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THE DNA BINDING PARAMETERS OF HYBRID TETRAMERS OF LACTOSE REPRESSOR AND THE TRYSIN-RESISTANT CORE PROTEIN: A MODEL FOR REPRESSOR-OPERATOR INTERACTION

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by

MARIETTA DUNAWAY

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

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HOUSTON, TEXAS

APRIL, 1980
For Steve,

Wanda,

Stewart, in memorium
The DNA Binding Parameters of Hybrid Tetramers of Lactose Repressor and the Trypsin-Resistant Core Protein: A Model for Repressor-Operator Interaction

Marietta Dunaway

The nonspecific DNA binding capacity of the lac repressor protein has been assessed by two different methods. Boundary sedimentation of repressor and calf thymus DNA fragmented by shearing yielded dissociation constants in good agreement with values previously reported in the literature. The binding activity was also assayed by nitrocellulose ultrafiltration employing labelled λplac DNA in the presence of inducer concentrations sufficient to insure dissociation of repressor from the operator region of the DNA. This method gave values comparable with those obtained by the more rigorous boundary sedimentation method and is in addition a fast, convenient method which allows determination of a dissociation constant using only ~0.2 mg of protein.

Kinetic studies of inducer binding to the repressor-operator complex demonstrate that the inducer association rate is decreased from 4.6 x 10^{4} \text{ M}^{-1}\text{s}^{-1} to 1.0 x 10^{4} \text{ M}^{-1}\text{s}^{-1} by the presence of saturating amounts of operator DNA fragments. The inducer dissociation rate was measured by dilution of repressor-operator-inducer complexes and by displacement of the sugar molecules from repressor by the binding of operator DNA, yielding a value of 0.8 \text{s}^{-1}. These kinetic results suggest that repressor should exhibit a 20-fold lower affinity for inducer when operator DNA fragments are bound; this prediction is in agreement with previous equilibrium measurements.
Determination of the apparent association rate for inducer binding to repressor in the presence of varying concentrations of operator DNA fragments indicates that DNA molecules dissociate from the protein-operator-inducer complex at intermediate stages during the sugar binding reaction. The exact point at which dissociation occurs is a complex function of the concentration of operator and inducer and the relationship between the rates for sugar binding and for operator fragment dissociation.

Hybrid tetramers of lac repressor and its trypsin-resistant core protein were isolated by chromatography on phosphocellulose. The purity of each tetramer was demonstrated by physical characterization and measurement of the binding activities. The affinity of each hybrid tetramer for λlac DNA and a 29 base pair operator DNA fragment was determined. The affinities of the hybrids for operator DNA indicate that two NH₂-termini supply major operator contacts while the remaining two contribute only minor contacts. Competition studies with nonspecific DNA indicate that the repressor has two operator binding sites and that the contacts of a given NH₂-terminus are not identical for both operator binding sites. In contrast, the protein appears to bind to nonspecific DNA with four independent, non-interacting sites under the conditions used for assay.

Although the rate of dissociation of the repressor-operator complex is increased by a factor of two upon removal of each NH₂-terminus, calculations using the measured dissociation rate and equilibrium dissociation constant indicate that the rate of association of repressor to operator is decreased 5-fold. These data suggest that
the NH₂-terminus is a key feature in the rapid association rate of repressor to operator DNA.

A model is presented for the structure of the lactose repressor protein and for its interaction with inducer, operator DNA, and nonspecific DNA. The proposed structure is based on experimental evidence from this laboratory and from the literature. The features of this model include: 1) plane rectangular arrangement of subunits (dihedral symmetry); 2) two operator DNA binding sites; 3) four inducer binding sites; 4) contact of the NH₂-terminal regions with the operator DNA sequence involving largely ionic forces; 5) asymmetrical interaction of the core region with operator DNA utilizing primarily nonionic forces; 6) perturbation of the core-operator contacts on inducer binding with little or no disruption of NH₂-terminal regions; 7) significant flexibility and independent structure in the NH₂-terminal regions.
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TABLE OF CONTENTS

Chapter I. Introduction
   The Lactose Operon
   The \textit{Lac} Repressor Protein

Chapter II. Assays for the Nonspecific DNA Binding
   Activity of the \textit{Lac} Repressor
   Introduction
   Methods and Materials
   Results and Discussion

Chapter III. Kinetic Studies of Inducer Binding to
   \textit{Lac} Repressor-Operator Complex
   Introduction
   Materials and Methods
   Results and Discussion

Chapter IV. DNA Binding Parameters of Hybrid Tetramers
   of Native and Core \textit{Lac} Repressor Protein
   Introduction
   Hybrid Tetramers - Materials and Methods
   Results
   Discussion

Chapter V. A Model for the \textit{Lac} Repressor Protein and
   its Interaction with Ligands
   The Model
   Evidence for the Model

Chapter VI. Conclusion - Implications of the Model
LIST OF TABLES

I. Association Constants for Sugar Ligands with Repressor 25
II. Dissociation Constants for Repressor-Ligand Interactions 29
III. Dissociation Constants for Repressor and Nonspecific DNA Determined by Boundary Sedimentation Using $^{14}$C-IPTG 39
IV. Dissociation Constants for lac Repressor and Nonspecific DNA Determined by Boundary Sedimentation and Ultrafiltration 48
V. Summary of Kinetic and Equilibrium Constants 68
VI. Dns-Cl End Group Analysis of Hybrid Tetramers 96
VII. Binding Parameters for Hybrid Tetramers and Operator DNA 104
VIII. Kinetic Constants for Hybrid Tetramers and Operator 108
IX. Binding Parameters for Hybrid Tetramers and Nonspecific DNA 115
X. Some Genetic Regulatory Proteins and Their Characteristics 157
LIST OF FIGURES

1. Regulation of expression of the lactose operon 5
2. The amino acid sequence of the Lac repressor 9
3. Missense and nonsense mutations 13
4. Operator DNA sequence 20
5. Sedimentation pattern for repressor-calf thymus DNA complex 37
6. Modified Scatchard plots for repressor-DNA interaction 41
7. Determination of the site size for repressor binding to DNA 43
8. Salt dependence of nonspecific DNA binding using nitrocellulose filtration 46
9. Fluorescence emission spectra of Lac repressor 56
10. Binding of inducer to repressor-operator complex 58
11. Determination of the second order rate constant for binding of inducer to repressor and repressor-operator complex 60
12. Dissociation of inducer from repressor and repressor-operator DNA fragment complex by rapid dilution 63
13. Dissociation of inducer from repressor after binding to operator 66
14. Dissociation of operator DNA fragment from repressor 70
15. Observed rates for the binding of inducer to repressor-operator complex as a function of operator concentration 72
16. Monod-Wyman-Changeux scheme for repressor 80
17. Separation of hybrid tetramers on phosphocellulose 90
18. Electrophoresis of hybrid tetramers 93
19. Operator binding activity of hybrid tetramers under stiochiometric conditions

20. Titration of λpac DNA with hybrid tetramers

21. Titration of operator fragment with hybrid tetramers

22. Determination of dissociation rates of hybrid tetramer-operator DNA complexes

23. Saturation curves for binding of hybrid tetramers to nonspecific DNA

24. Scatchard treatment of hybrid tetramer-nonspecific DNA binding data

25. Competition curves for hybrid tetramer binding to operator DNA in the presence of increasing quantities of poly-[d(A-T)]

26. Lac repressor protein bound to operator DNA

27. Lac repressor protein free and bound to inducer

28. Operator DNA sequence

29. Repressor protein in the presence of operator DNA and inducer

30. Symmetry diagram of possible operon control systems involving a regulatory protein and a controlling metabolite
CHAPTER I

Introduction to the Lactose Operon and Its Repressor Protein
Genetic regulation has stirred the curiosity and imagination of scientists for some time, but only recently have the mechanisms of these regulatory processes begun to be understood. The first integrated hypothesis on control of a particular genetic system was advanced by Jacob and Monod (1961 a) to explain their studies on mutants of lactose metabolism. Their theory proposed a regulatory unit named the operon, and one of the details of this model was verified by Gilbert and Müller-Hill (1966) with the isolation of the lac repressor protein. Subsequently, the investigation of genetic control proteins in other prokaryotic and, recently, eukaryotic systems has led to the general understanding of the role of protein-DNA interactions in control of transcription. The control of mechanism for the synthesis of enzymes of both anabolic and catabolic pathways have been elucidated in prokaryotes, and recent technological innovations are making the understanding of eukaryotic organisms a hopeful prospect. However, the physical chemistry of protein-DNA interaction is still poorly understood.

**THE LACTOSE OPERON**

*Escherichia coli* mutants whose lactose metabolism is altered were studied by Monod as early as the late 1940's. Although other "adaptive enzymes" were being studied at the time, the lac system was chosen because of the relative ease of preparing chemical derivatives of the sugar lactose. These derivatives were tested for their ability to induce β-galactosidase activity, affinity for the enzyme, and suit-
ability as substrates of β-galactosidase. Four classes of sugars were found (Monod and Cohn, 1952): 1) poor non-metabolizable competitive inhibitors which were excellent inducers (isopropyl-β, D-thiogalactoside) 2) excellent substrates which were not necessarily inducers (o-nitrophenyl-β, D-galactoside) 3) good non-metabolizable competitive inhibitors that were not inducers (phenyl-β, D-thiogalactoside) 4) noncompetitive inhibitors which were excellent inducers (melibiose).

These studies established an I gene which was distinct from the Z (β-galactosidase) gene. By this kind of analysis coupled with mutant studies and genetic experiments using merodiploids (Pardee et al., 1959; Jacob and Monod, 1961a), Jacob and Monod postulated the operon model (1961a). This model of negative control called for a repressor which bound to an operator region, thereby restricting synthesis of the messenger RNAs for the enzymes required for utilization of β-galactosides. Under conditions of repression the levels of enzyme in the cell were very low, while in the presence of a metabolite (inducer) the enzyme levels increased a thousand-fold. This process was termed induction, and the inducer presumably interacted with the repressor causing a conformational change and a reduced affinity of the repressor for the operator region. Experiments with inhibitors of protein synthesis led Jacob and Monod to the conclusion that the repressor was likely not a protein when the operon model was first proposed; however, the isolation and purification of the repressor protein (Gilbert and Müller-Hill, 1966; Riggs and Bourgeois, 1968) and subsequent in vitro studies demonstrating its functions (Riggs et al., 1968) established the protein nature of the repressor. A schematic of this model as it is currently understood is
presented in Figure 1.

The repressor protein binds to a specific region of the genome, the operator, preventing synthesis of the mRNA coding for the structural genes β-galactosidase, lactose permease, and thiogalactoside transacetylase. Some sugar ligands, inducers, reduce the affinity of the repressor for the operator, allowing the remainder of the genome to compete effectively for repressor binding. In the absence of the repressor, RNA polymerase binds to the promoter and initiates synthesis of the mRNA coding for the enzymes required for lactose metabolism. The natural inducer for the system is 1,6-allolactose, a by-product of β-galactosidase action on lactose (Jobe and Bourgeois, 1973). When lactose is depleted or withdrawn from the cells, the internal levels of inducer decrease, resulting in dissociation of the sugar from the repressor. The repressor assumes the conformation having high affinity for operator, and binds to the operator site to terminate synthesis of the structural genes. It is important to recognize that the repressor-operator binding is dynamic, so that even in the absence of inducer the repressor dissociates from the operator for discrete units of time. This dissociation allows synthesis of the low levels of β-galactosidase and lactose permease required for the initial induction steps.

In addition to this negative control, the lac operon is also subject to catabolite repression (Monod, 1947; Magasanik, 1962). Catabolite repression is a conservation measure employed by the cell to insure that glucose will be used preferentially as the carbon source in the presence of other sugars which require inducible enzyme systems for utilization. Catabolite repression is modulated by cyclic adenosine
Figure 1. Regulation of the expression of the lactose operon. 

(p,o) contains the promoter (p) or RNA polymerase binding and initiation site and the operator (o) region. The structural genes are (z), β-galactosidase; (y), lactose permease; (a), thio-galactoside trans-acetylase. (i) is the structural gene for the lac repressor protein and (p_1), the promoter for the lac i gene. I is an inducer; (○) or (□) symbolize conformational states of the repressor. The drawing is not to scale.
monophosphate (cAMP) levels and their effect on the affinity of the catabolite activator protein (CAP). In the presence of glucose, cAMP levels decrease (Makman and Sutherland, 1965), and the catabolite activator protein does not bind to the lac promoter region. As glucose levels drop, cAMP concentrations rise, and this ligand binds to CAP. This complex has a higher affinity for the promoter, and it binds to the promoter, increasing transcription. The role of cyclic AMP in regulation was demonstrated by the relief of catabolite repression on addition of exogenous cAMP (Perlman and Pastan, 1968; Ullman and Monod, 1968). Thus, full induction of the lac enzymes requires both inducer binding to repressor, removing it from the operator, and cAMP binding to CAP and subsequent binding of this protein to the promoter (de Crombrugghe et al., 1971). Sequencing work has shown that the CAP binding site is upstream on the DNA from the RNA polymerase site (Dickson et al., 1975; Reznikoff and Abelson, 1978); the mechanism of CAP-RNA polymerase-DNA interaction is not well understood.

The lac repressor protein remains one of the few stable repressors which have been isolated, and it is also available in sufficient quantities for detailed physical studies. Consequently, the lac repressor has been actively investigated. While there is indeed an impressive library of data on this protein, the mechanism of its binding to DNA is still not well understood and continues to be the subject of further study.
THE LAC REPRESSOR PROTEIN

Structural Features. The structure of thelac repressor protein has been probed through genetic, physical and chemical methods. The repressor is a tetrameric protein with a molecular weight of 150,000 daltons, consisting of four identical subunits (Müller-Hill, 1971). The amino acid sequence of the protein is not unusual (Beyreuther, 1975, 1978; Farabaugh, 1978) (Figure 2), and the protein has no detectable RNA or DNA components. The protein exhibits an isoelectric point of 5.6. Powder X-ray studies and electron microscopy on microcrystals of the protein indicate that the repressor is an elongated molecule (140 x 60 x 45 Å ) with three 2-fold axes of symmetry (Steitz et al, 1974). However, electron microscopy on the native protein employing negative staining (Ohshima et al, 1975; Barkley and Bourgeois, 1976) show a U-shaped tetramer composed of parallel bundles. Dimensions of the protein from these studies are approximately 90 x 100 x 90 Å . There appears to be one operator binding site (Müller-Hill, 1971; Riggs et al, 1968) and a maximum of four inducer/anti-inducer binding sites per tetramer (Müller-Hill, 1971; Ohshima et al, 1974; Friedman et al, 1977).

