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Binding of E.coli lac repressor to non-operator DNA*

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ABSTRACT

It is shown by melting profile analysis of lac repressor-DNA complexes that repressor binds tightly and preferentially (relative to single-stranded DNA) to double-stranded non-operator DNA. This binding stabilizes the DNA against melting and the repressor against thermal denaturation. Analysis of the extent of stabilization and the rate of dissociation of repressor from non-operator DNA as a function of sodium ion concentration shows, in confirmation of other studies,[3,4] that the binding constant (K_{RD}) is very ionic strength dependent; K_{RD} increases from $\sim 10^6$ M^{-1} at ~ 0.1 M Na^+ to values in excess of 10^{10} M^{-1} at 0.002 M Na^+. Repressor bound to non-operator DNA is not further stabilized against thermal denaturation by inducer binding, indicating that the inducer and DNA binding sites probably represent separately stabilized local conformations. Transfer melting experiments are used to measure the rate of dissociation of repressor from operator DNA. These experiments show that most of the ionic strength dependence of the binding constant is in the dissociation process; the estimated dissociation rate constant decreases from greater than 10^{-1} sec^{-1} at $[Na^+] \geq 0.02$ M to less than 10^{-4} sec^{-1} at $[Na^+] \leq 0.002$ M. Competition melting experiments are used to show that at 0.02 to 0.002 M Na^+ the affinity of lac repressor for various natural DNAs and synthetic double-stranded polynucleotides (including poly[d(m^6A-T)], which carries a methyl group in the large groove) are approximately independent of base composition, except that the affinity of repressor for poly[d(A-T)] is $\sim$ 2- to 3-fold greater than for the other DNAs tested. The affinity for single-stranded polynucleotides is at least 50-fold less than for the double-helical forms.

INTRODUCTION

An area of research which greatly interested the late Jerome Vinograd, and to which he made major contributions, is the explication of the molecular mechanisms whereby proteins direct and control the expression of DNA in both lower and higher organisms. In this communication we outline some studies from our laboratory in this area, focusing on recent developments in our understanding of the interactions of lac repressor with non-operator DNA which may have implications for the functional control of the lactose operon of E. coli.

The observation that lac repressor binds to DNA sequences other than the operator region was first made by Lin and Riggs,[5] who used a filter binding tecnhique[6] to partially quantitate this "non-specific" binding. Under most conditions the binding of repressor to non-operator DNA is too weak to be measured directly by this procedure; instead the strength of the interaction has been estimated by a competition assay.[5] This involves measuring the decrease in the extent (or rate) of binding of ^{32}P-labeled operator-containing DNA to the filter by repressor, as a consequence of the addition of various non-operator DNAs which compete for the available repressor molecules.[6]

While the competitive filter binding technique has been very useful as a qualitative tool, it is subject to a number of assumptions and artifacts[6,7,8] which make its use suspect as a primary quantitative procedure for determining binding parameters. For this reason we have characterized the binding of lac repressor to non-operator DNA by direct physico-chemical methods.

The existence of an appreciable non-sequence-dependent affinity of lac repressor for DNA is not surprising when one considers that association of DNA-binding proteins with the nucleic acid must involve charge-charge interactions with the (more or less) monotonically structured sugar-phosphate backbone of the DNA. On this basis we picture most native DNA-protein interactions as involving a rather non-specific (largely electrostatic) component superimposed on specific interactions between protein side-chains and the functional groups (exposed via the major and minor grooves of the DNA structure) of a particular sequence of base pairs.

Quantitative information on the binding of repressor to non-operator DNA is important to an ultimate understanding of the repressor-operator interaction in at least three ways:

(1) Molecular Insight. A variety of genetic and physico-biochemical approaches have demonstrated that the same portions of the repressor subunits, and probably the same binding sites, are involved in both interactions (for a recent review, see ref. 9). Analysis of the molecular details of non-specific binding will thus help in elucidation of the operator-repressor interaction at the molecular level.

