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INDUCER BINDING TO LAC REPRESSOR: EFFECTS OF POLY[d(A-T)] AND TRYPSIN DIGESTION

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SUMMARY

The parameters for inducer association with the tryptic core protein of the lactose repressor have been measured. The second-order rate constant and the equilibrium dissociation constant are unchanged from the native protein. The association rate of interaction for inducer and repressor is also unaffected by the presence of poly[d(A-T)] and/or various Mg^{++} concentrations. From this information in concert with results of a number of other studies, we offer the hypothesis that the core region of the repressor contains determinants for the specific DNA binding capacity of the protein, while the NH_2-terminus possesses primarily nonspecific DNA binding determinants.

The lactose repressor protein binds specifically to the operator region of the Escherichia coli DNA with high affinity and with lower affinity to nonspecific sequences of DNA. While the exact mechanism by which repressor binds to DNA is not known, the involvement of the NH_2-terminal regions of the protein has been strongly indicated by genetic data (1-3). Furthermore, removal of the NH_2-terminal 59 residues from each repressor subunit by mild tryptic digestion results in loss of both operator and nonspecific DNA binding activities (1,4,5). The remaining protein molecule, termed the core protein, binds inducer ligands with the same affinity as native repressor (1). In addition, the difference in the ultraviolet spectrum of the protein produced on binding inducer is essentially identical for core protein and native repressor (6). In order to examine the relationship between loss of DNA binding capability and the repressor protein's inducer binding activity, measurements of the parameters of inducer binding to core protein and to repressor-poly[d(A-T)] were undertaken.

When inducer binds to the repressor protein, the specific affinity of the protein for the operator DNA region is reduced by several orders of magnitude (7).

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While this decrease is significant, it is the ability of the remainder of the genome (nonspecific DNA) to compete effectively with operator for binding the induced protein that results in release of the operator DNA and subsequent transcription (8). In contrast, the affinity of repressor for nonspecific sites on the DNA is unaffected by the presence of inducer molecules (5,9). The presence of nonspecific DNA does not affect the binding of inducer to the repressor protein (10), while, as would be expected, operator DNA fragments significantly perturb inducer binding (11). The differential binding of the repressor and repressor-inducer complex to operator versus nonspecific DNA has been viewed as a difference in degree of binding affinity. Recent evidence suggests that the difference between specific and nonspecific binding may represent more than just the favorability of the substrate. The two types of binding can be distinguished both genetically (12) and chemically (13,14). Mutants and chemically modified proteins have been isolated whose operator binding activity is abolished but whose nonspecific DNA binding activity is retained. This distinction between the two binding functions suggests that separate determinants for specific versus nonspecific binding must exist. Nonspecific binding may be a necessary prerequisite for the tight specific binding observed, but specific binding has separate determinants which govern the interactions between the protein and the operator site on the DNA.