Initial genetic analysis of the lac I gene classified repressor mutants according to their in vivo phenotype. Although more sophisticated methods are now used to analyze mutants, the original designations have been retained. Mutants which manifest constitutive lac phenotypes fall into two major classes: operator constitutive (o^c) and transdominant (i^-d) mutants. The o^c mutants have mutations
Figure 2. The amino acid sequence of the lac repressor protein (Beyreuther, 1978).
Met-Lys-Pro-Val-Thr-Leu-Tyr-Asp-Val-Ala-Glu-Tyr-Ala-Gly-Val-
10
Ser-Tyr-Gln-Thr-Val-Ser-Arg-Val-Val-Asn-Gln-Ala-Ser-His-Val-
20
Ser-Ala-Lys-Thr-Arg-Glu-Lys-Val-Glu-Ala-Ala-Met-Ala-Glu-Leu-
40
Asn-Tyr-Ile-Pro-Asn-Arg-Val-Ala-Gln-Gln-Leu-Ala-Gly-Lys-Gln-
50
Ser-Leu-Leu-Ile-Gly-Val-Ala-Thr-Ser-Ser-Leu-Ala-Leu-His-Ala-
70
Pro-Ser-Gln-Ile-Val-Ala-Ala-Ile-Lys-Ser-Arg-Ala-Asp-Gln-Leu-
80
Gly-Ala-Ser-Val-Val-Val-Ser-Met-Val-Glu-Arg-Ser-Gly-Val-Glu-
100
Ala-Cys-Lys-Ala-Ala-Val-His-Asn-Leu-Ala-Gln-Arg-Val-Ser-
110
Gly-Leu-Ile-Ile-Asn-Tyr-Pro-Leu-Asp-Asp-Gln-Asp-Ala-Ile-Ala-
120
Val-Glu-Ala-Ala-Cys-Thr-Asn-Val-Pro-Ala-Leu-Phe-Leu-Asp-Val-
130
Ser-Asp-Gln-Thr-Pro-Ile-Asn-Ser-Ile-Ile-Phe-Ser-His-Glu-Asp-
140
Gly-Thr-Arg-Leu-Gly-Val-His-Leu-Val-Ala-Leu-Gly-His-Gln-
150
Gln-Ile-Ala-Leu-Leu-Ala-Gly-Pro-Leu-Ser-Ser-Val-Ser-Ala-Arg-
160
Leu-Arg-Leu-Ala-Gly-Trp-His-Lys-Tyr-Leu-Thr-Arg-Asn-Gln-Ile-
170
Gln-Pro-Ile-Ala-Glu-Arg-Glu-Gly-Asp-Trp-Ser-Ala-Met-Ser-Gly-
180
Phe-Gln-Gln-Thr-Met-Gln Met-Leu-Asn-Glu-Gly-Ile-Val-Pro-Thr-
200
Gln-Pro-Ile-Ala-Glu-Arg-Glu-Gly-Asp-Trp-Ser-Ala-Met-Ser-Gly-
210
Phe-Gln-Gln-Thr-Met-Gln Met-Leu-Asn-Glu-Gly-Ile-Val-Pro-Thr-
220
Val-Glu-Ala-Ala-Cys-Thr-Asn-Val-Pro-Ala-Leu-Phe-Leu-Asp-Val-
240
Ala-Met-Leu-Val-Ala-Asn-Asp-Gln-Met-Ala-Leu-Gly-Ala-Met-Arg-
250
 Ala-Ile-Thr-Glu-Ser-Gly-Leu-Arg-Val-Gly-Ala-Asp-Ile-Ser-Val-
260
Val-Gly-Tyr-Asp-Asp-Trp-Glu-Ser-Asp-Ser-Cys-Tyr-Ile-Pro-Pro-
280
Leu-Thr-Ile-Lys-Gln-Asp-Phe-Arg-Leu-Gly-Gln-Thr-Ser-
290
Val-Asp-Arg-Leu-Gln-Leu-Ser-Gln-Gly-Gln-Ala-Val-Lys-Gly-
300
Asn-Gln-Leu-Leu-Pro-Val-Ser-Leu-Val-Lys-Arg-Lys-Thr-Leu-
310
Asn-Gln-Leu-Leu-Pro-Val-Ser-Leu-Val-Lys-Arg-Lys-Thr-Leu-
320
Ala-Pro-Asn-Thr-Gln-Thr-Ala-Ser-Pro-A arg-Ala-Leu-Ala-Asp-Ser-
340
Leu-Met-Gln-Leu-Ala-Arg-Gln-Val-Ser-Arg-Leu-Glu-Ser-Gly-Gln
360
in the operator region itself and will be discussed in more detail later. The \textit{i}^{-d} \textit{mutants} are defective in operator binding, but have normal inducer binding activity. The \textit{i}^{-d} designation originated from the cis-trans test (Jacob and Monod, 1961 b) used to distinguish \textit{o}^{C} and \textit{i}^{-} mutants. The \textit{i}^{-d} symbol has been largely replaced by \textit{i}^{-}. \textit{Variants} which have defective inducer binding but have normal operator binding (\textit{i}^{S}) have a negative \textit{lac} phenotype. Temperature sensitive mutants are designated as \textit{i}^{ts}. Both \textit{i}^{-} and \textit{i}^{S} mutants have been shown to form stable tetramers. \textit{Early mapping studies} of these mutants showed that \textit{I}^{-} mutations mapped predominately in the left of the gene or amino-terminal region of the protein, while \textit{i}^{S} mutations mapped in the middle of the \textit{I} gene (Pfahl, 1972; Pfahl \textit{et al}, 1974; Miller-Hill \textit{et al}, 1975; Weber \textit{et al}, 1972 b; Bourgeois and Jobe, 1970; Jobe \textit{et al}, 1972).

Elegant fine structure mapping of the \textit{lac} \textit{I} gene has been accomplishd by Jeffrey Miller and his co-workers. These investigators have been able to correlate a large number of missense and nonsense mutations to the amino acid sequence of the protein. By deriving a fine structure deletion map containing approximately 100 intervals, or deletion groups, and using this in conjunction with the amino acid sequence and mutagens with known specificity, missense mutants have been correlated to the specific amino acid change, or very close to this change (Miller \textit{et al}, 1977; Schmeissner \textit{et al}, 1977; Miller and Schmeissner, 1979). In addition, nonsense mutants crossed into suppressor strains have been used to systematically examine the protein by substituting a variety of amino acids at the nonsense mutation sites (Coulondre and Miller, 1977; Miller \textit{et al}, 1979). These methods allow
the rapid analysis of any mutant of interest. A composite map containing both the nonsense and missense mutants which have been analyzed is presented in Figure 3. Examination of the map reveals several key features of the primary structure and its correspondence to protein function. The I\(^-\) mutations appear throughout the genome, but predominantly in the regions 1-60 and 120-310. There appear to be silent regions from amino acid 70 to 120 and in the carboxy-terminus from 310-360. In addition, two mutants which bind operator more tightly than wild-type repressor map at amino acid residues 3 and 61 (Schmitz et al, 1978). However, I\(^S\) mutations map only in the region 60-300; that is, those mutations which affect inducer binding are not observed in the amino terminal region at all. Miller (1979) has pointed out that these I\(^S\) mutations fall in clusters and has carefully tested these clusters by suppressing all possible nonsense mutations with a series of amino acids. The effect of suppression mutations in the remainder of the I gene on inducer binding has been measured in order to rule out any effects due to hotspots in the DNA. He has concluded that the amino acid residues at the sites of these mutation clusters in the sequence may form the turns of \(\beta\)-pleated sheets which face directly into the inducer binding site.

Gentle treatment with trypsin and other proteases removes the first 60 amino acids of the repressor yielding a trypsin resistant core protein (Platt et al, 1973; Files and Weber, 1976). The core protein has normal inducer binding activity but aberrant operator binding activity. Conditions have been established which leave the amino-terminal peptide (amino acids 1-51 or 1-59) intact and the peptides
Figure 3. The combined results from studies of missense mutations (lines A and C) and suppressed nonsense mutations (lines B and D) are shown for the entire gene protein map. The upper part of the diagram considers the $i^-$ phenotype, and the lower portion the $i^S$ phenotype. For lines A and C, bars pointed upwards indicate temperature-independent mutations, and bars pointed downward depict temperature-sensitive mutations. Open bars indicate weaker effects: weaker $i^{-}\text{ts}$ effects in line A, and weaker $i^S$ effects in line C. (The open bars in line A pointing upwards represent mutations that affect aggregation. These have the letter A inside the bar.) Parentheses indicate a slight ambiguity in positioning, where, in general, every bar is within 3 residues of the corresponding position in the protein. Although many temperature-sensitive mutations confer partial $i^S$ character at low temperature, only the mutants selected for this character are shown in line C. For lines B and D, each replacement produced by suppression is represented by a box. In line B, (☐) indicates replacements which do not produce the $i^-$ phenotype (loss of repression), (■) $i^S$ exchanges, and (□) partial $i^-$ proteins. A dot in the half-filled box indicates temperature sensitivity. In line D, (☐) represent exchanges that do not result in $i^S$ proteins, (■) $i^S$ proteins, and (□) weaker effects. A dot in a half-filled box represents a temperature-sensitive $i^S$ protein. The asterisks in the boxes at positions 3 and 61 depict proteins that bind operator more tightly than wild-type (figure and legend from Miller, 1979).
can be isolated, (Geisler and Weber, 1977). Both peptides have significant secondary structure (Jovin et al., 1977) and each binds to both λ and λplac DNA with equal affinity. Ogata and Gilbert (1979) have shown that the longer peptide exhibits operator specificity in studies of operator methylation protection/enhancement patterns in the presence of the peptide. Matthews (1979) has recently demonstrated that the core protein has operator-specific binding which is IPTG sensitive and cannot be attributed to repressor contamination. In addition, this core protein has no detectable nonspecific DNA affinity (>1 x 10⁻⁴ M).

Fanning (1975) has shown that iodination of repressor at low iodine excesses is confined to tyrosines 7, 12 and 17 with concomitant loss of operator activity. Nitration of tyrosines via the reagent tetranitromethane (TNM) at high excesses results in reaction of tyrosines both in the core and in the amino-terminus, while reaction at ten to fifteen-fold excess results in nitration of tyrosines 7 and 17 only (Alexander et al., 1977). Operator binding activity was lost at these low excesses implicating tyrosines 7 and 17 in DNA binding. However, more recent work using this reagent (Hsieh and Matthews, manuscript in preparation) has shown that loss of operator activity at these low excesses is due primarily to oxidation of cys 107. Nitration of repressor with cysteines protected by N-ethyl maleimide showed operator activity loss corresponding to nitration at tyr 7 and 17; nonspecific DNA binding was not affected as drastically as operator binding. Reduction of the nitro group to an amino group resulted in recovery of nonspecific binding to near control levels and significant recovery of operator activity.
The cysteine residues of repressor have been modified with a variety of reagents, including 2-chloromercuri-4-nitrophenol (MNP), 2-bromoacetamido-4-nitrophenol (BNP), N-ethylmaleimide (NEM), fluorescein mercuric acetate (FMA), and N-bromosuccinimide (NBS). Reaction with MNP, BNP, or NEM resulted in no loss in either DNA or inducer binding activity (Yang and Matthews, 1976; Yang et al, 1977; Brown and Matthews, 1979). Modification with FMA and mild oxidation with NBS affected operator activity exclusively (Burgum and Matthews, 1978; Manly and Matthews, 1979).

Repressor has also been modified with N-bromosuccinimide, which resulted in the oxidation of one of the two tryptophans per monomer (O'Gorman and Matthews, 1977a). Inducer protected the tryptophan from oxidation while anti-inducer did not, and oxidation did not affect inducer binding. Operator binding was lost at low levels of reagent and was subsequently correlated to oxidation of cys 107 and possibly 140 (Manly and Matthews, 1979). At levels of NBS at which operator binding was lost, nonspecific binding was retained; this was an early indication that the nonspecific and operator DNA binding sites are functionally separable.

The DNA Binding Activities of Repressor. The sequence specific, or operator, binding activity of the lac repressor is characterized by its extremely low dissociation constant, $10^{-13}$ M, and its sensitivity to inducer. Non-sequence specific, nonspecific, DNA binding is not affected by the conformational change elicited by inducer and is characterized by dissociation constants ranging from $10^{-6}$ to $10^{-9}$ M, depending on the base composition of the DNA and the salt concentration.
Because of these observations, inducer sensitivity has become the most critical indication of specific binding. In the induction process, inducer binds to the repressor, reducing its affinity for the operator from $10^{-13}$ M to $10^{-10}$ M (Barkley et al, 1975). Since nonspecific DNA is present in such excess over the operator sequence in the E. coli genome, this reduction in operator affinity is sufficient to allow the nonspecific DNA to compete effectively for the repressor, thereby removing the protein from the operator region. Thus, nonspecific DNA binding is not an insignificant or artifactual activity of the protein but is essential for the in vivo function of the repressor.

The repressor-operator interaction has been thoroughly studied by equilibrium and kinetic methods. The dissociation constants for the repressor-operator interaction have been measured using a variety of synthetic operators (Bahl et al, 1977; Goeddel et al, 1977). Operator fragments shorter than 16 base pairs have significantly reduced affinity; the 29 base pair operator fragment used in studies by O'Gorman et al (1980 a, b) has a 100-fold lower affinity for the repressor ($\approx 10^{-11}$ M) than λplac DNA used in the routine operator binding assay. These differences in affinity are due primarily to increases in the dissociation rate of the repressor-operator complex (Goeddel et al, 1977). The half-time of dissociation for the repressor-λplac complex is 40 minutes; in the presence of inducer this rate decreases to four minutes while anti-inducer increases the rate 2 to 8-fold (Riggs et al, 1970 b, c; Jobe and Bourgeois, 1973). The bi-molecular rate constant for the association of repressor to operator has been measured at $7 \times 10^9$ M$^{-1}$s$^{-1}$, about an order of magnitude greater than the value normally expected for a dif-
fusion controlled reaction between molecules of this size (Riggs et al. 1970 c). This rate has stimulated considerable speculation in the literature regarding the mechanism by which the protein recognizes and interacts with its target DNA sequence. Early models proposed the repressor making a one dimensional search down the DNA to the operator; however, the association rate of repressor to chemically synthesized operator 21 base pairs in length is not significantly altered by the length of fragment (Goeddel et al., 1977). von Hippel et al. (1975) has proposed a model in which the repressor is transferred between the strands of DNA; this model would require the existence of a second operator DNA binding site on the protein. Recent studies have demonstrated the existence of two such sites (O'Gorman et al., 1980 a, b; Chapter IV).

The lac operator is duplex DNA, and the size of the fragment protected from nuclease digestion by repressor is 27 base pairs (Gilbert and Maxam, 1973). The operator DNA segment unwinds slightly on binding to repressor (Wang et al., 1974). The extent of unwinding, \(\sim 90^\circ\), is insufficient to produce cruciform or other unusual DNA structures, but it is not known whether the unwinding is uniform over the entire stretch of the operator sequence or confined to produce kinks in the helix structure.