(2) Thermodynamics of Repression. The concentrations (activities) of free repressor (R) and repressor-induced complex (RI) in the bacterial cell, and their binding equilibria with operator (O), determine the level of repression in vivo. Since non-operator DNA (D) provides the primary competitive binding sites for the R and RI species in the cell, the relative magnitudes of

the binding affinities of R and RI for D and O will control the concentrations of free R and RI, and thus ultimately the level of repression of the lac operon.[10,11,3]

(3) Kinetics of Repression. The kinetics of the interaction of repressors with their target operators in very dilute solution in vitro has indicated that RO complex formation appears to proceed much faster than the calculated diffusion-controlled limiting rate.[12] This apparent anomaly has been explained by a two-step kinetic mechanism involving a second-order free diffusion of repressor to non-operator DNA, followed by a rapid first-order intramolecular transfer of the DNA-bound repressor to the operator site.[11,13] Study of the kinetics of non-specific repressor binding should yield insight into in vivo mechanisms for the transfer of DNA-binding proteins from one DNA site to another, a process which may be crucial for the functioning of lac repressor as well as other genome-regulating proteins.

In the following sections we summarize recent studies on the binding of lac repressor to non-operator DNA which provide background information for the further exploration of these aspects of the interaction of repressor with operator.

MATERIALS AND METHODS

The preparation, purification, and characterization of lac repressor, various DNAs, and poly[d(m^6A-T)] have been described elsewhere.[4,14-16] Other synthetic polynucleotides were obtained from Miles or P.L. Biochemicals. All experiments were performed in buffer adjusted to pH 7.5, and containing 10^{-4} M Na_2EDTA and 10^{-3} M phosphate. The total Na^+ concentration was adjusted as required by adding NaCl.

Repressor-nucleic acid complexes were formed as follows: purified repressor (stored at -70° in 30% glycerol, 1 M Tris) was dialyzed at 4°C into a buffer containing at least 0.1 M Na^+ and centrifuged briefly to remove aggregates. Repressor concentrations were determined using an extinction coefficient (ε_{280}) of 9 x 10^4 M^{-1} cm^{-1} (per repressor tetramer).[14] DNA and repressor were mixed, at moderately high ionic strength, to the desired DNA base-pair:repressor tetramer (D:R) ratio. The mixed complexes were then dialyzed to the experimental salt concentration.

Melting experiments on repressor-DNA complexes were conducted in a Gilford 2000 recording spectrophotometer.[17] Initial sample temperatures were generally 10° to 20°, and the rate of heating of the samples was usually

0.5°/min. Controls were run to demonstrate that the observed optical changes reached equilibrium at all temperatures at this heating rate. Absorbance changes were followed at several wavelengths, including 320 nm to monitor solution turbidity. Since experiments were generally conducted over rather restricted temperature ranges, corrections for thermal expansion of the solvent were not required. For Figures, the melting profiles have all been normalized to constant poly[d(A-T)] or poly[d(m^6A-T)] concentration.

Ultracentrifugation of DNA-repressor complexes was performed in a Beckman-Spinco Model E analytical ultracentrifuge, as described elsewhere.[4]

RESULTS AND DISCUSSION

Solution Properties and Thermal Denaturation of Repressor. In the absence of polynucleotides, native lac repressor tends to aggregate irreversibly in solution. The rate and extent of this aggregation can be minimized by maintaining the protein at low concentration ($\sim 10^{-6}$ M), moderate ionic strength ($\sim$ 0.1 M Na^+) and low temperature; under these conditions the native protein can be kept totally in solution for hours or days.

If a solution of repressor under these conditions is heated (at $\sim$ 0.5°/min), it undergoes a sharp and irreversible increase in turbidity starting at $\sim$ 44° (± 1°). It can be shown (see below) that this turbidity increase represents a real protein denaturation followed by aggregation of the denaturated product, since in the presence of polynucleotide the aggregation phase can be prevented and the protein denaturation step (characterized by appreciable hyperchromicity at 280 nm) can be isolated. Thus $\sim$ 44° may be taken as the melting (denaturation) temperature of free repressor. This denaturation temperature is not markedly affected by small changes in either solution ionic strength or repressor concentration.

