; r is calculated from the ratio OD(230)/

Figure 4. Plot of protein to poly d(A-T) ratio r versus the
fraction of hyperchromicity $(h_i + h_2)$ 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 $(h_i + h_2)$, we can get a value for
the maximum of r by extrapolating to $(h_i + h_2)$ = 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 insensivity of T_2 to r (fig. 3) is a local pro-
perty of repressor bound d(A-T).

Thermal CD transition curves

        The CD melting curves were obtained for the same solu-
tions 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 λ = 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 λ = 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 tran-
sitions : the first and major one with its maximum slope around
60°C and the second and minor one with its maximum slope around
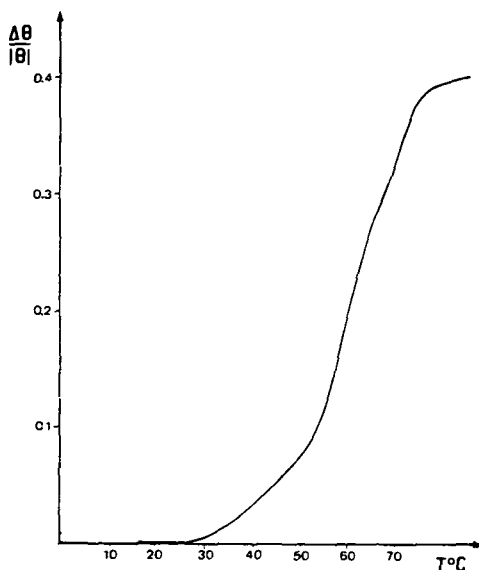70°C (fig. 5). By comparing the OD melting (poly d(A-T)) and

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 λ = 220 nm (corrected for

poly d(A-T) contribution when necessary), and around 40% for

λ = 230 nm. Actually these relative values correspond to abso-

lute 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 (β - α) 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 diffe-

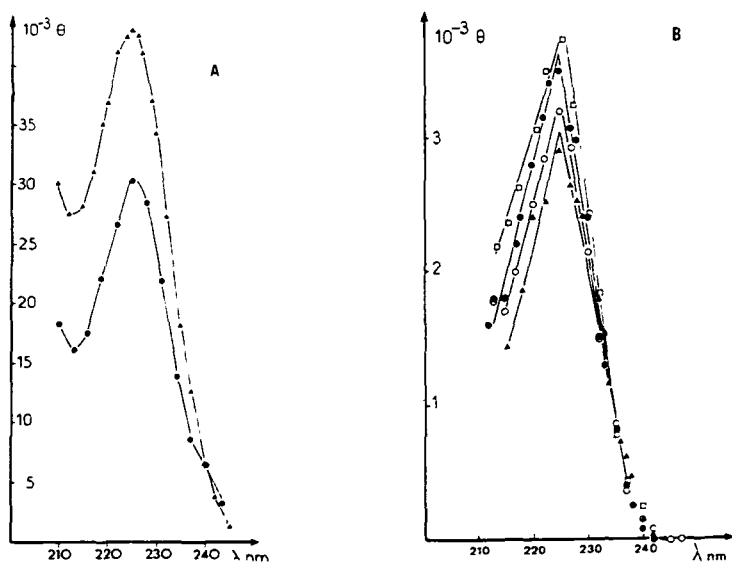rence spectra for melting of lactose repressor and for an α to