The operator sequence displays an impressive degree of symmetry; 16 of the 21 base pairs are symmetric about a 2-fold axis (Gilbert et al., 1975 a). This symmetry, coupled with the tetrameric structure of the repressor, led to the expectation of a highly symmetric repressor-operator interaction. However, the majority of operator con-
stitutive mutations lie on the promoter proximal side of the operator (Gilbert et al, 1975 b) (Figure 4). Furthermore, no highly constitutive mutations map on the right side of the operator. Base substitutions in chemically synthesized operators at symmetrical points in the outer regions of the sequence have equal effects on repressor affinity, but base substitutions in the symmetry equivalent base pairs in the central region result in dissimilar changes in the measured binding free energies (Goeddel et al, 1978). Repressor-operator contacts have been probed by DNA methylation and ethylation in the presence of protein (Gilbert et al, 1975 a; Ogata and Gilbert, 1979; von Hippel, 1979) and protein crosslinking with BrdUrd-substituted operator (Ogata and Gilbert, 1977). These studies have shown that there is a sidedness to the repressor-operator interaction, which is especially evident in the ethylation (von Hippel, 1979). Operator-protein crosslinking has demonstrated major groove contacts, while methylation studies have shown contacts in both major and minor grooves.

The nonspecific DNA binding parameters of the repressor have been examined under a variety of conditions using a potpourri of techniques. The apparent dissociation constant for repressor and nonspecific E. coli DNA is on the order of $10^{-7}$ M (Lin and Riggs, 1975 b). The dissociation constant for binding of repressor to nonspecific DNA varies in the range of $10^{-6}$ to $10^{-9}$ M as determined by competition using DNA of varying base composition with operator-containing λplac DNA at low ionic strength (Riggs et al, 1972; Lin and Riggs, 1970, 1971, 1972). The only exception studied was poly[d(A-T)] which was bound 3-fold more tightly than the other DNA's examined. Repressor-nonspecific DNA binding
Figure 4. Operator DNA sequence. The wild-type sequence of the operator is shown (Gilbert et al., 1975) and the positions numbered for reference in the text. The arrow indicates the central base pair of the symmetric region of the operator, and the bars show the symmetric sequences. The negative and positive signs near the bases indicate either protection (−) or enhancement (+) of methylation in the presence of repressor (Ogata and Gilbert, 1979). Operator constitutive mutations are indicated (Gilbert et al., 1975) as well as the thymines which can be cross-linked to the repressor protein (Ogata and Gilbert, 1977). Phosphate linkages which exhibit altered reactivity in the presence of inducer are indicated by asterisks between the base pairs (von Hippel, 1979).
5' - T G T G G A A T * T * C T G A G C A G * G A T A T A C A G A T T
A C A C C T T A A C A C T * C * G C C T A T T G * T * T A A - 5'

0°C Mutations

5' - A T G T T A C T
T A C A A T G A - 5'

Cross-linked bases

5' T T T
T T T T T T - 5'
is 5 to 10 times more sensitive to pH than binding to operator DNA; apparently, increasing the negative charge on the protein results in electrostatic repulsion from the negatively charged DNA phosphate backbone (Reuzin and von Hippel, 1977; de Haseth et al, 1977). Boundary sedimentation methods have been used to directly assess the binding of repressor to nonspecific DNA (Reuzin and von Hippel, 1977; Chapter II), this method has demonstrated significant salt dependence of repressor binding to calf thymus and λphage DNA. Elution of repressor from calf thymus DNA-cellulose columns at various ionic strengths has also been utilized to determine dissociation constants and the effects of ionic strength and ionic composition on the binding parameters (de Haseth et al, 1977). At constant ionic strength, the binding interaction is quite sensitive to Mg^{++} concentration, apparently due to the competition of this ion for binding to the phosphate backbone. Theoretical analysis of the ionic strength dependence of the repressor-DNA dissociation constant suggests involvement of approximately 12 ion pairs between nonspecific DNA and the repressor protein, and also suggests that two ionizable amino acids are protonated on DNA binding (Record et al, 1977). Synthetic DNA's containing different repeating sequences have been employed to determine the effects of such sequences on repressor binding. Poly[d(A-T)] binds more tightly than random sequences of DNA and has the second highest affinity of a nonspecific sequence for the repressor protein (Lin and Riggs, 1971). Although it has been suggested that the A-T rich character mimics the operator sequence, the binding is not inducer sensitive (Lin and Riggs, 1972). The repetitive sequence dTTG:dAAC provides the TGT sequence found in
the outer regions of the symmetric region of the operator; this synthetic DNA has the highest known nonspecific affinity of the repressor, but again the binding is not inducer sensitive (Riggs et al., 1972; Lin and Riggs, 1975).

The size of the nonspecific DNA region bound by repressor has been measured by boundary sedimentation techniques (Rezvin and von Hippel, 1977), by titration methods utilizing circular dichroism and light scattering (Butler et al., 1977; Maurizot et al., 1974), and by elution from calf thymus DNA-cellulose (de Haseth et al., 1977). The apparent site size measured by these methods is 12 to 15 base pairs, about half the value obtained for the operator site. However, electron microscopy of calf thymus DNA-repressor complexes has revealed that the protein occupies both sides of the DNA (Zingsheim et al., 1977). Thus, the actual site size must be 24-30 base pairs, approximately similar to the operator binding site value.

The effects of substituents in the major and minor grooves of the DNA helix on repressor-nonspecific DNA binding have been investigated. The substitution of the thymine 5'-methyl group with bromine increases the affinity of repressor for DNA; this result suggests the possibility of major groove contacts, although a general increase in affinity of DNA binding proteins for DNA's substituted with BrdUrd has been noted (Lin and Riggs, 1971). Blocking the minor groove with actinomycin D inhibits repressor-DNA binding, indicative of interactions between the protein and phosphate backbone (Lin and Riggs, 1975, 1976). Blocking the major groove of the poly[d(A-T)] analog by conversion of the thymine 5'-methyl group to a mercury mercaptan
(poly d(a-U-HgX)) has no effect on binding, nor does the presence of glucosylated 5'-hydroxymethyl cytosine residues of bacteriophage T DNA's (Richmond and Steitz, 1976). Thus, the major sites of interaction of nonspecific DNA appear to be the polyphosphate backbone and the minor groove of the helix.

**Inducer Binding to Repressor and the Mechanism of Induction.**

The inducer binding activity of the repressor has been studied in great detail. The protein has varying affinities for sugar ligands which can be classified according to their effect on the operator binding activity of the protein. Inducers lower the affinity of the protein for operator, anti-inducers increase the affinity of the protein for operator, and neutral sugars bind to the protein but have no effect on operator binding. The affinities of inducers and anti-inducers for repressor and their classification are presented in Table I. The gratuitous inducer IPTG has a high affinity for the repressor \( (2 \times 10^{-6} \text{ M}) \) and has been used in many studies of inducer binding. Varying the pH and salt concentrations has little effect on the repressor-inducer affinity, and the presence of \( \text{Mg}^{++} \) has no effect on inducer binding to free repressor (Friedman et al, 1977). Nonspecific DNA has no effect on the affinity of repressor for inducer. Inducer binding to free repressor is essentially noncooperative. Kinetic studies have shown that sugar binding is the rate limiting step in the transition from the uninduced to induced state (Friedman et al, 1976).

Operator DNA decreases the affinity of repressor for inducer. Barkley et al (1975) noted a 100-fold increase in dissociation constant using \( \lambda \)plac DNA, while only a 20-fold decrease was observed in the pre-
<table>
<thead>
<tr>
<th>Sugar</th>
<th>( K_A ) (M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inducers</strong></td>
<td></td>
</tr>
<tr>
<td>Methyl (\beta), D-galactoside</td>
<td>(1.0 \times 10^4)</td>
</tr>
<tr>
<td>Methyl (1)-thio-(\beta), D-galactoside</td>
<td>(1.1 \times 10^5)</td>
</tr>
<tr>
<td>(n)-Propyl (1)-thio-(\beta), D-galactoside</td>
<td>(1.3 \times 10^6)</td>
</tr>
<tr>
<td>Isopropyl (\beta), D-galactoside</td>
<td>(1.0 \times 10^4)</td>
</tr>
<tr>
<td>Isopropyl (1)-thio-(\beta), D-galactoside</td>
<td>(1.2 \times 10^6)</td>
</tr>
<tr>
<td>(n)-Butyl (\beta),D-galactoside</td>
<td>(1.1 \times 10^5)</td>
</tr>
<tr>
<td>Benzyl (\beta), D-galactoside</td>
<td>(1.0 \times 10^3)</td>
</tr>
<tr>
<td>2-Phenylethyl-(\beta), D-galactoside</td>
<td>(5.1 \times 10^3)</td>
</tr>
<tr>
<td>(o)-Nitrophenyl-(1)-thio-(\beta),D-galactoside</td>
<td>(4.3 \times 10^2)</td>
</tr>
<tr>
<td>D-Fucose</td>
<td>(2.2 \times 10^2)</td>
</tr>
<tr>
<td>Galactose</td>
<td>(1.0 \times 10^3)</td>
</tr>
<tr>
<td>Allolactose</td>
<td>(1.7 \times 10^6)</td>
</tr>
<tr>
<td><strong>Neutral</strong></td>
<td></td>
</tr>
<tr>
<td>(o)-Nitrophenyl-(\beta), D-galactoside</td>
<td>(9.2 \times 10^3)</td>
</tr>
<tr>
<td><strong>Anti-inducers</strong></td>
<td></td>
</tr>
<tr>
<td>Phenyl-(\beta), D-galactoside</td>
<td>(1.1 \times 10^3)</td>
</tr>
<tr>
<td>Phenyl-(1)-thio-(\beta), D-galactoside</td>
<td>(2.3 \times 10^2)</td>
</tr>
<tr>
<td>(o)-Nitrophenyl (\beta), D-fucoside</td>
<td>(7.1 \times 10^3)</td>
</tr>
<tr>
<td>(o)-Nitrophenyl-(1)-thio-(\beta), D-fucoside</td>
<td>(1.5 \times 10^2)</td>
</tr>
<tr>
<td>Glucose</td>
<td>(1.4 \times 10^1)</td>
</tr>
</tbody>
</table>
sence of a 29 base pair operator DNA fragment (O'Gorman et al., 1980 b). Inducer binding to repressor-operator fragment complex is a positive cooperative process.

Central to understanding the mechanism of induction in vivo is the elucidation of the conformational change occurring in the protein upon induction. This conformational change has been investigated by various methods including ultraviolet spectroscopy, fluorescence spectroscopy, circular dichroism, and sedimentation velocity centrifugation. Changes in the ultraviolet spectrum of repressor have been observed in the presence of inducers (Ohshima et al., 1972; Matthews et al., 1973), but not anti-inducers (Matthews et al., 1973). Solvent perturbation studies on repressor and on core protein indicate that inducers elicit a change in exposure of both tryptophan and tyrosine (Matthews et al., 1973; Matthews, 1974). The inducer difference spectrum for the core protein is identical to intact repressor protein, and the glycerol perturbation spectrum indicated that tyrosines in the amino-terminal region of the protein were exposed to solvent (Matthews, 1974).

It has been demonstrated that the repressor exhibits a shift in wave-length maximum of the fluorescence emission spectrum but no change in intensity of the signal upon binding inducer (Laiken et al., 1972), and that this change is due to alteration of the tryptophan environment. In addition the tryptophan fluorescence is quenched more effectively in the presence of inducer, but repressor exhibits the same shift in fluorescence on binding inducer in the presence of poly [d(A-T)] or free in solution. Oxidation of tryptophan 220 with N-
bromosuccinimide and subsequent quenching studies on the modified protein allowed assignment of the portions of the fluorescence spectrum to specific tryptophan residues (O'Gorman and Matthews, 1977). Other fluorescence studies using pulse fluorimetry (Brocham et al., 1977) and acrylamide quenching in mutants containing only one tryptophan residue (Bandyopadhyay and Wu, 1979) have resolved the spectra of the two tryptophans. Both studies led workers to conclude that trp 220 is affected by the inducer conformational change and that the two tryptophans contribute unequally to the fluorescence spectrum. Circular dichroism spectra have been recorded in several laboratories with disagreement over whether a change in the spectrum occurs on binding inducer (Laiken et al., 1972; Ohshima et al., 1972; Matthews et al., 1973).

The conformational change associated with induction has been successfully demonstrated by sedimentation studies (Ohshima et al., 1972; F. Kwok, personal communication), and the chemical reactivity of some amino acids in the protein is altered by induction. Addition of inducer caused decreased reactivity of core tyrosines toward tetranitromethane, but this alteration was not observed in the presence of anti-inducers (Alexander et al., 1977). The tyrosine which demonstrably changed its reactivity upon inducer binding was identified as 204, and the change in reactivity apparently reflects the conformational change. Treatment of repressor protein with N-bromosuccinimide resulted in oxidation of one of the two tryptophans in each monomer, but in the presence of inducer, tryptophan 220 was protected from oxidation (O'Gorman and Matthews, 1977 a). The studies on repressor containing oxidized tryptophan show that both tryptophan 201 and 220 are affected by the
conformational change accompanying induction.

When the studies reported in this thesis were begun, the putative nonspecific DNA and operator binding site consisted of the amino-terminal 60 amino acids. Ongoing research in the laboratory had focused on the conformational change and the inducer binding site. Since it was known that the inducer conformational change did not affect nonspecific DNA binding but had a dramatic effect on operator binding, studies probing the differences between the two DNA binding activities were begun. The first step in this process was the development of a nonspecific DNA binding assay which was not dependent on operator binding activity. By the time the details of this assay were established, chemical modification studies (O'Gorman and Matthews, 1977 a; Burgum and Matthews, 1978; M. Dunaway, unpublished results) had indicated that there were probably operator binding determinants in the core region of the protein. Soon after this Matthews (1979) showed that the core protein had an appreciable affinity for operator. These results indicated that a domain study of the protein using hybrid tetramers of intact repressor and trypsin-resistant core protein would be an effective probe of the relationship of the amino-terminal and core region in operator and nonspecific DNA binding. These studies provided the missing puzzle pieces which allowed us to fit most of the available library of data on the lac repressor into a cohesive model.
TABLE II

Dissociation Constants for Repressor-Ligand Interactions

<table>
<thead>
<tr>
<th>Interaction</th>
<th>$K_D$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repressor - IPTG</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>Repressor-Operator (λplac)</td>
<td>$10^{-13}$</td>
</tr>
<tr>
<td>Repressor-Operator (plac)-IPTG</td>
<td>$10^{-10}$</td>
</tr>
<tr>
<td>Repressor-DNA (E. coli)</td>
<td>$10^{-7}$</td>
</tr>
<tr>
<td>Repressor-DNA (E. coli)-IPTG</td>
<td>$10^{-7}$</td>
</tr>
<tr>
<td>Repressor-Poly[d(A-T)]</td>
<td>$10^{-9}$</td>
</tr>
</tbody>
</table>
CHAPTER II

Assays for the Nonspecific DNA Binding Activity of the Lac Repressor
To understand the DNA binding characteristics of the lac repressor protein, several key questions must be answered. The number of subunits which interact with operator and with nonspecific DNA must be determined along with the relationship of the nonspecific DNA and operator binding sites on the protein. One of the major problems in studies which would answer these questions is the assay of the nonspecific DNA binding activity. As discussed in Chapter I the term nonspecific binding should not be construed to mean low affinity or artifactual binding of the protein to DNA; the term nonspecific DNA binding means only that the binding is not sequence specific.