Stabilization of Repressor on Binding to DNA. In the presence of native DNA, lac repressor is stabilized against thermal denaturation. Solutions containing $\sim 10^{-6}$ M repressor plus either native calf thymus or native M. lysodeikticus DNA at a DNA base pair:repressor tetramer ratio of $\sim$ 13:1 were made 0.2, 0.1, 0.06, 0.02 and 0.002 M in Na^+, and subjected to melting analysis. [The melting temperatures of the free DNAs under all but the last of these salt conditions fall well above the temperature range investigated, and furthermore (see below) binding of repressor to native DNA stabilizes DNA against melting.] Repressor melting was monitored at 280 and 320 nm. In 0.2 M Na^+, repressor melting began at 44-48°; in 0.1 M Na^+, the onset of

repressor denaturation was shifted to 47-52°; in 0.06 M Na^+, denaturation began in the 50-55° range; and in 0.02 and 0.002 M Na^+, little or no repressor denaturation was observed until the experimental temperature reached 60° or above. At the higher ionic strengths the thermal denaturation of the repressor could be monitored at either 280 or 320 nm, indicating that heat-denatured repressor aggregates as in the absence of DNA. However in 0.02 and 0.002 M Na^+, no change in OD_{320} is seen, though a clear transition is observed at 280 nm. Thus the denatured repressor apparently remains bound to the DNA (and thus fails to aggregate) at these lower ionic strengths.

By monitoring the quenching of the intrinsic protein fluorescence of repressor,[15] we have shown that inducer binding to repressor is independent of whether or not the repressor is bound to non-operator DNA. The presence of saturating concentrations of the inducer isopropyl-β-D-thiogalactoside (IPTG) also does not change the observed thermal denaturation behavior of either free or non-operator DNA-bound repressor. This suggests that the thermally-induced unfolding and aggregation processes described above involve the DNA-binding portion of the molecule; and confirms (see ref. 9) not only that the inducer and DNA-binding sites of repressor are separate, but also shows that they involve essentially independently stabilized local conformations. As expected, the addition of small amounts of urea lowers the transition-temperature of both free and DNA-bound repressor. Heat-denatured repressor, even that still bound to DNA at low ionic strengths, shows no inducer-binding affinity.

Assuming a simple binding equilibrium, and approximating the complications introduced by overlap of potential binding sites,[18] this stabilization of repressor conformation by binding to non-operator DNA can be used to estimate very crudely an association constant (K_{RD}) for the system. At $\sim$ 0.1 M Na^+ we estimate that $K_{RD} \simeq 10^6\ M^{-1}$, in reasonable accord with results by filter binding[5] and direct physico-chemical methods.[4] In further accord with these studies, the repressor stabilization induced by DNA binding also shows that the affinity of repressor for DNA increases markedly with decreasing ionic strength.

Stabilization of DNA on Binding to Repressor. Since binding to DNA stabilizes repressor conformation, repressor binding must also stabilize DNA. Whether the single- or double-stranded form of DNA is stabilized will depend on which is bound more tightly. Figure 1 shows UV melting profiles of repressor-poly[d(A-T)] complexes at various DNA:repressor ratios. Clearly repressor binding increases the melting temperature of this double-stranded