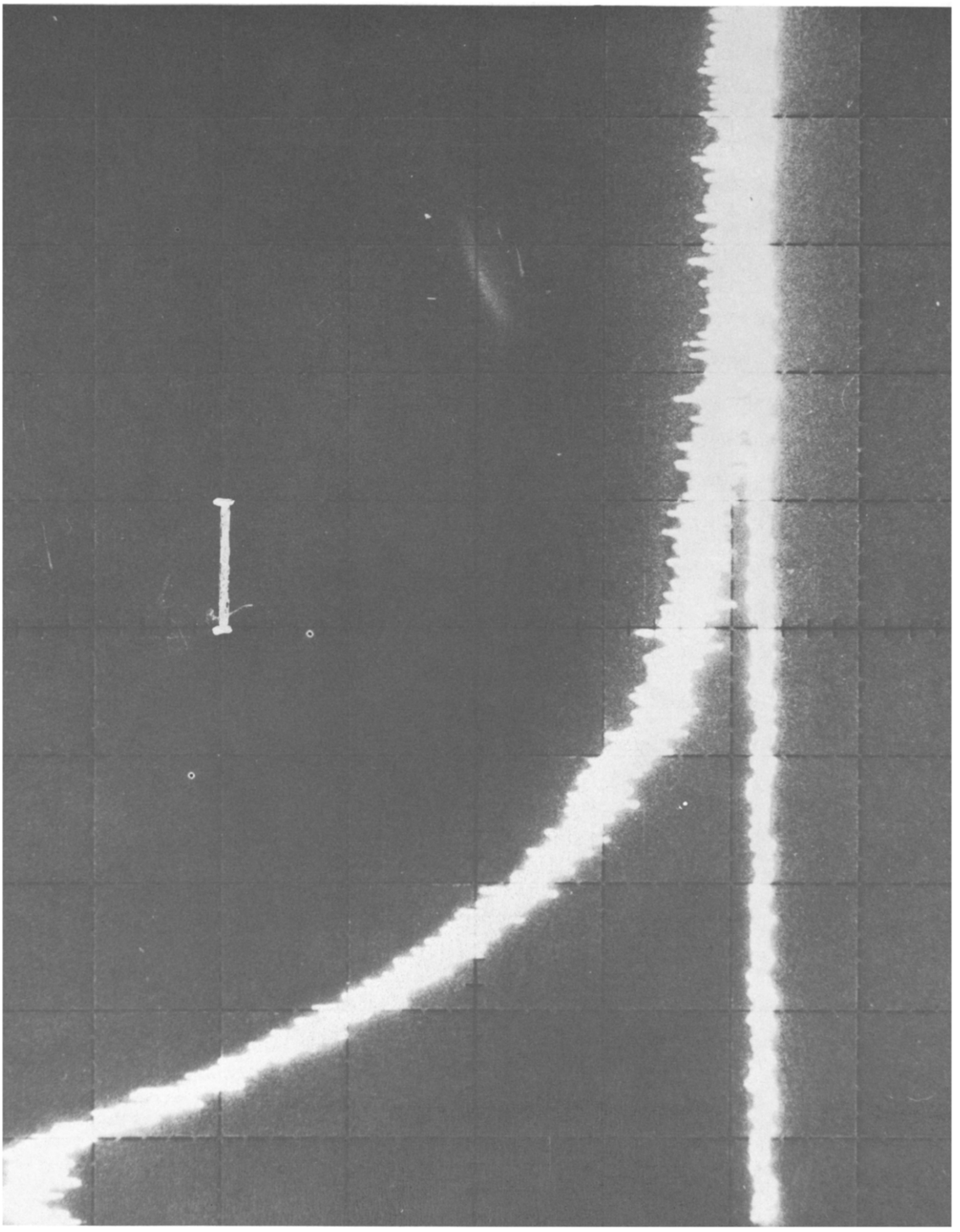
Chemical modification studies have indicated the location of the site(s) affecting specific binding affinity. Reaction of the protein with the thiol reagent fluorescein mercuric acetate yields a modified repressor with normal nonspecific DNA binding characteristics but lacking the high affinity for operator DNA (14), and the sites of modification are all in the core region of the molecule. N-Bromosuccinimide oxidation of the protein also results in loss of operator DNA binding activity with no effect on the nonspecific binding affinity (13). Recent work in this laboratory (Manly and Matthews, manuscript in preparation) has shown that the sites responsible for operator binding loss

upon oxidation are the cysteine residues, which are located in the core region of the molecule.

In earlier studies, stopped-flow rapid mixing spectral techniques were employed to monitor changes in tryptophan fluorescence which accompany the association or dissociation of inducer with repressor (10). The change in protein conformation in response to ligand binding results in perturbations of the environment of the tryptophan residues; these perturbations are reflected in the observed alteration in protein fluorescence. In this manner, the rates of binding of inducer (isopropyl-β,D-thiogalactoside)[1] to the protein were determined. The presence of calf thymus DNA did not affect the rate of inducer binding to repressor (10). In a similar manner, we have measured the parameters for inducer binding to core protein and to the repressor-poly[d(A-T)] complex to determine whether removal of the putative DNA binding site through tryptic digestion or the presence of tightly-bound nonspecific DNA affects the affinity or rate of inducer binding to the core repressor.