Both genetic analysis of mutant proteins and chemical modification of repressor can be used to test whether the nonspecific DNA binding site overlaps or coincides with the operator DNA binding site. However, it is first necessary to have an assay for nonspecific DNA binding which is not dependent on the ability of the protein to bind to operator DNA. The dependence on operator binding is a grave inadequacy of the competition assay used to probe the effect of base composition and sequence on nonspecific binding (Riggs et al., 1972; Lin and Riggs, 1970, 1971, 1972). The protein-DNA crosslinking assay using BrdUrd substituted DNA is not dependent on operator binding, but it is too laborious for routine use (Lin and Riggs, 1975 a). Thus, an ideal assay would be quick, yield quantitative results, use reasonably small quantities of protein, be reproducible, and be independent of operator activity.

Two different types of assays will be presented in this section. Neither has all the properties of the ideal assay, but
each has advantages for different uses. The development of these assays has allowed the members of this laboratory to examine the effects of different agents on the nonspecific DNA binding activity of the protein, and the assays have also been used in studies to be described in this thesis (Chapter IV).

METHODS AND MATERIALS

Isolation of Repressor. The lac repressor protein is isolated from a strain of E. Coli which carries a mutation in the promoter for the I gene. This mutation has been crossed onto a temperature sensitive lac-transducing prophage to increase the dosage of the I gene in the cell (Miller-Hill et al., 1975). Under optimal conditions repressor may constitute 7% of the cell protein. The cells are grown in a rich tryptone-yeast extract broth and when the optical density measured by light scattering at 650 nm is 1.0, the temperature of the media is raised to 45°C to induce phage production. The cells are harvested in a continuous flow centrifuge and frozen before use.

The repressor protein is purified by methods described in the literature (Miller-Hill et al., 1971; Platt et al., 1972), using the recent modifications reported by Rosenberg et al. (1977) which result in 100% operator active repressor. The cells are broken in 0.2 M Tris-Cl, pH 7.6, 0.2 M KCl, 0.01 M Mg acetate, 3 x 10^{-4} M dithiothreitol, 5% glucose and treated with DNase to allow the release and recovery of the repressor protein. The homogenate is centrifuged to remove debris, and ammonium sulfate is added to the supernatant to 33% saturation to pre-
cipitate the protein. After centrifugation, the pellet is redissolved in 0.12 M potassium phosphate, pH 7.5, 5% glucose, 3 \times 10^{-4} M dithiothreitol; the column is washed until the A_{280} is less than 0.20. A salt gradient from 0.12 to 0.24 M potassium phosphate, 5% glucose, 3 \times 10^{-4} M dithiothreitol is started, and fractions are collected. The protein peak is assayed for inducer activity, and this value is compared to the concentration of protein determined by Lowry assay. The purity of the protein preparation is further assessed by electrophoresis on sodium dodecyl sulfate gels (Weber et al., 1972a). The repressor prepared by this method is judged to be >95% pure, and impurities in the preparation do not have detectable operator binding activity.

Assay of Repressor. Repressor was routinely assayed for inducer activity by the ammonium sulfate precipitation method using \[^{14}C\]-IPTG as described by Bourgeois (1970). Operator assays were performed on nitrocellulose filters using \lambda plac DNA isolated from the prophage carrying strain MBC 5 (obtained from Mary Barkley, University of Kentucky). Cells were grown in defined medium, and \[^{3}H\]-thymidine was added to the medium at induction to label the DNA. The cells were lysed and cell debris was cleared from the solution by centrifugation. This supernatant is further centrifuged at 12,000 rpm for 90 minutes, and the phage-containing pellet is resuspended in 0.01 M Tris-Cl, 0.01 M MgSO\textsubscript{4}, pH 7.4. The suspension is put on a cesium chloride density gradient and spun at 40,000 rpm for 24 to 40 hours. The visible phage bands are collected by an Auto Densi-flow gradient collection device (Buchler), and the major, operator-
containing phage band located and pooled. The phage are deproteinized by extraction with phenol followed by extensive dialysis to remove the phenol.

**Determination of Nonspecific DNA Binding Activity by Boundary Sedimentation.** Calf thymus or salmon sperm DNA (Sigma) or poly[d(A-T)] (Sigma) was prepared by first dissolving in 0.2 M sodium acetate, pH 8.0 followed by fragmentation via hydrodynamic shearing. This process was executed by pushing the solution through a syringe fitted with a 16 gauge needle 10 times using maximum thumb pressure. Fragments were precipitated by addition of ethanol and redissolved in 0.01 M Tris-Cl, pH 7.5, 3 x 10^{-4} M dithiothreitol with varying concentrations of NaCl. This buffer was used in the assay, and DNA and repressor were added in appropriate amounts. The uniformity of the size of the DNA (M_r = 15 x 10^6 daltons) allowed direct determination of the bound protein concentration. Repressor and fragmented DNA were mixed homogeneously throughout a 5 to 20% glycerol gradient and subsequently sedimented. The gradients were spun for approximately 1 to 2 hours at 45,000 rpm in a Beckman SW 50.1 swinging bucket rotor. At the end of the run the centrifuge was allowed to stop without the brake and the gradients were fractionated via an Auto Densi-flow gradient collection device (Buchler).

Two protein detection methods were used. In early experiments, \(^{14}\text{C}\) IPTG was included in the gradient, and the gradients were fractionated directly into scintillation vials. Scintillation fluid was added to the vials, and the samples were counted. The resulting counts per minute were plotted versus fraction number. Alternatively, the fractions were deposited into test tubes and buffer added to a volume
of 1 ml. The relative fluorescence of these samples was measured in a SLM Instruments Series 400 spectrofluorometer and plotted against the fraction number.

**Determination of Nonspecific DNA Binding Activity by Ultra-Filtration.** The nonspecific DNA binding activity was measured using a variation of the operator assay. Samples containing DNA (λplac or λ) labelled with [3H]-thymidine and increasing concentrations of repressor were prepared. IPTG (10⁻⁴ M) was included in the assay mixture with λplac DNA to assure dissociation of the repressor from the operator region. The buffer utilized for the assay contained 0.01 M Tris-Cl, 10⁻⁴ M dithiothreitol, 5% glycerol, pH 7.5, and varying concentrations of NaCl. The mixtures (0.5 ml total volume) were incubated for 30 minutes and then filtered through 25 mm nitrocellulose filters at a constant rate (0.05 ml/s). The filters were prewashed with 0.5 ml of buffer and rinsed following sample filtration with 0.25 ml buffer. The filters were dried for 30 minutes at 45°C and immersed in scintillation fluid for determination of adsorbed radioactivity. All protein concentrations were filtered in duplicate or triplicate. At the lowest salt concentration employed, the dissociation constant for repressor-inducer/operator is ~10⁻¹⁰ M; the dissociation constant for repressor/nonspecific DNA is ~10⁻⁷ (sites). The concentration of operator sites is 3 x 10⁻¹¹ M, versus 1 x 10⁻⁷ M (sites) for nonspecific sites. Thus, the binding of induced repressor to operator does not contribute significantly to the observed binding. The dissociation constants were determined by the repressor concentration at half-saturation minus the DNA concentration and by a Scatchard plot of the low saturation data as described by Bailey (1979).
RESULTS AND DISCUSSION

The Boundary Sedimentation Assay. A typical sedimentation profile for assays using the radioactive IPTG detection system is presented in Figure 5. The method used for determining the fractional saturation from each gradient is shown in the figure. Since the total amount of protein in the gradient is known, the concentrations of free and bound repressor can be determined. The fluorescence detection system described in Methods and Materials yields a curve of the same shape (Figure 5). However, the dissociation constants obtained from the two methods varied dramatically (Tables III and IV). The reason for these discrepancies is not clear; equilibrium studies on repressor-inducer binding indicate that nonspecific DNA does not affect the repressor-inducer dissociation constant (Friedman et al, 1977), and O'Gorman et al (1980 a) have shown there is no detectable DNA absorbance at 285 nm. Therefore, one would expect fractional saturation values obtained using the radioactive detection system to be consistent with those obtained using other detection methods. The values obtained using the fluorescence detection system are comparable to those reported by Revzin and von Hippel (1977) using a similar assay.

Classical data treatment for protein-ligand binding is not applicable to protein-DNA interactions because of several inherent differences in the nature of this binding process which violate the assumptions fundamental to traditional methods. McGhee and von Hippel (1974) discuss the differences in the considerations of a macromolecule binding to a one-dimensional lattice versus small ligands binding to discrete protein binding sites. In protein-DNA binding it is necessary to
Figure 5. Sedimentation pattern for repressor-calf thymus DNA complex. A. A uniform mixture of repressor (3.5 x 10^-7 M) and calf thymus DNA (3.4 x 10^-6 M base pairs) was sedimented through a 5 to 20% glycerol gradient containing 0.075 M NaCl, 0.01 M Tris-Cl, 10^-4 M dithiothreitol, pH 7.5, at 4° C. The sample was centrifuged for 1.75 hours at 48,000 rpm; 11 drop fractions were collected directly into scintillation vials utilizing a Buchler Auto-Densi-flow with peristaltic pump. Scintillation fluid was added and the samples counted. The fractional saturation of repressor was determined as indicated in the figure. B. A uniform mixture of repressor (3.5 x 10^-7 M) and calf thymus DNA (3.4 x 10^-6 M base pairs) was sedimented through a 5 to 20% glycerol gradient containing 0.10 M NaCl, 0.01 M Tris-Cl, 10^-4 M dithiothreitol, pH 7.5, at 4° C. The sample was centrifuged for 1.75 hours at 48,000 rpm; 11 drop fractions were collected utilizing a Buchler Auto-Densi-flow with peristaltic pump and a drop counting fraction collector. These were diluted to 1 ml with 1 M NaCl, 0.1 M Tris-Cl, pH 7.5, 10^-4 M dithiothreitol. The fluorescence of the fractions was measured using an SLM Series 400 Spectrofluorometer with excitation at 285 nm and measuring emission at 345 nm. The concentration of repressor was determined using the fluorescence of the plateaus against a repressor standard curve.
A

CPM vs. FRACTION NUMBER

B

RELATIVE FLUORESCENCE vs. FRACTION NUMBER

[Rep]_{BOUND} and [Rep]_{FREE}
### TABLE III

Dissociation Constants for Repressor and Nonspecific DNA Determined by Boundary Sedimentation Using $[^{14}C]$-IPTG

<table>
<thead>
<tr>
<th>DNA</th>
<th>Salt Conditions</th>
<th>$K_D$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf thymus</td>
<td>0.075 M</td>
<td>1.6 x $10^{-4}$</td>
</tr>
<tr>
<td>Salmon sperm</td>
<td>0.075 M</td>
<td>1.0 x $10^{-4}$</td>
</tr>
<tr>
<td>Poly[d(A-T)]</td>
<td>0.075 M</td>
<td>2.4 x $10^{-5}$</td>
</tr>
</tbody>
</table>

The dissociation constants were determined graphically as described by McGhee and von Hippel (1974).
take into account that the DNA is a macromolecule itself, that the sites on the DNA are connected and not separate and distinct, that the sites are overlapping and there may be gaps on the DNA between two bound proteins in which another protein cannot bind. The difficulties encountered in dealing with this type of binding data can perhaps best be illustrated by considering the binding equation:

\[ K_{R\text{DNA}} = \frac{[R]\text{free} \cdot [\text{DNA}]\text{free}}{[R-D\text{NA}]} \]

How will the concentration of each component be expressed? Furthermore, how will the usual Scatchard quantities \( v \) and \( v/L \) be expressed? The McGhee and von Hippel model system deals with these problems by treating the protein as the ligand and the DNA as a uniform one-dimensional lattice. Therefore, \( v = R_B/\text{DNA}_T \); the binding data have been plotted using this method (Figure 6). The intercept on the ordinate is the dissociation constant and the intercept on the abscissa is the site size. Because of the conditions necessary for determination of the dissociation constant, the site size can only be estimated by this method.

**Determination of the Nonspecific DNA Site Size by Boundary Sedimentation.** A good estimate of the site size can be obtained by sedimenting increasingly high ratios of repressor to DNA in gradients and plotting the concentration of bound repressor versus the total repressor. The curve is extrapolated to the saturation point; this point corresponds to the number of sites (Figure 7). By dividing this number into the DNA concentration, the site size is obtained. This determination indicates that the site size is 11.5 base pairs, assuming that the protein binds to only one side of the DNA. Recent electron
Figure 6. Modified Scatchard Plots for Repressor-DNA Interaction. The fractions of repressor bound and free were calculated from sedimentation patterns similar to that shown in Figure 5. Using the modified Scatchard treatment (McGhee and von Hippel, 1974), the dissociation constants were calculated from the plots shown by fitting the data points with the theoretical curves shown. The theoretical curves were determined using the equation:

\[
\frac{\bar{v}}{L} = K(1 - n\bar{v}) \cdot \left( \frac{1 - n\bar{v}}{1 - (n - 1)\bar{v}} \right)^{n - 1}
\]

The \( K_a \) is given by the \( y \) intercept. -●-, Repressor (7.1 x \( 10^{-8} \) M to 3.6 x \( 10^{-6} \) M) was sedimented with calf thymus DNA (5 x \( 10^{-6} \) M) as described in Figure 5; the buffer was 0.13 M NaCl, 0.01 M Tris-HCl, pH 7.5, 10\(^{-4}\) M dithiothreitol, 20° C. -■-, Repressor (2.8 x \( 10^{-7} \) M to 6.4 x \( 10^{-6} \) M) was sedimented with calf thymus DNA (1.8 x \( 10^{-5} \) M); the buffer was 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5, 10\(^{-4}\) M dithiothreitol, 20° C.
Figure 7. Determination of the site size for repressor binding to DNA. Repressor (9.8 x 10^{-7} M to 4.9 x 10^{-6} M) was sedimented with calf thymus DNA (2 x 10^{-5} M base pairs) in 0.10 M NaCl, 0.01 M Tris-HCl, 10^{-4} M dithiothreitol, pH 7.5, 4° C, as described in Figure 5. The concentration of repressor bound was determined by the difference between the fluorescence of free repressor observed and the fluorescence if all the protein was free (as determined by sedimentation without DNA present). This value was then plotted versus the total repressor concentration. The site size calculated by this method was 11.5 base pairs of DNA per repressor tetramer.
microscopic data (Zingsheim et al., 1977) indicates that nonspecific binding occurs on both sides of the DNA. This would correspond to site size of 23 base pairs, similar to the size of the operator fragment protected from DNase by repressor.