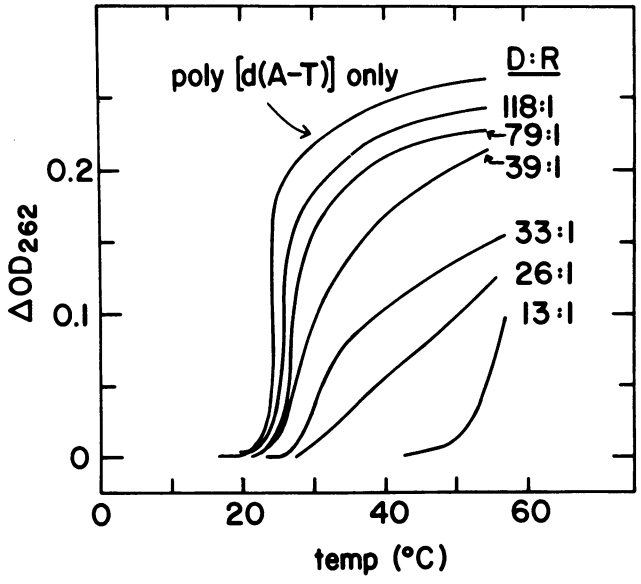

Figure 1. Melting profiles of poly[d(A-T)] complexed with lac repressor at the base pair:repressor tetramer (D:R) ratios indicated. Repressor concentration ≃ 10^{-6} M, $[Na^+]$ = 0.002 M. These profiles represent non-equilibrium (no transfer) conditions; see text and Figure 2.

polynucleotide, indicating that the protein binds preferentially to this form of DNA.

Poly[d(A-T)] is used in this experiment because at low ionic strengths (even in repressor-stabilized complexes) its melting is virtually complete prior to heat denaturation of the bound repressor itself (at 55 to 60°). Similar experiments with natural DNAs at low ionic strength show that repressor binding stabilizes the double-helical form of these moieties as well, though denaturation of the repressor prevents visualization of the complete transitions.

Figure 1 shows that at high DNA:repressor (D:R) ratios most of the poly[d(A-T)] melts at temperatures not very different from uncomplexed poly[d(A-T)] at the same temperature. As the D:R ratio is decreased, more of the poly[d(A-T)] is stabilized against melting, and at D:R ratios ≃ 13:1 virtually all the poly[d(A-T)] melts fairly sharply at a temperature elevated by ∿ 35° from that of poly[d(A-T)] alone. Lower D:R ratios (data not shown) reveal little further increase in transition temperature, and, indeed, are partially obscured by aggregation of free repressor beginning at 45-48°. This suggests that under these tight-binding conditions virtually all the repressor

is bound to the poly[d(A-T)], and that the DNA lattice saturates at a D:R ratio of ∿ 13 base pairs per repressor tetramer. [It should be borne in mind that because of overlap of potential binding sites, this site size will represent a slight over-estimate.[18,14]] This site size (n) for repressor binding is very close to the value of n = 12 base pairs per repressor molecule obtained directly by titration methods.[14]

Repressor Transfer Experiments. Melting profiles of nucleic acid-protein complexes such as those of Figure 1 can, in principle,[19] be interpreted to obtain considerable additional quantitative information on binding parameters. First, however, one must determine whether these melting experiments represent equilibrium or non-equilibrium processes in a molecular sense.

In an equilibrium melting experiment, in which the ligand (protein) is preferentially bound to the double-helical form of the DNA and the DNA is not initially saturated, protein which dissociates from melted DNA sequences (below the melting temperature of the protein ligand itself) will rebind to DNA sequences which remain double-stranded.[20,19] This requires, however, that the rate of dissociation of protein from the DNA be large relative to the rate at which the melting experiment is conducted. Alternatively, if dissociation of the protein is slow, the melting experiment portrays the ligand distribution characteristic of the original unmelted system.

Many instances of both types of behavior are known: thus the melting of DNA-netropsin complexes[19] represents an equilibrium process, while the melting of DNA-polylysine systems at low ionic strength is essentially non-equilibrium and mirrors the initial distribution of polylysine ligands on the DNA lattice.[17]

Repressor transfer experiments can be designed to distinguish these possibilities. Thus one can form repressor-DNA complexes under conditions close to lattice saturation (D:R ≃ 13:1), and after dialysis to low ionic strength (and just prior to melting) add sufficient additional DNA to approximately double (to ∿ 26:1) the overall D:R ratio. If transfer to equilibrium takes place, the entire system will melt like a 26:1 complex (Figure 1); if transfer is very slow, one-half the DNA will melt as if it were free, and the other one-half like a 13:1 complex. Figure 2 represents the latter type of experiment under the conditions ($[Na^+]$ = 0.002 M) of Figure 1, showing that the melting profiles of Figure 1 represent a totally non-equilibrium (low transfer rate) system.