The core protein was prepared according to the procedure described by Platt *et al.* (1). Repressor protein was treated with trypsin (0.5% by weight) for 20 minutes at 30° C. The reaction was stopped by addition of phenylmethyl-sulfonyl fluoride. The core protein was isolated by passage through a Sephadex G-75 column. The homogeneity of the protein was evaluated on sodium dodecyl sulfate polyacrylamide gels, which exhibited one band for the core protein (of molecular weight ∿30,000). Kinetics of the interaction of IPTG with the core were measured as previously reported (10). The binding of inducer to native repressor and to the core protein were monitored under the same conditions. The exponentially decreasing fluorescence signal observed when IPTG was mixed with repressor was used to determine the apparent rate of reaction under pseudo first-order conditions (Figure 1). A plot was made of the second-order observed rates *versus* the concentrations of inducer at which they were measured (Figure 2); the slope of the line thus generated was used to calculate the second-order

[1] Isopropyl-β,D-thiogalactoside is abbreviated IPTG.



rate constant. In 0.1 M Tris-HCl, 1.0 M NaCl buffer, pH 8.0, 3×10^{-4} M dithiothreitol at 20° C the rate was 10.9×10^{4} $M^{-1}s^{-1}$ for core protein, as compared to 9.8×10^{4} $M^{-1}s^{-1}$ for a sample of native repressor. The association rate for the binding of inducer is essentially unaffected by the presence (or absence) of the NH_2-terminal region and thus normal DNA-binding capacity. Similarly, the equilibrium dissociation constant of the repressor (2.0×10^{-6} M in 50 mM Tris-HCl, 10^{-4} M dithiothreitol, pH 7.8) and inducer was found to be unchanged for the core protein (2.3×10^{-6} M), as previously shown by Platt et al. (1). These equilibrium constant determinations were made by titrating the shift in tryptophan fluorescence of the proteins on binding to inducer. Poly[d(A-T)] and repressor were prepared in a buffer containing 0.01 M Tris-HCl and 0.02 M NaCL, pH 7.5. The mixture was reacted with various concentrations of IPTG in the stopped-flow spectrometer. The rate determined from these experiments was 10.1×10^{4} $M^{-1}s^{-1}$ (Figure 2). The presence of Mg^{++} up to 10 mM had no effect on the rates observed.

The experimental results summarized above coupled with observations from previous studies suggest that there are determinants in the protein structure which are required for operator DNA binding but which are not necessary for nonspecific DNA binding. The data indicate that these operator-specific determinants may reside in the core region of the repressor protein. It is only this region of the molecule whose conformation is sensitive to inducer binding, as demonstrated using ultraviolet difference spectra, chemical modification techniques, fluorescence spectral alterations, and kinetic measurements. Furthermore, chemical reactions with residues located only in the core region result in selective loss of operator DNA binding capacity. The fact that the typical

Figure 1: Time course for the interaction of inducer with the core protein. The buffer was 0.1 M Tris-HCl, with 1.0 M NaCl and 3.0×10^{-4} M dithiothreitol, adjusted to pH 8.0, at 25° C, and the rate was measured as described in the text. Excitation was at 290 nm, using a 75 W xenon lamp and a slit-width of 1 mm. A glass cut-off filter was employed so that fluorescence emission at wavelengths longer than 350 nm could be recorded. Concentrations given are before mixing. A solution of core protein (1.3×10^{-6} M) was mixed with a solution of IPTG (0.5×10^{-3} M). A time constant of 0.1 ms was used. Marker corresponds to 20 ms.