The Ultrafiltration Assay. While the boundary sedimentation assay accurately measures the dissociation constant using rigorous data treatment, it has the disadvantages of being tedious and requiring rather large amounts of protein since six gradients are required to determine one dissociation constant. Because of the time necessary for preparing, centrifuging, fractionating and analyzing the gradients, the comparison of multiple samples in a day's time is impossible. This disadvantage is particularly acute in chemical modification experiments which require the comparison of samples modified under different conditions or to various extents and a control. These needs led to the development of an ultrafiltration assay using nitrocellulose filters. This method gives dissociation constants comparable to those in the literature and to those determined by the boundary sedimentation assay. Saturation curves at varying salt concentrations using this method are shown along with their respective Scatchard plots of the low-saturation binding data. The dissociation constants obtained using this method are listed in Table IV.

The data resulting from the saturation curves must be treated differently than the data from boundary sedimentation. In this assay the raw data are counts per minute from the DNA. Consequently, the data indicates the amount of DNA bound to protein and thus retained on the filter, but gives no direct information about the protein
Figure 8. Salt dependence of nonspecific DNA binding using nitrocellulose filtration. Repressor was mixed in increasing quantities with $[^3H]$-λplac DNA (8.9 x 10^{-8} \text{ M} \text{ sites}) in the presence of 10^{-4} \text{ M} \text{ IPTG} and filtered through nitrocellulose filters as described in Materials and Methods. The amount of DNA retained was determined by scintillation counting. A. – ● –, 0.05 \text{ M} \text{ NaCl}; – □ –, 0.10 \text{ M} \text{ NaCl}; – △ –, 0.13 \text{ M} \text{ NaCl}; – ○ –, 0.15 \text{ M} \text{ NaCl}. All buffers were pH 7.5, 0.01 \text{ M} \text{ Tris}-\text{HCl}. A, The binding curves indicated for each salt concentration were derived from multiple DNA and protein preparations and have been averaged and reported as per cent saturation to allow comparison. The dissociation constants were determined from the half-saturation values, and the averages of separate determinations were reported in Table IV. B. Scatchard-type plots for binding data in A at saturation values below 50% were generated according to Bailey (1979). In calculating $\bar{V}(=[R]_{\text{bound}}/[\text{DNA}]_{\text{total}})$, the per cent saturation was assumed to be equal to the fractional saturation with respect to repressor; the dissociation constants determined from these plots are reported in Table IV.
### TABLE IV

Dissociation Constants for *Lac* Repressor and Nonspecific DNA\(^a\) Determined by Boundary Sedimentation and Ultrafiltration

<table>
<thead>
<tr>
<th>Salt Conditions</th>
<th>Calf Thymus DNA(^b)</th>
<th>λDNA(^c)</th>
<th>([R]_{1/2} - 1/2[DNA])(^d)</th>
<th>Scatchard(^e) λplac DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 M NaCl</td>
<td>7.5 x 10(^{-8})</td>
<td>-</td>
<td>1 x 10(^{-7})</td>
<td>1 x 10(^{-7})</td>
</tr>
<tr>
<td>0.13 M NaCl</td>
<td>8.3 x 10(^{-8})</td>
<td>7.5 x 10(^{-8})</td>
<td>4 x 10(^{-7})</td>
<td>2 x 10(^{-7})</td>
</tr>
<tr>
<td>0.15 M NaCl</td>
<td>4.2 x 10(^{-7})</td>
<td>3.3 x 10(^{-7})</td>
<td>6 x 10(^{-7})</td>
<td>3 x 10(^{-7})</td>
</tr>
</tbody>
</table>

\(^{a}\) The K\(_d\)'s are given in molar concentration of sites.

\(^{b}\) The dissociation constants were calculated as described in the text (see Figures 5 and 6); the values were converted to M sites by dividing by 12.

\(^{c}\) The dissociation constants were calculated from the association constants reported by Revzin and von Hippel (1977); the values were converted to M sites by dividing by 12.

\(^{d}\) Half the concentration of DNA is subtracted from the half-saturation concentration of repressor protein determined as described in the text.

\(^{e}\) The dissociation constants were calculated from data at less than 50% saturation as described by Bailey (1979).
bound to that DNA. Bailey (1979) has suggested that the slope of a
Scatchard plot of the low saturation data (<35%) reflects the disso-
ciation constant for the protein-DNA interaction. This investigator
points out that at low saturation the probability of having only one
protein molecule bound per DNA is much greater than at higher satu-
ration. Unfortunately, accurate low saturation data is technically
difficult to obtain due to the scatter inherent in nitrocellulose
filter assays. We have used the data up to 50% saturation and found
little deviation from the dissociation constants determined from the
data under 35% saturation. It should also be noted that the DNA con-
centration is expressed in sites.

These considerations clearly show that the boundary sedi-
mentation method gives the most accurate and rigorous determination
of the dissociation constant. In addition it does not require radio-
active DNA so that any type of DNA may be used. The fluorescence de-
tection system is used exclusively in this laboratory. However, this
method is tedious and requires rather large amounts of protein, making
it unmanageable for routine use. Although the ultrafiltration assay
yields data that is ambiguous, it has the advantages of being quick,
requiring small amounts of protein, and resulting in reasonably accurate
dissociation constants. This assay is excellent for comparing the non-
specific DNA binding activity of multiple samples under identical con-
ditions (Chapter IV).
CHAPTER III

Kinetic Studies of Inducer Binding to

Lac Repressor-Operator Complex
The conformational change associated with inducer binding to the lac repressor is manifest in alterations of both the ultraviolet absorption spectrum and the fluorescence emission and excitation spectra of the protein (Ohshima et al., 1972; Laiken et al., 1972; Matthews, 1974; O'Gorman and Matthews, 1977 b); these spectral changes have been used to measure the association rates for sugar binding in stopped-flow rapid mixing experiments (Friedman et al., 1977; Friedman et al., 1976; Friedman and Matthews, 1978). The observed rates are identical for all the probes utilized. Apparently the second order interaction of inducer with repressor causes a rapid conformational change which is transmitted throughout the protein molecule. The removal of the NH$_2$-terminus by mild trypsin treatment to produce core protein does not affect the extent of the observed spectral changes, the rate of inducer binding, or the equilibrium dissociation constant. Thus, the second order binding of inducer appears to be the rate-limiting step in the sequence of events in which the protein assumes a new conformation with decreased affinity for operator DNA. It has also been demonstrated that no cooperativity is exhibited during the binding of the inducer IPTG to repressor protein and that the rates and equilibrium constants for this process are not affected by the presence of nonspecific DNA bound to the protein.

O'Gorman et al (1980) have studied both the interaction of the repressor protein with a 29 base pair operator fragment and the effect of the inducer IPTG on this interaction. Their results indicate that the presence of operator DNA fragments decreases the affinity of the protein for inducer, causing a 20-fold increase in the concentration of
inducer necessary for half-saturation of the protein. The non-linearity of the Scatchard-type plots and the slopes of the Hill plots of the binding data indicate that inducer binding exhibits cooperative behavior in the presence of operator DNA fragments. The Hill coefficient of the repressor-operator interaction with inducer is 1.45, compared to a coefficient of 1.02 for repressor interaction with inducer.

The experimental data from these studies were compared to simulated curves obtained using the theoretical models developed by Monod-Wyman-Changeux and Koshland (Monod et al, 1965; Koshland et al, 1966). Using reasonable parameters for the equilibrium constants, the experimental data was best fit by Monod-Wyman-Changeux and Koshland models with the following features: 1) Positive co-operativity in the binding of inducer to the repressor-operator complex; 2) two sites for the binding of the operator DNA fragment to repressor protein; 3) all four sub-units of the repressor affected by the binding of each operator DNA fragment.

In order to further characterize the binding of inducer to repressor-operator complexes and understand the mechanism of co-operativity, we have measured the rate of IPTG binding to the repressor protein in the presence of varying concentrations of operator DNA fragments. In the past, kinetic measurements of repressor-inducer binding in the presence of operator DNA were not possible due to the limiting amounts of operator DNA (λplac) available and the significant viscosity problems associated with concentrated DNA solutions. The successful synthesis and subsequent cloning of the operator sequence have allowed the preparation of large quantities of 29 base pair operator DNA fragments.
However, even with the operator DNA fragments, protein concentrations on the order of $1 \times 10^{-8}$ M are required for these experiments; this low concentration of protein renders monitoring rates in the millisecond range by fluorescence a challenging task. On the other hand, the concentrations of both repressor and operator DNA used in these experiments closely reflect the concentrations which have been calculated to be present in the cell, $2 \times 10^{-8}$ M and $2 \times 10^{-9}$ M, respectively (Kao-Huang et al, 1977). Thus, these kinetic studies potentially yield information regarding the mechanism of cooperative inducer binding to the repressor-operator DNA complex under physiological conditions.

**MATERIALS AND METHODS**

**Isolation and Assay of Repressor.** Repressor protein was isolated as described previously (Chapter II) and assayed for inducer binding by the techniques described by Bourgeois (1971) and for operator DNA binding by Riggs et al (1968). The dissociation rate of repressor from operator was measured by nitrocellulose filter assays (Riggs et al, 1970) with the following modifications: 1) The buffer used was 0.01 M Tris-HCl, pH 7.5, 0.05 M NaCl, 5% glycerol, and $3 \times 10^{-4}$ M dithiothreitol, the standard buffer used in these experiments; 2) Poly[d(A-T)] was added in excess instead of unlabelled operator fragment in order to conserve operator DNA.

**Isolation of Operator DNA Fragments.** The lac operator DNA fragments were isolated from plasmid pBR345 (Bolivar et al, 1977), a small (1,219 base pair) plasmid with two identical operator DNA fragments derived from chemically synthesized DNA originally cloned in
pMB9 (Lin et al., 1976). The central 21 base pairs of the operator fragments have the sequence of the natural lac operator and are bounded by Eco RI restriction site sequences. Thus, the operator DNA fragments can be excised from the plasmid by treatment with Eco RI endonuclease. Plasmid (pBR345, 500 mg) DNA was purified, cleaved with Eco RI endonuclease, and the lac operator fragment separated from linear plasmid by gel filtration (Kallai et al., 1980). Homogenous lac operator was obtained. Concentration of fragment was determined using $e^{260nm} = 20$ and a fragment molecular weight of 17,400.

**Kinetic Measurements.** The association or dissociation of inducer with repressor was monitored by the decrease or increase of the fluorescence emission of the tryptophans associated with the conformational change. All of the rapid-mixing experiments were carried out in a Gibson-Durrum (Dionex Model 110) stopped-flow spectrometer which is interfaced to a high speed, 12-bit A/D converter (OLIS, Athens, Georgia) and a Nova 2 minicomputer system. The data were collected as absolute voltage signals and then converted to fluorescence changes by the computer software. Typically 7 to 8 replicate time courses were measured and then averaged to improve signal to noise ratios. The resultant traces were stored as files on a floppy disk for future analyses. All the curves were then fitted to a single exponential expression using an iterative least squares program (Bevington, 1969) to obtain apparent first order rate constants. The rates were measured in 0.01 M Tris-HCl, pH 7.5, 0.05 M NaCl, 5% glycerol, and $3 \times 10^{-4}$ M dithiothreitol.
RESULTS AND DISCUSSION

Measurement of the Rate of Inducer Binding to Repressor and Repressor-Operator Complex. The fluorescence emission spectrum of repressor and repressor-inducer is shown in Figure 9. The fluorescence change which occurs on inducer binding is not affected by operator fragment binding and has been used to measure the rate of inducer binding to the repressor-operator complex. A sample time course for the binding of IPTG to native repressor bound to operator fragments is shown in Figure 10 along with the corresponding log plot of the data points. It should be noted that the experiments were performed at extremely low concentrations of repressor \(1 \times 10^{-8} \text{ M}\) in order to saturate the protein with operator; the amount of operator available was the limiting factor in these experiments. The second order rates for inducer binding were determined by plotting the observed pseudo first order rates versus the concentration of IPTG (Figure 11). The rate determined for unliganded repressor was \(4.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}\); this value is in good agreement with previously measured rate constants (Friedman et al, 1977). The rate for inducer binding to repressor-operator complex under conditions at which no dissociation of DNA occurs during the reaction (i.e., a 10-fold excess of operator DNA) was \(1.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}\).

Measurement of the Dissociation Rate of Inducer From Repressor and Repressor-Operator Complex. To measure the rate of dissociation of inducer from repressor and repressor-operator complex, protein partially saturated with IPTG was mixed with a 6.5-fold larger volume of buffer in the stopped-flow spectrometer. Dilution of the protein solution resulted in dissociation of the sugar molecules with a concomitant increase in
Figure 9. Fluorescence emission spectra of lac repressor (O’Gorman et al., 1980b). Concentration of repressor was $2.2 \times 10^{-8}$ M (tetramer) in 0.05 M NaCl, 0.01 M Tris-HCl, $3 \times 10^{-4}$ M dithiothreitol, 5% glycerol, pH 7.8, 20°C. Fluorescence emission was recorded on an SLM Instruments Series 400 Spectrofluorometer with excitation at 285 nm. Solid line spectra represent repressor; dashed line spectra represent repressor in the presence of $10^{-3}$ M IPTG. (A) Repressor. (B) Repressor in the presence of $2.2 \times 10^{-7}$ M operator DNA fragment.
Figure 10. Binding of inducer to repressor-operator complex.