This experiment has been repeated (data not shown) in solutions containing progressively higher concentrations of Na^+, and it has been shown that the

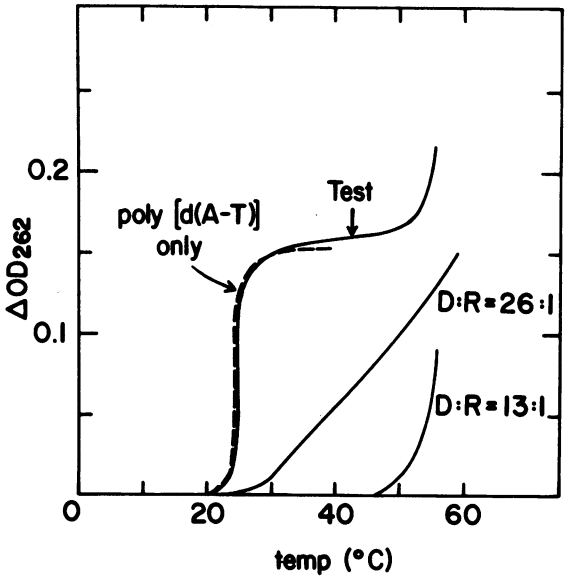

Figure 2. Transfer test for $\sim 10^{-6}$ M lac repressor mixed with poly[d(A-T)] at a D:R ratio of 13:1 at 0.1 M Na^+ and dialyzed to 0.002 M Na^+. Just before melting, an equal amount of poly[d(A-T)] was added. The "Test" curve indicates no transfer takes place under these conditions; the added poly[d(A-T)] melts like the free species, and the remainder follows the D:R = 13:1 curve (see text).

rate of repressor transfer becomes comparable to the rate of heating at $[Na^+] \simeq 0.01$ M, and that totally equilibrium melting profiles are obtained at $[Na^+] \geq 0.02$ M.

These findings were confirmed by similar (isothermal) transfer experiments conducted in the analytical ultracentrifuge with repressor and either λ phage DNA or poly[d(A-T)]. In these experiments the repressor-DNA complexes sediment much more rapidly than poly[d(A-T)] alone, and thus equivalent transfer determinations can be made. Comparison of these sedimentation experiments with the equivalent melting profile transfer experiments also confirms that the rate of change with temperature of the affinity constant of lac repressor for DNA is small.[5,4] Thus melting profiles representing non-equilibrium transfer conditions at low temperatures correspond to such conditions at high temperatures as well.

These results can be used to estimate kinetic parameters for the repressor-non-operator DNA interaction. The association rate constant for the interaction of repressor with a non-operator DNA site can be estimated at $\sim 10^7$ M^{-1} sec^{-1} (refs. 11 and 8) and is approximately independent of ionic

strength. Thus for equilibrium constants (K_{RD}) > 10^9 M^{-1}, the dissociation rate constant would be expected to be smaller than ∿ 10^{-2} sec^{-1}, corresponding to dissociation (and thus transfer) half-times comparable to the heating rates in the melting experiments. This suggests that K_{RD} > 10^9 M^{-1} at ∿ 0.01 M Na^+, in reasonable accord with values obtained by extrapolation of directly measured association constants to this salt concentration.[4]

Repressor Binding to DNA is Non-Cooperative. Based on the above transfer experiments, the non-equilibrium melting profiles of Figure 1 can be interpreted further. These curves show that all the poly[d(A-T)] is partially stabilized against melting by repressor when the DNA lattice is ∿ one-half saturated (D:R ≃ 26:1). Thus binding is noncooperative (i.e., contiguous repressor binding is not favored over isolated binding), since even low levels of positive binding cooperativity would tend to isolate virtually all the repressor on approximately one-half of the poly[d(A-T)] molecules.[18] Direct analysis of binding isotherms also shows that repressor binding to poly[d(A-T)] and to natural DNAs is noncooperative.[4]

Figure 1 shows that at saturation (in 0.002 M Na^+) repressor binding stabilizes the poly[d(A-T)] lattice against melting by at least 30 to 40°. This is probably an under-estimate since repressor itself undergoes partial heat denaturation at the top end of the D:R ≃ 13:1 melting profile. Therefore repressor binding stabilizes the double-helix by at least 0.6 to 0.8 kcal per base pair under these conditions, corresponding to a minimum total free energy of stabilization of 8 to 10 kcal (at 0.002 M Na^+) per repressor tetramer covering 13 base pairs.