Figure 6. Part A : CD difference spectra for an α to β struc-
ture change obtained form 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 dif-
ferent 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 irreproduci-bility 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 compo-sition now available (4). This composition permits us to eva-luate 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_1 (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_1 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 tetra-
meric 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 $\overset{\circ}{A}$
of DNA length ; in other words 40 base pairs are sterically
forbidden for further binding, unless lactose repressor tetra-
mers 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 tetra-
mer 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 coope- rative meltings separated by an intermediary temperature range with almost no melting ; we observe an intermediary non coope- rative 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_i) 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 invol- ved in the binding. Since 10 phosphates are protected by one repressor subunit, and since the experimental values of h_2 and h_i 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_i) 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) altough the repressor is a tetra- meric 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 phe-
nomenon 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 ν of
amino acids, shifting from α to β structure when a poly d(A-T)
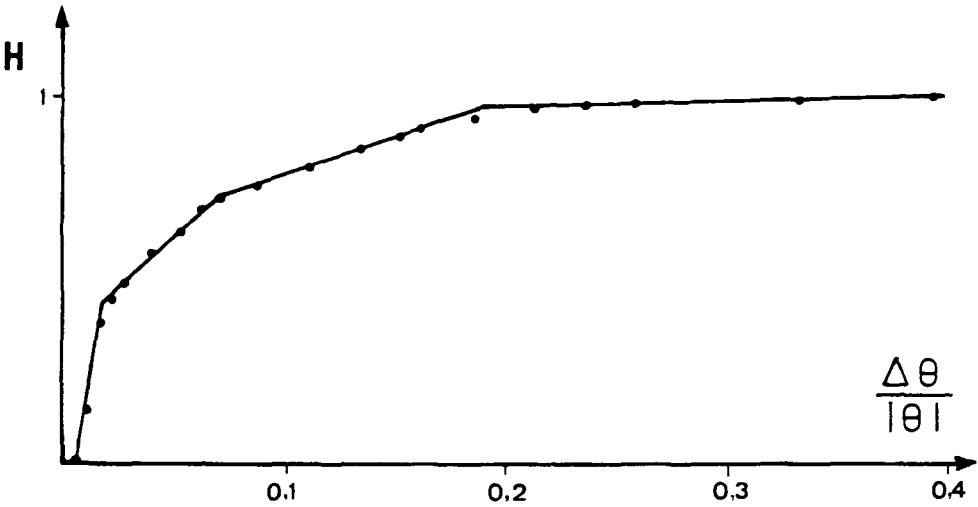pair melts according to a simple relation :

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

$$\nu \;=\; \frac{\Delta\rho}{\Delta OD} \;\times\; \frac{\varepsilon_H}{\rho_{\alpha\beta}}$$

where $\Delta\rho$ 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 ;

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

to β structure.

We find, as expected, that ν 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 ν to be 1.5 and for the melting around 60°C ν =
3.8 ; using the results of stoichiometry and the fact that the
total hyperchromicity for each segment is about the same, the
ν 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 possi-
ble 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.

REFERENCES

1. Müller-Hill, B., Beyreuther, K., Gilbert, W. (1971)
   Methods in Enzymology 21 (D) 484-501.

2. Barth, G., Bunnenberg, E., Djerassi, C. (1972) Analytical Biochemistry 48, 471-479.
3. Sober (1970) Handbook of Biochemistry, 2nd edn, the Chemical Rubber Co., Cleveland.
4. Beyreuther, K., Adler, K., Geisler, N., Klemm, A. (1973) Proc. Nat. Acad. Sci. USA, 70 (12), 3576-3580.
5. Laiken, S.L., Gross, C.A., von Hippel, P.H. (1972) J. Mol. Biol. 66, 143-155.
6. Matsuura, M., Ohshima, Y., Horiuchi, T. (1972) Biochem. Biophys. Res. Comm. 47 (6), 1438-1443.
7. Chen, Y.H., Yang, J.T., Martinez, H. (1972) Biochemistry 11 (12), 4120-4131.
8. Lemieux, G., Lefèvre, J.F., Daune, M. (1974) Eur. J. Biochem. 49, 185-194.
9. Gilbert, W., Maxam, A. (1973) Proc. Nat. Acad. Sci. USA 70 (12) 3581-3584.
10. Steitz, T.A., Richmond, T.J., Wise, D., Engelman, D., (1974) Proc. Nat. Acad. Sci. USA, 71 (3) 593-597.
11. Garel, A., Kovacs, A.M., Champagne, M., Daune, M. (1975) Biochim. Biophys. Acta, in press.

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 λ = 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.