Concentrations of reactants after mixing were: repressor, 
$1 \times 10^{-8}$ M; operator fragment, $1 \times 10^{-7}$ M; and inducer, $2 \times 10^{-4}$ M, 
in 0.01 M Tris-HCl, pH 7.5, 0.05 M NaCl, 5% glycerol, $3 \times 10^{-4}$ M 
dithiothreitol. Fluorescence excitation was at 290 nm, and the 
reaction was monitored by recording all fluorescence emission at 
wavelengths greater than 350 nm. A. The time course represents 
the average of eight experiments. B. The corresponding log plot 
of the observed fluorescence changes. Note that the reaction ap-
ppears to be a simple pseudo first order process since a straight 
line is observed in B.
Figure 11. Determination of the second order rate constant for
the binding of inducer to repressor and repressor-operator com-
plex. Buffer conditions are given in Figure 10. The observed
pseudo first order rates were plotted versus the inducer con-
centration after mixing. -○-, Repressor; -△-, repressor-
operator DNA complex.
tryptophan fluorescence (Friedman et al., 1977). Dilution of repressor in the absence of IPTG yielded no fluorescence change. The observed rate constant \( k_{\text{obs}} \) for the dissociation of ligand from protein is:

\[
k_{\text{obs}} = k'[\text{IPTG}] + k
\]

where \( k' \) is the association rate constant, and \( k \) is the dissociation rate constant. To minimize effects of the association rate on the observed rates, it was necessary to utilize initial IPTG concentrations which resulted in less than 50% saturation of inducer binding sites. The appropriate sugar concentrations were determined from the previously measured equilibrium dissociation constants for repressor alone and for the repressor-operator complex. Time courses for dissociation of IPTG from the inducer-repressor and inducer-repressor/operator complex are shown in Figure 12. The dissociation of inducer from the repressor-operator complex is more rapid than from repressor protein alone. The rates determined by computer fitting were 0.2 \( \text{s}^{-1} \) for inducer dissociation from repressor and 0.5 \( \text{s}^{-1} \) for inducer dissociation from operator bound protein.

The dissociation rate of inducer from the repressor-operator complex was also determined by displacement of sugar molecules from repressor by the binding of operator DNA fragments. Repressor-IPTG complexes were mixed with operator fragments in the stopped-flow spectrometer, and the fluorescence increase observed under these conditions was monitored and recorded. The rate observed should be a combination of the association rate of repressor-inducer with operator DNA fragments and the dissociation rate for inducer from the repressor-operator DNA complex. The association rate of repressor with similar operator
Figure 12. Dissociation of inducer from repressor and repressor-operator DNA fragment complex by rapid dilution. The increase in fluorescence concomitant with inducer dissociation was monitored as described in Figure 10. A mixture of $2 \times 10^{-7}$ M repressor, $5 \times 10^{-5}$ M IPTG, and, where present, $6 \times 10^{-7}$ M operator fragment, in 0.01 M Tris-HCl, pH 7.5, 0.05 M NaCl, 5% glycerol, $3 \times 10^{-4}$ M dithiothreitol was diluted 1:6.5 with buffer. The unlabelled curves represent the experiment carried out in the absence of operator DNA. A. The time courses shown represent the average of eight experiments and are fit by a single exponential curve. B. The corresponding plot of the log of the fluorescence change versus time is presented.
DNA fragments has been measured by Goeddel et al. (1977), and was found to be $2 \times 10^9 \text{M}^{-1}\text{s}^{-1}$. Under the conditions of this experiment, the observed rate would be $200 \text{ s}^{-1}$, about 1000 times greater than the expected rate of sugar dissociation (Fig. 13). Consequently, the observed displacement should be first order and reflect the rate of inducer dissociation from the repressor-operator DNA fragment complex. The time course is shown in Figure 13, and the observed rate constant obtained by the computer fit for a single exponential function was $1.0 \text{s}^{-1}$. This rate is comparable to that measured by dilution of the repressor-inducer/operator complex (Fig. 12, $k_{\text{obs}} = 0.5 \text{s}^{-1}$).

The equilibrium association constant calculated from the rate constants for the binding of IPTG to repressor alone is $2.3 \times 10^5 \text{M}^{-1}$, which is in good agreement with the previously measured value of $2.5 \times 10^5 \text{M}^{-1}$ (Friedman et al., 1977; O'Gorman et al., 1980 b). The apparent equilibrium association constant for IPTG binding to the repressor-operator complex calculated from the rate constants is $1.3 \times 10^4 \text{M}^{-1}$, and this value also corresponds well with the concentration of inducer required for half saturation of the repressor-operator complex which was determined from previous equilibrium binding measurements (O'Gorman et al., 1980 b). The 4 to 5-fold difference between both the inducer association and dissociation rates for free repressor and repressor-operator complex results in a 20-fold difference in the observed equilibrium constants for IPTG binding (Table V).

**Measurement of the Dissociation Rate of Operator Fragment from Repressor.** The rate of dissociation of operator DNA fragments from the repressor protein can be measured using a nitrocellulose filter technique. Labelled operator fragments are equilibrated with repressor,
Figure 13. Dissociation of inducer from repressor after binding of operator. Repressor, $1 \times 10^{-8}$ M, and IPTG, $3 \times 10^{-5}$ M, were mixed with $2 \times 10^{-7}$ M operator DNA fragment (final concentrations) in 0.01 M Tris-HCl, pH 7.5, 0.05 M NaCl, 5% glycerol, $3 \times 10^{-4}$ M dithiothreitol. The increase in fluorescence was monitored as described in Materials and Methods. A. The observed time course is an average of eight experiments. B. The corresponding log plot of observed data is presented.
### TABLE V

Summary of kinetic and equilibrium constants

<table>
<thead>
<tr>
<th></th>
<th>Equilibrium $K_a$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k'$ M$^{-1}$s$^{-1}$</td>
</tr>
<tr>
<td>Repressor + Inducer</td>
<td>$4.6 \times 10^4$</td>
</tr>
<tr>
<td>Repressor/Operator DNA Fragment + Inducer</td>
<td>$1.0 \times 10^4$</td>
</tr>
<tr>
<td>Repressor + Operator DNA Fragment$^d$</td>
<td>$2 \times 10^9$</td>
</tr>
<tr>
<td>Repressor/Inducer + Operator DNA Fragment$^e$</td>
<td>$2 \times 10^9$</td>
</tr>
</tbody>
</table>

$^a$Calculated from $K_a = k'/k$.

$^b$K's for inducer binding were measured by fluorescence titrations (O'Gorman et al, 1980 b);

$^c$K's for operator binding were measured by nitoro-cellulose filtration (O'Gorman et al, 1980 a);

$^d$This value was determined by averaging values obtained from dilution and DNA displacement experiments.

$^e$The value for $k'$ was measured by Goeddel et al (1977) for a slightly different fragment.

$^f$The value for $k'$ was assumed to be the same as for free repressor, and the value for $k$ was calculated from the measured $K_a$; $k = k'/K_a$. 
and then at \( t_0 \) a large excess of poly[d(A-T)] is added to replace labelled operator DNA molecules which were previously bound to the repressor protein. Aliquots are filtered at designated time intervals, and the amount of repressor-operator complex remaining is determined by scintillation counting. Using this technique, the half-time of dissociation for operator DNA fragments from repressor has been reported to be on the order of 30-45 sec (Lin et al., 1976; Goeddel et al., 1977). In our work, the rate for dissociation of the operator DNA fragment was observed to be 0.04 s\(^{-1}\), which corresponds to a half-time of 17 s (Figure 14). The lag observed in the semi-log plot in Fig. 5 is due primarily to the time required for addition of poly[d(A-T)] and subsequent mixing prior to filtration. The association rate for the formation of the repressor-operator DNA complex calculated from the measured equilibrium association constant (O'Gorman et al., 1980 a) and the dissociation rate was found to be \( 4 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \), which is about two-fold higher than the rate measured by Goeddel et al. (1977) for a similar DNA fragment (see Table V).

Since the operator-repressor complex is stabilized primarily by electrostatic interactions, it is unlikely that inducer binding significantly affects the rate of its formation. For example, differences in the equilibrium association constants for the binding of \( \lambda \)lac operator to repressor in the presence and absence of bound IPTG have been shown to be due to changes in the dissociation rate. The rate of dissociation of operator fragment from repressor in the presence of IPTG is too fast to measure by the nitrocellulose filter assay. However, this rate can be estimated to be about 40 s\(^{-1}\) from the equili-
Figure 14. Dissociation of operator DNA fragment from repressor.

Initial concentrations of repressor and labelled operator fragment were both $1 \times 10^{-9}$ M in 0.01 M Tris-HCl, pH 7.5, 0.05 M NaCl, 5% glycerol, and $3 \times 10^{-4}$ M dithiothreitol. Aliquots of the starting reaction mix were filtered to obtain values at 0 time. At $t=0$, 10 µg/ml poly[d(A-T)] was added to the reaction mixture, and the aliquots were filtered at designated times on nitrocellulose filters at a rate of 1 ml/s. Background was determined by adding IPTG ($10^{-4}$ M) to the reaction mix and filtering aliquots; the background was subtracted from the data before plotting.
Figure 15. Observed rates for the binding of inducer to repressor-operator complex as a function of the operator concentration. Inducer at $2 \times 10^{-4}$ M (after mixing) was mixed with repressor, $1 \times 10^{-8}$ M (after mixing), and varying concentrations of operator DNA fragment in the stopped flow spectrometer, and all fluorescence greater than 350 nm was recorded as described in Materials and Methods. The buffer was 0.01 M Tris-HCl, pH 7.5, 0.05 M NaCl, 5% glycerol, and $3 \times 10^{-4}$ M dithiothreitol. The solid theoretical line was calculated using the Monod-Wyman-Changeux model assuming rapid equilibration of the DNA molecules. The dashed line was calculated assuming that there is no DNA re-equilibration during sugar binding.

The solid theoretical curve in Figure 15 was calculated from the Monod-Wyman-Changeux model (2 operator DNA binding sites, 4 inducer binding sites (O'Gorman et al, 1980 b)) by assuming that the operator DNA fragments equilibrate very rapidly with all the repressor-inducer species. Under these conditions the effective allosteric constant which describes the ratio of T and R states is given by:

$$L_{eff} = \frac{L(1 + 2K_{TO}[0] + K_{TO}^2[0]^2)}{(1 + 2K_{RO}[0] + K_{RO}^2[0]^2)}$$

where $[0]$ is the free concentration of operator fragments. The apparent association and dissociation rates for the binding of the third sugar molecule are then given by:
\[ k' = k'_{RI} \frac{1}{1 + L_{eff}c^2} + k'_{TI} \frac{L_{eff}c^2}{1 + L_{eff}c^2}, \]

\[ k = k_{RI} \frac{1}{1 + L_{eff}c^3} + k_{TI} \frac{L_{eff}c^3}{1 + L_{eff}c^3}, \]

\[ k_{obs} = k' [IPTG] + k, \]

where \( c \) is the ratio of the T to R equilibrium constants for sugar binding \((K_{TT}/K_{RR})\).

The dashed curve in Figure 15 represents the other extreme where the operator DNA fragments do not dissociate or re-equilibrate at all during the course of the sugar binding reaction. In this case, the distribution of total protein among free repressor, [R]; repressor-operator, [RO]; and repressor-operator\(_2\), [RO\(_2\)] is fixed by the concentrations of DNA and protein present initially before mixing with IPTG:

\[ [R] = \frac{1 + L}{(1 + K_{RO}[0])^2 + L(1 + K_{TO}[0])^2} \]

\[ [RO_1] = \frac{2K_{RO}[0] + L(2K_{TO}[0])}{(1 + K_{RO}[0])^2 + L(1 + K_{TO}[0])^2} \]

\[ [RO_2] = \frac{K_{RO}^2[0]^2 + LK_{TO}^2[0]^2}{(1 + K_{RO}[0])^2 + L(1 + K_{TO}[0])^2} \]

where \( L \) is the allosteric constant in the absence of both DNA and inducer and \( K_{RO} \) and \( K_{TO} \) are the R and T state operator DNA equilibrium binding constants. The fraction of T and R states in each of these 3
protein species containing \( n \) bound IPTG molecules is given by:

\[
f_{T,i,n} = \frac{\text{Lc}^n_{\text{cDNA}}}{1 + \text{Lc}^n_{\text{cDNA}}} ; \quad f_{R,i,n} = 1 - f_{T,i,n}
\]

where \( c_{\text{DNA}} \) is the ratio of T to R DNA association equilibrium constants \( (K_{T0}/K_{R0}) \), \( i \) is the number of bound DNA molecules, and \( c \) is the ratio of T to R inducer association equilibrium constants \( (K_{TI}/K_{RI}) \). The observed association and dissociation rates for the binding of the third inducer molecule to each of the 3 protein species is given by:

\[
k'_i = k'_R f_{R,i,2} + k'_T f_{T,i,2}
\]

\[
k_i = k_{R,i,3} + k_{T,i,3}
\]

\[
k_{\text{obsi}} = k'_i (\text{IPTG}) + k_i.
\]

Then the overall observed rate is given by:

\[
k_{\text{obs}} = \frac{k_{\text{obs0}[R]} + k_{\text{obs1}[RO_1]} + k_{\text{obs2}[RO_2]}}{[R] + [RO_1] + [RO_2]}
\]

where \( k_{\text{obsi}} \) represents the observed rates for protein containing 0, 1, and 2 bound DNA fragments.

In both sets of calculations, \( L \), \( c \), \( c_{\text{DNA}} \), \( K_{T0} \), \( K_{R0} \) and the sugar binding rates were set at previously determined values (see (O'Gorman et al, 1980 b) and Table V):
\[ L = 0.7, \quad c = 0.03, \quad c_{\text{DNA}} = 500, \]

\[ K_{\text{TO}} = 1.25 \times 10^{10} \text{ M}^{-1}, \quad K_{\text{RO}} = 2.5 \times 10^{7} \text{ M}^{-1}, \quad k' = 4.6 \times 10^{4} \text{ M}^{-1}\text{s}^{-1}, \]

\[ k_\text{T} = 1.0 \times 10^{4} \text{ M}^{-1}\text{s}^{-1}, \quad k_R = 0.2 \text{ s}^{-1}, \quad \text{and} \quad k_T = 0.8 \text{ s}^{-1}. \]
brium constant for operator binding (O’Gorman et al. 1980 b), and the
association rate constant (Goeddel et al., 1977). This calculation
assumes that the association rate of repressor to operator is not
changed; this assumption is quite reasonable since electrostatic
forces are primarily involved in the association reaction (O’Gorman
et al., 1980 a; Chapter IV).

Effect of Operator DNA Concentration on Inducer Binding to
Repressor: Intermediate Cases for Sugar Binding. The cooperativity
observed in the equilibrium studies of IPTG binding to repressor-
operator DNA complex is not manifest in the time courses of inducer
binding or release in the presence of saturating concentrations of
operator DNA fragment (Figures 10 and 12). In neither case are acce-
lerating fluorescence traces observed within the limits of the noise
levels. However, the expected interaction between IPTG and operator
binding is evidenced when the rate of inducer binding is measured as
a function of operator DNA concentration (Figure 15). Furthermore,
these results suggest that the DNA molecules do dissociate rather
rapidly from repressor intermediates containing only one or two in-
ducer molecules with increasing DNA concentration. If DNA dissociation
were very slow with respect to inducer binding, the observed IPTG bind-
ing rates would be expected to decrease in a linear fashion until the
protein is saturated with operator, at which point the rate would no
longer change (dashed line, Figure 15). Under the conditions in
Figure 15, the DNA concentration at which no further decrease in rate
would be observed would be predicted to be \(2 \times 10^{-8} \text{ M}\). Clearly the
measured rates do not follow this pattern; the rate of IPTG binding to
repressor-operator DNA is faster than expected for intermediate concentrations of operator assuming all the DNA remained bound and maintained the protein in the slowly reacting (T) state. The predicted curve (solid line) in Figure 15 corresponds to the rate calculated by the Monod-Wyman-Changeux model (O'Gorman et al, 1980 b) for the binding of the third inducer molecule to the repressor protein in the presence of the indicated total operator DNA concentration. The rate for the binding of the third inducer molecule is approximately that expected by measuring the half-time of the reaction. In these calculations it was assumed that all the rates of DNA association and dissociation are much greater than those for sugar binding. The correspondence between predicted and observed rates is reasonable considering experimental error.