Stabilization of Poly[d(m^6A-T)] by Repressor Binding. The transfer experiments discussed above (Figure 2) show that higher ionic strength conditions [Na^+] ≥ 0.02 M) must be used to obtain equilibrium melting profiles of repressor-DNA complexes. Yet at higher salt concentrations most DNAs melt at temperatures above the denaturation temperature of even DNA-bound repressor. To circumvent this problem we used poly[d(m^6A-T)], which has a structure like that of double-stranded poly[d(A-T)] except that the N_6-amino group carries a methyl residue which protrudes into the large groove of the Watson-Crick double-helix. The presence of this methyl group destabilizes the double-helix,[16] and as a consequence lowers the melting temperature of the double-stranded polynucleotide to ∿ 27° at 0.02 M Na^+ (Figure 3), making it an ideal substrate for an equilibrium melting experiment. The results of such an experiment are shown in Figure 3. In addition to representing an equilibrium

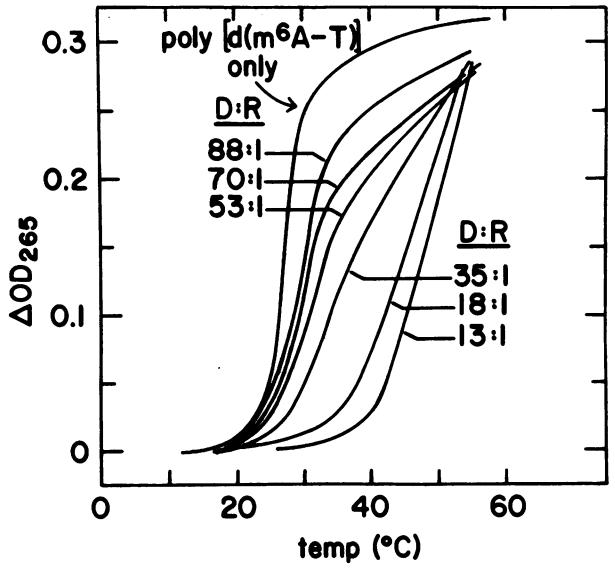

Figure 3. Equilibrium melting profiles of poly[d(m^6A-T)] complexed with lac repressor at the base pair:repressor tetramer (D:R) ratios indicated. Repressor concentration ≃ 10^{-6} M; [Na^+] = 0.02 M.

melting situation these curves are fully reversible (to at least ∿ 45-50°), in that the system can be recooled and the melting profiles retraced in the opposite direction.

Again we observe a marked stabilization of the double-helical polynucleotide structure, and the shapes of the melting profiles are in good qualitative accord with theoretical expectations for the reversible noncooperative binding of a large ligand covering a number of base pairs and binding preferentially to the native DNA lattice (see Figure 8, ref. 19). We have not attempted a quantitative fit to theory[19] because of complications introduced by a small unexplained residual hypochromicity in the complexes relative to poly[d(m^6A-T)] alone, possible denaturation of small amounts of repressor, etc.

From these curves we see that repressor binding stabilizes the poly[d(m^6A-T)] lattice by ∿ 20-25°, corresponding to a stabilization free energy of ∿ 6 to 7 kcal per bound repressor molecule. Using a rough estimate of the concentration of free repressor supporting the equilibrium under these conditions, this leads to a binding constant of about 10^{-9} M^{-1} for this system (at 0.02 M Na^+), again in reasonable agreement with values obtained by extrapolation of directly measured binding constants for comparable systems (see below and ref. 4).