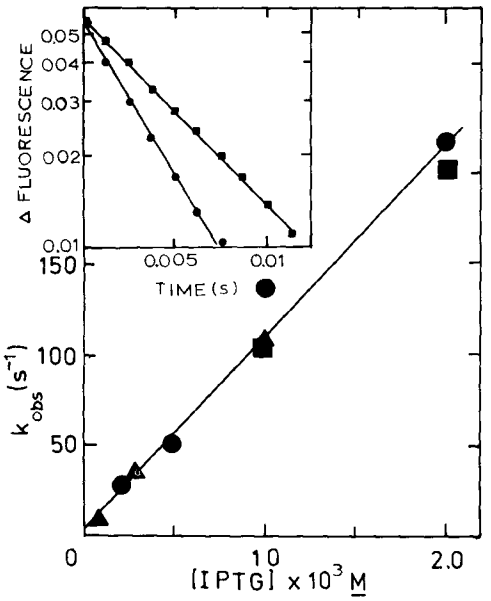


Figure 2: Determination of association rate for inducer binding to repressor, repressor-poly[d(A-T)] and core protein. The second order rate constants for the binding of IPTG to the proteins were derived from a plot of the observed pseudo first-order rate constants versus the concentration of IPTG, using data such as that shown in Figure 1. Inset shows sample logarithmic plots of fluorescence change versus time used to determine the pseudo first-order rate constants for the core protein. -■-, Repressor protein (1.3 x 10^{-6} M); -▲-, Repressor (2.3 x 10^{-6} M) in the presence of 5 x 10^{-4} M poly [d(A-T)]; -●-, Core protein (1.3 x 10^{-6} M). All concentrations are before mixing. The second order rate constants were determined by a least squares fit of the respective lines: Repressor, 9.8 x 10^4 $M^{-1}s^{-1}$; Repressor plus poly[d(A-T)], 10.1 x 10^4 $M^{-1}s^{-1}$; Core protein, 10.9 x 10^4 $M^{-1}s^{-1}$. The buffers used were: 0.1 M Tris-HCl, 1 M NaCl, pH 8.0, 3 x 10^{-4} M dithiothreitol for repressor and core protein. Lower ionic strength was required for the DNA experiment: 0.1 M Tris-HCl, 0.02 M NaCl, pH 7.5, 3 x 10^{-4} M dithiothreitol. The rate for repressor at the lower ionic strength was measured to be 9.6 x 10^4 $M^{-1}s^{-1}$.

ultraviolet difference spectrum observed on inducer binding to core is identical to that found for repressor indicates that removal of the NH_2-termini does not alter the spectral changes accompanying the conformational isomerization on inducer binding. From a thermodynamic point of view, the observation that the affinity (1) and the rate of inducer binding are unchanged by removal of the NH_2-terminal region or the presence of nonspecific DNA suggests that the conformational alteration in the repressor protein structure in response to inducer binding is not translated into the NH_2-terminal region of the protein

to any significant extent. If the NH_2-termini underwent major conformational changes in response to inducer binding, the removal of these fragments or the presence of nonspecific DNA would be expected to modify the free energy state of the molecule; this alteration would be expressed as a difference in the equilibrium association constant and/or the second-order rate constant for inducer binding. However, no change in these parameters was observed experimentally. If there is not a major effect of inducer binding on the state of the NH_2-termini, then the interaction between these terminal regions of the protein and the DNA cannot account for the lowered affinity of protein binding to the operator DNA in the presence of inducer. Since this region is implicated in DNA binding, the role which the NH_2-terminus serves may be a general nonspecific binding, which is required for binding operator DNA, but is not sufficient for the specific interaction. The data suggest that the factors critical for the specific association with the operator DNA are located in the core region. Consistent with this hypothesis, the conformational change associated with inducer binding and decrease in operator DNA affinity is primarily limited to the core region of the repressor molecule. While it is not possible at this juncture to assess potential contributions of the NH_2-terminal region to operator selective binding, there is significant indirect evidence to favor the view that operator binding specificity is a property of the core protein. This interesting possibility is currently being further explored.

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