A more rigorous treatment of the theory would require taking into account operator DNA dissociation rates as well as inducer association and dissociation rates, since the DNA molecules probably do not equilibrate instantaneously after the binding of inducer molecules to repressor, particularly at the beginning of the reaction. The rate of operator dissociation in the absence of inducer is 0.04 s\(^{-1}\) and increases to approximately 40 s\(^{-1}\) after four inducers have bound. For the first steps of inducer binding, the rate of DNA dissociation (0.4 to 4 s\(^{-1}\) for 1 and 2 bound inducers) is comparable both to the association rate and the dissociation rate of inducer binding. However, it is evident from the experimental results shown in Figure 15 that the binding of inducer molecules does result in dissociation of DNA from repressor at concentrations of operator DNA below 5 x 10\(^{-8}\) M. Using the Monod-Wyman-Changeux model, the repressor has two states, the R (high affinity for
inducer, low affinity for operator) and the T (low affinity for inducer, high affinity for operator) (Figure 16, from O'Gorman et al, 1980 b). The free repressor population exists in an equilibrium of these two states, but the addition of either inducer or operator shifts this equilibrium toward the R or T state, respectively. At the initiation of the experiment the repressor bound to operator is in the T state. If the DNA molecules remained bound and caused the protein to remain in the T state throughout the experiment, the inducer binding rate would decrease as a function of operator concentration until roughly two equivalents of operator DNA per repressor were present (dashed line Figure 15). However, the experimental results suggest that at relatively low concentrations of operator DNA, most of the repressor shifts to the R or more rapidly reacting state during the course of the inducer binding reaction. The net result is that the apparent rate is faster than that predicted by assuming that all the DNA remains bound. In theory, this change in inducer binding rate from the T to the R values should be observed in a single experiment as an accelerating time course. However, since the R and T inducer association rates are only different by 5-fold and since very low concentrations of protein were requisite in these experiments, the resultant fluorescence changes were simply not large enough compared to background noise to resolve multiple rates from each individual time course.

In summary, the rates of binding of inducer molecules to the lactose repressor protein are significantly influenced by the presence of operator DNA fragments. The association rate is decreased, while
Figure 16. Monod-Wyman-Changeux scheme for inducer and operator DNA fragment binding with two sites for operator DNA binding. R and T states are defined in the text. I refers to inducer, and O to operator DNA fragment. The indicated transitions are allowed.
the dissociation rate is increased; the extents of the effects are approximately the same on both rates (4-5-fold). The overall effect predicted on the equilibrium association constant is 20-fold, a value which is in agreement with the inducer half-saturation concentration and with the theoretical value calculated for the model derived from equilibrium measurements of inducer binding (O'Gorman et al, 1980 b). The equilibrium between R and T states and thus the pathway for inducer binding to repressor-operator complexes is dependent upon the concentration of the operator. At concentrations of operator-DNA which are not saturating in the presence of inducer, both R and T states are present and will bind inducer. The measured rates are a mixture of the rate for inducer binding to repressor alone, the rate for inducer binding to the intact repressor-operator complex, and probably also the rates for operator DNA fragment dissociation from the protein-inducer complex.
CHAPTER IV

DNA Binding Parameters of Hybrid Tetramers
of Native and Core Lac Repressor Protein
Modification of the tetrameric structure of the lac repressor protein has been used previously to investigate the requirements for operator binding (Sadler and Tecklenberg, 1976; Geisler and Weber, 1976; Kania and Müller-Hill, 1977). Such studies have been used primarily to determine the number of subunits involved in the repressor-operator interaction. Sadler and Tecklenberg (1976) constructed hybrid tetramers composed of subunits from wild-type repressor and tight binding mutants by denaturing the purified proteins and subsequently renaturing tetramers using the appropriate ratios of the two types of monomers. Their results indicate that more than one repressor subunit is involved in the operator interaction. Similar experiments to form hybrids between wild-type repressor and homogeneous tryptic core proteins also indicate that more than one subunit is required for operator binding and verify the assumption that the NH$_2$-terminus is not required for tetramer formation (Geisler and Weber, 1976). Hybrid tetramers composed of β-galactosidase and a chimaera of lac repressor and β-galactosidase have been isolated; tetramers which have the repressor moieties of two chimaeric subunits in close enough proximity for repressor-repressor crosslinking have detectable, though not native, operator binding activity (Kania and Müller-Hill, 1977).

Recently, Matthews (1979) has demonstrated that the trypsin-resistant core protein binds to operator with an affinity of \(~10^{-7}\) M, and Geisler and Weber (1976) have reported that isolated NH$_2$-terminal peptides, amino acids 1-59 or 1-51, bind to DNA with an affinity of \(~10^{-6}\) M. Alterations in the pattern of methylation of the operator sequence by these peptides suggests that the NH$_2$-terminus has some se-
quence specificity (Ogata and Gilbert, 1979). Thus, detailed studies of the operator binding characteristics of hybrid tetramers of the tryptic core protein of the lac repressor and native repressor can be used to probe the relationship of the NH$_2$-terminal and core domains in operator DNA binding. In addition, variation of the tetrameric structure of the protein can be used to study both the binding of the protein to nonspecific DNA and differences in nonspecific and operator DNA binding. These experiments require larger amounts of the hybrid proteins than can be obtained easily by methods reported in the literature. We have therefore isolated the hybrid tetramers by chromatography on phosphocellulose in adequate amounts for extensive DNA binding experiments and for physical characterization.

**HYBRID TETRAMERS - MATERIALS AND METHODS**

**Preparation of Hybrid Tetramers.** Repressor (20-50 mg) was thawed and concentrated via an Amicon ultrafiltration cell. The protein was dialyzed into 1.0 M Tris-HCl, pH 7.5, 30% glycerol, 3 x 10$^{-4}$ M dithiothreitol. Hybrid tetramers were generated by subjecting the native repressor to limited tryptic digestion; the protein was divided into aliquots, and trypsin was added in concentrations from 0.01% to 0.5% trypsin (w/w) for five minutes at 4°C. One aliquot was left untreated as a control. The reaction was stopped by addition of phenylmethylsulfonylfluoride (PMSF) in ethanol. Both trypsin and PMSF were purchased from Sigma. The aliquots were pooled and dialyzed against 0.075 M potassium phosphate, pH 7.5, 5% glycerol, 3 x 10$^{-4}$ M dithiothreitol. The sample was applied to a phosphocellulose (Whatman) column, equilibrated in 0.075 M potassium
phosphate. The column was washed with the equilibration buffer to
elute core protein, NH₂-terminal peptides and impurities in the re-
pressor preparation. After elution of core, the column was washed
with 0.12 M potassium phosphate to elute the hybrid tetramer composed
of one native and three core monomers (1:3). The other hybrid tetra-
mers were eluted with a salt gradient from 0.18 M to 0.30 M potassium
phosphate. All column buffers were pH 7.5 and contained a 5% glycerol
and 3 x 10⁻⁴ M dithiothreitol. The column effluent was monitored with a
Gilson ultraviolet flow meter linked to an Omniscribe recorder. After all
the tetramers had been collected from the column, the peaks were assayed
for IPTG binding activity and the appropriate fractions were pooled.

Characterization of Hybrid Tetramers. Each hybrid tetramer
was characterized by disc gel electrophoresis, sodium dodecyl sulfate
gel electrophoresis, and Dns-Cl end group analysis. Disc gel electro-
phoresis was performed using a Tris-HCl-glycine system (Gabriel, 1969).
Sodium dodecyl sulfate electrophoresis was done according to the method
of Weber et al (1972), and gels were scanned. The NH₂-terminal amino
acid residues were determined by reaction with the Dns-Cl using sodium
dodecyl sulfate as a denaturing agent (Gray, 1972). After hydrolysis
samples were analyzed by thin layer chromatography on silica gel de-
veloped in chloroform:ethanol:acetic acid (38:4:3).

Operator Binding Activity of Hybrid Tetramers. Each hybrid
tetramer was assayed for operator binding activity in three ways. Oper-
ator assays under stiochiometric conditions were done as described by
Bourgeois (1971). The operator binding activity of each hybrid tetramer
was further analyzed by titration of λplac DNA under conditions at which
the dissociation constant can be determined (i.e., with DNA concentration at or below the $K_d$ for the interaction with the protein). For these determinations λplac DNA was purified from strain MBC 5 (obtained from Mary Barkley) and labelled with $^3$H-thymidine to high specific activity so that less than 30 ng of DNA can be detected by scintillation counting. Equilibrium titrations were performed on nitrocellulose filters. The dissociation constants for the interaction of hybrid tetramers with a 29 base pair operator fragment [obtained from Drs. Dickerson (California Institute of Technology), Riggs, Rosenberg, Kallai and Itakura (Kallai et al., 1980)] were measured using labelled fragment under equilibrium conditions on nitrocellulose filters. Labelled operator fragments were prepared using the method described by Maxam and Gilbert. Alkaline phosphatase was purchased from P-L Biochemicals, polynucleotide kinase from Boeringer-Mannheim, and $[\gamma-\text{32P}]$ATP from Amersham-Searle. Purified operator fragment (20 μg/ml) in 10 mM Tris-HCl, 1 mM magnesium acetate, pH 8.0, was treated with alkaline phosphatase (6 ng/ml) for one hour at 37°C. The solution was extracted with phenol, and the DNA precipitated with 95% ethanol. Recovered fragments were incubated with 300 μCi $[\gamma-\text{32P}]$ATP and 5 units polynucleotide kinase in 0.05 M glycine, 5 mM magnesium acetate, 0.05 mM spermidine, 5 mM dithiothreitol, 0.005 mM ethylenediaminetetraacetic acid, 5% glycerol, pH 9.5, at 37°C for one hour. After addition of sodium acetate to 0.3 M, the fragments were precipitated with 95% ethanol. All DNA binding assays were performed in 0.01 M Tris-HCl, 0.01 M magnesium acetate, 0.01 M potassium chloride, 3 x 10^{-4} M dithiothreitol, 5% dimethyl sulfoxide, 50 μg/ml bovine serum albumin, pH 7.5.
Kinetics of Hybrid Tetramer-Operator Dissociation. The half time for dissociation of the protein-operator complex was determined as described by Riggs et al (1970) with some modification. Because the dissociation constant of each hybrid tetramer for operator is different, care was taken to insure that the initial, equimolar concentrations of protein and operator were ten-fold above their respective dissociation constant. At time zero a 7- to 10-fold excess of unlabelled operator was added to the equilibrated mixture of protein and [3H]-λplac DNA. Aliquots of this mixture were filtered at designated times. The filters were dried, immersed in scintillation fluid, and counted.

Nonspecific DNA Binding Activity in Hybrid Tetramers. Each hybrid tetramer was assayed for nonspecific DNA binding activity using the nitrocellulose filter binding assay previously described (Chapter II). When required, samples were concentrated via Amicon ultrafiltration cells before use in this assay. The number of ion pairs involved in the interaction of one amino terminus with nonspecific DNA was determined by assaying the 1:3 hybrid for nonspecific DNA binding activity under a variety of salt conditions (de Hase Th et al, 1977).

Competition of Poly[d(A-T)] with Operator Binding to Hybrid Tetramers. Competition assays were performed under equilibrium conditions as described by Lin and Riggs (1972) with modifications to take into account the difference in the dissociation constants of the hybrid tetramers for operator. As in the determination of the half time of dissociation for hybrid tetramers from operator, it was necessary that the concentrations of protein and labelled operator were above the dissociation constants for that interaction. Increasing amounts of
poly[d(A-T)] were added to the protein-operator mixtures, and the assays were filtered on nitrocellulose filters. The filters were dried and counted as for all other filter assays.

RESULTS

Preparation and Isolation of Hybrid Tetramers. The hybrid tetramers were prepared by limited trypsin proteolysis as described. By utilizing several different concentrations of trypsin, sufficient quantities of each tetramer were obtained for characterization and assay. Each aliquot was handled identically with the exception of the trypsin concentration. Reaction was stopped by addition of PMSF, and the samples were pooled and dialyzed against 0.075 M potassium phosphate, pH 7.5, prior to separation on phosphocellulose.

Fresh phosphocellulose was used in each preparation reported here since we observed poor resolution of the hybrids and reduced operator binding activity in all of the tetramer species with repeated use of phosphocellulose. The elution of the hybrid tetramers from the phosphocellulose column is illustrated in Figure 17. Since native repressor is isolated on phosphocellulose but tryptic core protein has no detectable affinity for phosphocellulose (Matthews, 1979), the composition of each hybrid tetramer can be deduced from its position in the elution profile. The expected compositions are noted on the figure. The designations for the tetramers are given as native:core ratios; therefore the 1:3 tetramer is composed of one native and three core subunits. The only major variance in the two elution profiles shown is in the pattern for the 2:2 tetramer. This peak on occasion appears with a leading
Figure 17. Separation of hybrid tetramers on phosphocellulose. Hybrid tetramers of repressor and trypsin-resistant core were prepared as described in Materials and Methods. After dialysis against 0.075 M potassium phosphate, pH 7.5, 5% glycerol, $10^{-4} \text{ M}$ dithiothreitol, the sample was applied to a phosphocellulose column equilibrated in the same buffer. The column was washed with the equilibration buffer to elute the core protein, and the salt concentration of the buffer was stepped to 0.12 M potassium phosphate to elute the 1:3 tetramer. A gradient from 0.18 to 0.30 M potassium phosphate was used to elute the remaining tetramers. The solid line is the elution profile recorded with the column monitor using the absorbance at 280 nm, and the dotted line corresponds to the ionic strength. A. Column profile showing a symmetrical peak for the 2:2 tetramer with no leading shoulder. B. Column profile showing the elution of the 2:2 tetramer with a leading shoulder.
shoulder, suggesting that there are two arrangements of this hybrid tetramer. However, we could detect no differences in the shoulder and main peak during subsequent assay and physical characterization. As will be discussed later, the apparent identity of these two species may be due either to the assay conditions or to rapid rearrangement of the tetramers. Because of the possibility of rearrangement, assays were routinely performed the same day as the samples were isolated. IPTG activity was determined for each peak and was found to be equivalent to native repressor for all tetramers.