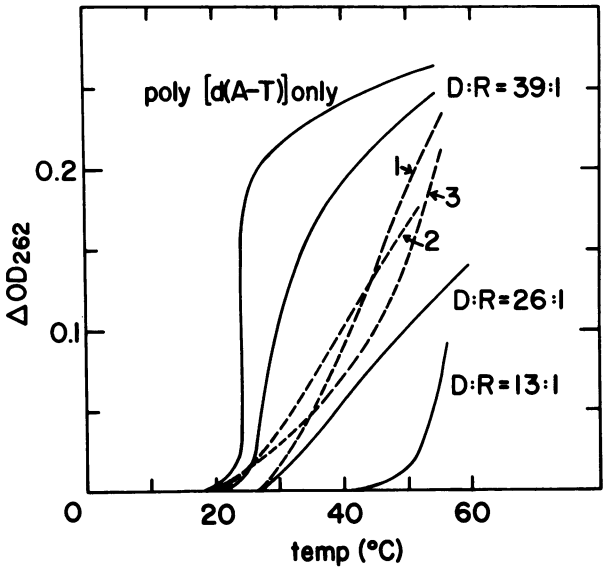

Figure 4. Competition (no transfer) melting profile experiments. The solid curves represent poly[d(A-T)]-repressor melting experiments at the indicated D:R ratios. The dashed lines represent poly[d(A-T)] repressor melting profiles to which various natural DNAs (in a 2:1 ratio relative to poly[d(A-T)]) had been added prior to dialysis to 0.002 M Na^+ and melting. Curve 1: calf thymus DNA; curve 2: phage λ DNA; curve 3: M. lysodeikticus DNA; (see text).

Competition Experiments. We can take advantage of the marked changes in shape of the repressor-DNA melting profiles as the D:R ratio is varied in experiments such as those of Figure 1 (and Figure 3) to determine relative binding constants for various types of natural and synthetic DNA. For example (Figure 4), using the melting of poly[d(A-T)] as a test probe we can mix various concentrations of higher melting DNAs with an aliquot of poly[d(A-T)] at 0.1 M Na^+, add repressor to a predetermined ratio, and dialyze the system to low salt (0.002 M Na^+). (In this case the non-equilibrium behavior of the melting system is an advantage, since no repressor is transferred to the added DNA as the poly[d(A-T)] melts, and the entire melting profile reflects the initial distribution of repressor between the poly[d(A-T)] and the test DNA.) Thus in the experiment of Figure 4, if the affinity of the various test DNAs for repressor had been equal to that of poly[d(A-T)], we would expect the melting profile to follow a D:R = 39:1 curve; while if the affinity of repressor for poly[d(A-T)] were (e.g.) 10-fold greater than for the test DNA, we might expect the D:R = 13:1 profile to be followed. (If the affinity of the test DNA for repressor were much greater

than that of poly[d(A-T)], the synthetic polynucleotide would melt as the uncomplexed polymer.)

In carrying out these experiments we have adjusted the ratios of test DNA to poly[d(A-T)] to maximize the sensitivity of the assay. Figure 4 shows that calf thymus DNA (42 mole % G-C), phage λ DNA (50 mole % G-C), and M. lysodeikticus DNA (72 mole % G-C) all have virtually equivalent affinities for lac repressor, and that poly[d(A-T)] binds two- to three-fold more tightly to repressor than do the various natural DNAs at this ionic strength.

A series of similar experiments were carried out using poly dA•poly dT and poly[d(m^6A-T)] as the low melting DNA species. These experiments showed that the binding constants for both these synthetic double-stranded polynucleotides to lac repressor are about the same as those for native DNA in 0.002 M and 0.02 M Na^+.

Binding to Single-Stranded DNA. In order to obtain a complete description of the various repressor to DNA binding equilibria, we have also examined the interaction of lac repressor with single-stranded polynucleotides. Repressor and poly dT were mixed at D:R ratios (here moles polynucleotide bases to repressor tetramer) ranging from 13:1 to ∿ 50:1 at 0.1 M Na^+, and dialyzed to 0.002 M Na^+. No repressor aggregation was observed at this low ionic strength, indicating that repressor had bound to the polynucleotide. The samples were then subjected to thermal melting; no change in optical density was seen at 44°, again indicating that repressor binds to poly dT under these conditions and is stabilized against heat denaturation as a consequence. At ∿ 55° the optical density increase characteristic of the denaturation of polynucleotide-stabilized repressor was observed.

Repressor interacts similarly with single-stranded poly dA under these conditions. However in addition the binding of native lac repressor to poly dA stabilizes the stacked form of this polynucleotide. Poly dA heated alone shows a significant and progressive hyperchromic absorbance change with increasing temperature, while in the presence of saturating repressor concentrations this increase is virtually abolished until the repressor itself denatures. In keeping with the preference of lac repressor for double-stranded DNA, this suggests that poly dA is accommodated as a stacked structure in the DNA binding site of repressor.

Competition experiments against poly[d(A-T)] were carried out with poly dT and poly dA to assess the relative affinity of repressor for single- and double-stranded polynucleotides. The highest concentrations of single-stranded polynucleotides tested (four- to six-fold molar excesses over

poly[d(A-T)]) had no competitive effect whatsoever on the stabilization of the poly[d(A-T)] melting profiles by repressor, indicating that single-stranded DNA binds to repressor at least 50-fold more weakly than native DNAs at this ionic strength.

CONCLUSIONS

In addition to providing information about the lac repressor system, some of the procedures outlined in this paper, especially the ligand transfer and binding competition experiments, may be useful in studying transfer rates and relative binding affinities in other nucleic acid-protein interaction systems. Testing various histones for sequence-specific DNA binding affinity might be a good example. These approaches are particularly well suited to the study of complexes which are primarily electrostatically stabilized, since for such systems the rate of change of association constant (K) with ionic strength is usually much greater than the rate of change of this parameter with temperature, and thus the equilibrium is not much perturbed by the temperature change involved in the melting experiment. [Of course, if the experiment is done under non-equilibrium ("no transfer") conditions, the melting profiles will represent the equilibrium which applies to the conditions of dialysis.]

If this paper we have shown that lac repressor binds to double-helical non-operator DNA much more tightly than to single-stranded DNA sequences. Since considerable evidence (see ref. 9) suggests that the same repressor binding sites are involved in the interaction with operator and non-operator DNA, this result is also consistent with the demonstrated preference of repressor for double-stranded operator sequences.[6] Further insight into the relation of repressor structure to functional binding domains is derived from the finding that neither the affinity of repressor for double-helical non-operator DNA, nor the extent to which repressor is stabilized against heat denaturation by this interaction, is altered by inducer binding. Thus the DNA and inducer binding sites are not only located on different parts of the repressor molecule, but also presumably these binding domains represent independently stabilized local conformations.

The results presented in this paper also confirm that repressor binding to non-operator DNA is very ionic strength dependent; K_{RD} ranges from $\sim 10^5$ M^{-1} at $[Na^+] \simeq 0.15$ M to values in excess of 10^{10} M^{-1} at very low (e.g., 0.002 M) Na^+ concentrations. Since this binding is also virtually independent of DNA base composition at both low (this work) and intermediate[4] ionic strengths, it appears that the major part of the binding free energy of

repressor to non-operator DNA must be electrostatic in origin, involving primarily ionic interactions of DNA phosphates with basic amino acid residues of the repressor binding sites.[21]

The fact that binding of repressor to non-operator DNA is essentially unperturbed by introducing a methyl group into the large groove of the DNA (in poly[d(A-T)]) indicates further that nucleotide base-protein interactions, at least via the large groove, are not important in non-specific binding. This conclusion is consistent with the finding of Richmond and Steitz[22] that the addition of bulky groups in the large groove of synthetic double-stranded DNA models does not inhibit repressor binding, and the demonstration by Kolchinsky et al.[23] that non-specific binding of repressor does not protect the DNA functional groups located in the major groove against chemical methylation. Such methylation experiments have shown that functional groups in the major groove probably are involved in operator binding.[24]

A more detailed molecular picture of the various interactions involved in repressor-operator complex formation, as well as a determination of the groups involved in non-specific binding, must probably await crystallographic analysis of repressor-operator and/or repressor-non-operator DNA complexes.

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