Characterization of Hybrid Tetramers. Discontinuous polyacrylamide gels of each tetramer exhibited only one band (Figure 18). The electrophoresis was performed concurrently with the assays on the mixed tetramers and therefore reflects the condition of the tetramers at the time of assay. We observed rearrangement of the hybrid tetramers after prolonged storage at 4° C. The rearrangement reaction is enhanced significantly by exposing the mixed tetramers to temperatures above 4° C, particularly in buffers of low ionic strength. However, the tetramers remain stable and retain activity after freezing in the potassium phosphate column buffers; the frozen and thawed tetramers exhibit their characteristic operator dissociation constants. Repeated freezing and thawing, even in the column buffers, results in rearrangement of the tetramers.

Sodium dodecyl sulfate electrophoresis of the hybrid tetramers is shown in Figure 18. The composition of each tetramer predicted by the elution position from the phosphocellulose column is shown in the gels and further substantiated by gel scans. The smaller molecular
Figure 18. Electrophoresis of hybrid tetramers. A. Discontinuous gels of the hybrid tetramers are shown to demonstrate that only one protein species is present in each preparation. The gels were electrophoresed using the standard Tris-HCl-glycine system (Gabriel, 1969) fixed with 5% trichloroacetic acid, and stained with Coomassie Blue. A, 1:3 hybrid; B, 2:2 hybrid; C, 3:1 hybrid; D, 4:0 hybrid; E, a mixture of core, 2:2 hybrid, and repressor. B. Sodium dodecyl sulfate electrophoresis of the hybrid tetramers using the system of Weber et al confirms the subunit composition of each tetramer. A, 1:3 hybrid; B, 2:2 hybrid; C, 3:1 hybrid, D, 4:0 tetramer. R is repressor; C is core protein. C. Spectrophotometric scans of sodium dodecyl sulfate gels. A model 2400 Gilford Spectrophotometer fitted with model 2410 gel scanner was used to scan the fixed and stained gels at 600 nm. A 0.5 nm aperture was used. Areas designated in the figure were determined by triangulation and are expressed in arbitrary units. The symbols are those used in B.
weight core is the faster moving species; the separation between core and native monomers allows the comparison of the compositions of the hybrid tetramers.

Dns-Cl end group analysis yielded NH$_2$-terminal residues of Dns-methionine, the NH$_2$-terminal residue of native repressor, and a mixture of Dns-valine and Dns-gultamine (Table VI). These results indicate that cleavage occurs at amino acids 51 and 59 in the primary sequence and not exclusively at lysine 59 as in tryptic core characterized by Files and Weber (1976). The difference in the cleavage sites under these conditions is not clear. However, these workers report isolation of NH$_2$-terminal peptides of lengths 1-51 and 1-59 in the 1.0 M Tris-HCl, 30% glycerol buffer; the NH$_2$-terminal residue of their core protein byproduct was not analyzed.

**Operator Binding Activity of Hybrid Tetramers.** The differences in operator activity between the mixed tetramers using the standard operator assay under stoichiometric conditions is shown in Figure 19. Under these conditions ([$\lambda$plac] = 2 x 10$^{-11}$ M) all the tetramers show operator binding activity. While the 4:0 and 3:1 tetramers show the same activity under these conditions, a change in dissociation constant less than tenfold would not be detected. The 2:2 tetramers show intermediate activity, and the 1:3 tetramer displays least activity. The operator assay for 1:3 tetramer under these conditions can be used to determine an equilibrium dissociation constant for $\lambda$plac operator. Geisler and Weber (1977) observed no operator activity in the 1:3 tetramer and less activity for 2:2 than our data show. The reason for these differences is unclear, although the denaturation/renaturation process subjects the protein to a significant stress.
<table>
<thead>
<tr>
<th>Native:Core</th>
<th>Dns-amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:3</td>
<td>Dns-met</td>
</tr>
<tr>
<td></td>
<td>Dns-val, Dns-gln</td>
</tr>
<tr>
<td>2:2</td>
<td>Dns-met</td>
</tr>
<tr>
<td></td>
<td>Dns-val, Dns-gln</td>
</tr>
<tr>
<td>3:1</td>
<td>Dns-met</td>
</tr>
<tr>
<td></td>
<td>Dns-val, Dns-gln</td>
</tr>
<tr>
<td>4:0</td>
<td>Dns-met</td>
</tr>
</tbody>
</table>
Figure 19. Operator binding activity of hybrid tetrailomers under stoichiometric conditions. Protein was added to [3H]-labelled λplac DNA (2 x 10^{-11} M) in the ratios indicated in the figure in 0.01 M Tris-HCl, pH 7.5, 0.01 M KCl, 0.01 Mg(OAc)₂ 5% methyl sulfoxide, 10^{-4} M dithiothreitol, 50 µg/ml bovine serum albumin, and 0.5 ml aliquots were filtered through nitrocellulose filters. The radioactivity retained was determined by liquid scintillation counting. The background counts retained in the presence of 10^{-4} IPTG have been subtracted, and the curves were normalized to percent saturation to allow comparison between experiments. - ● -, untreated repressor, - ○ -, 4:0; - △ -, 3:1; - ■ -, 2:2; - △ -, 1:3.
Operator titrations using concentrations of λplac and protein close to the dissociation constant for native repressor yielded the saturation curves shown in Figure 20, and the dissociation constants derived from half-saturation concentrations of protein and their corresponding ΔG⁰ values are listed in Table VII. Although no difference in operator affinity between 4:0 and 3:1 tetramers was detected in the stoichiometric operator assay, a difference in dissociation constant is apparent under these assay conditions. This difference in affinity must be due to loss of contacts contributed by the fourth NH₂-terminal peptide; this result suggests that every subunit is involved in operator binding. However, it is also clear that the energy contributions of the amino termini are not equivalent. The contribution of a single NH₂-terminus to the core-operator interaction is reflected in the 1000-fold decrease in the dissociation constant for λplac DNA of the 1:3 hybrid tetramer compared to that of core. This difference in binding energy (ΔG = -RTlnK) reflected by the affinity increase between 0:4 and 1:3 represents approximately 50% of the total difference in the free energy of binding to λplac DNA between core protein (0:4) and native repressor (4:0). Thus, the first NH₂-terminus supplies 50% of the free energy of binding contributed by the 4 NH₂-terminal regions. The values for the energy differences can be easily determined from the ΔG⁰ values listed in Table VII.

Differences among the operator binding capacities of the hybrid tetramers are further evidenced by the affinities of the tetramers for a 29 base pair operator (Figure 21 and Table VII). Although the absolute difference between the affinities of core protein and the 1:3 tetramer
Figure 20. Titration of λplac DNA with hybrid tetramers. $[^3]H$-labelled λplac DNA (2 x $10^{-13} M$) was titrated with hybrid tetramers in the standard assay, and 0.5 ml aliquots were filtered through nitrocellulose filters. The radioactivity retained was determined by liquid scintillation counting, and the background counts were subtracted. $\bullet$, 4:0; $\bigcirc$, 3:1; $\square$, 2:2; $\triangle$, 1:3. The two 2:2 hybrids were isolated as the leading shoulder ($\square$) and the central portion ($\blacksquare$) and the 2:2 peak. No differences in activity were noted.
Figure 21. Titration of operator fragment with hybrid tetramers.
The 29 base pair operator fragment (1 x 10^{-11} M), labelled with
[^32]P as described in Materials and Methods, was titrated with
hybrid tetramers in the standard assay buffer, and 0.5 ml ali-
quots were filtered through nitrocellulose filters. The data
were collected and analyzed as in Figure 20. – ● –, 4:0; – ○ –,
3:1; – ■ –, 2:2; – △ –, 1:3.
<table>
<thead>
<tr>
<th>Tetramer:Native:Core</th>
<th>Binding Parameters</th>
<th>ΔG° (kcal)</th>
<th>λplac</th>
<th>K_d (M)</th>
<th>K_d (M)</th>
<th>ΔG° (kcal)</th>
</tr>
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<tbody>
<tr>
<td>0:4</td>
<td></td>
<td>-9.1</td>
<td>2 x 10^{-7}^a</td>
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<tr>
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</tr>
<tr>
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<td>-17.7</td>
<td>&lt;1 x 10^{-13}</td>
<td>2 x 10^{-11}</td>
<td></td>
<td>-14.6</td>
</tr>
</tbody>
</table>

^aDissociation constant reported by Matthews (1979)
for the fragment is smaller, 50% of the difference in free energy of binding between core (0:4) and intact repressor (4:0) is supplied by the first NH$_2$-terminus (see Table VII). The second NH$_2$-terminus is intermediate in its binding energy contribution, while the last 2 NH$_2$-termini make minor energy contributions to the operator binding process. These results can be interpreted by the following scheme: two NH$_2$-termini contribute major and approximately equal contacts with operator DNA while the remaining two make minor contributions. The reason for the larger increase in affinity with addition of the first NH$_2$-terminal interaction to the core binding is not clear; this could be due to anchoring of the operator DNA into a cleft on the protein (Chapter V), a conformational change in the protein associated with the presence of the first NH$_2$-terminus, a conformational change in the DNA such as a β-kink on binding to the protein, or alternatively, the increase in affinity could be due entirely to a dramatic increase in the association rate of the protein to operator. This last explanation is quite likely and will be discussed in more detail below.

**Rate of Dissociation of the Hybrid Tetramer-Operator Complex.**
The rate of dissociation of the tetramer-operator complex is not a function of the concentration of the binding species provided that the initial concentrations are such that the effect of the association rate on the observed dissociation rate is negligible. Thus, the dissociation rate is a fingerprint of the protein species and can be used as a criterion of purity. The rates of dissociation for the tetramers were followed by the retention of $^3$H-λplac DNA on nitrocellulose filters; the results are plotted in Figure 22 and tabulated in Table VIII.
Figure 22. Determination of the dissociation rates of hybrid tetramer-operator DNA complexes. Protein \(6 \times 10^{-11} \text{ M}\) and \(\lambda\)plac DNA \(8 \times 10^{-11} \text{ M}\) were equilibrated for 20 minutes in the standard assay buffer. At time zero a ten-fold excess of cold \(\lambda\)plac DNA \(6 \times 10^{-10} \text{ M}\) over protein was added, and 0.5 ml aliquots were filtered in triplicate. In the case of 2:2 tetramer the time zero counts were filtered before addition of cold operator DNA. Background counts were determined by addition of \(10^{-3} \text{ M}\) IPTG, and these counts were subtracted before plotting. Results were reported as counts per minute at the time of filtration divided by the counts per minute at zero time. \(\circ\), 4:0; \(\triangle\), 3:1; \(\blacksquare\), 2:2.
### TABLE VIII

**Kinetic Constants for Hybrid Tetr...**

<table>
<thead>
<tr>
<th>Native:Core</th>
<th>$t_{1/2}$ (m)</th>
<th>$k_d$ (s$^{-1}$)$^a$</th>
<th>$K_d$ (M)</th>
<th>$k_a$ (M$^{-1}$s$^{-1}$)$^b$</th>
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</thead>
<tbody>
<tr>
<td>1:3</td>
<td>--</td>
<td>--</td>
<td>$2 \times 10^{-10}$</td>
<td>--</td>
</tr>
<tr>
<td>2:2</td>
<td>7.5</td>
<td>$1.5 \times 10^{-3}$</td>
<td>$8 \times 10^{-12}$</td>
<td>$2 \times 10^{8}$</td>
</tr>
<tr>
<td>3:1</td>
<td>19</td>
<td>$6.1 \times 10^{-4}$</td>
<td>$7 \times 10^{-13}$</td>
<td>$9 \times 10^{8}$</td>
</tr>
<tr>
<td>4:0</td>
<td>30</td>
<td>$3.9 \times 10^{-4}$</td>
<td>$&lt;1 \times 10^{-13}$</td>
<td>$4 \times 10^{9}$</td>
</tr>
</tbody>
</table>

$^a$ $t_{1/2} = 0.693/k_d$

$^b$ The association rate is calculated from the dissociation rate constant and dissociation constant determined for each hybrid tetramer. $k_a = k_d/K_d$. 
Each curve shows only one rate, indicating that only one protein species is present. Occasionally, a second very slow rate was observed; this behavior did not appear to indicate an impurity since the second rate was displayed by all the hybrid tetramers when it was observed for one of them. Limitations in the amount of labelled and unlabelled operator available precluded the determination of the rate of dissociation of the 1:3 tetramer since the high dissociation constant for this tetramer's interaction with operator required the use of prohibitively high concentrations of λplac DNA.

The rate of association of each tetramer to λplac DNA has been calculated from the experimentally determined dissociation rate and dissociation constant; these rates are tabulated in Table VIII. Riggs et al (1970 b) have shown that the correlation between the measured dissociation constant for repressor-operator and that calculated from the association and dissociation rates is excellent. Although only a two-fold decrease is observed in the dissociation rates between species differing by the loss of each NH₂-terminus, a five-fold decrease is seen in the calculated rate of association between these species.

Nonspecific DNA Binding of Hybrid Tetramers. The nonspecific DNA binding capacity of each mixed tetramer has been examined (Figures 23, 24 and Table IX). As shown in Table IX, the increase in the free energy of binding as each NH₂-terminus is added is small. In general, if each moiety involved in binding to the same molecule can be assigned a value for its contribution to the free energy of binding, the measured affinity would be the product of the individual affinities (ΔG° = -Σ(RTlnKᵢ) = -RTlnK_{obs}). If the measured binding affinities increase
only as the concentration of added potential binding sites increase (e.g., the dissociation constant doubles as the concentration doubles), this result constitutes evidence that each moiety acts independently. The only increase observed in the affinities would be due to the increased concentration of these species. It is apparent that the free energy of the repressor tetramer binding to nonspecific DNA is not the sum of the free energies of binding of 4, or even 2, NH₂-termini as expected if all four NH₂-termini bind to one DNA molecule (Kₚ for isolated NH₂-termini is ~10⁻⁶ M, ΔG° = 18.2 kcal). Thus, either each NH₂-terminus can bind essentially independently to nonspecific DNA as discussed above, or one NH₂-terminus is primarily responsible for the binding of nonspecific DNA while the three other NH₂-termini contribute minor contacts. Since the DNA binding affinity of isolated amino termini (Geisler and Weber, 1977) suggests that each NH₂-terminus would be capable of binding independently, we favor the former explanation. We have tested the salt dependence of the nonspecific DNA binding activity of the hybrid tetramer 1:3 (data not shown). Within the limits of the nitrocellulose filter assay the 1:3 tetramer displays the same salt dependence for nonspecific binding as repressor, implying that the ionic contacts for the two tetramers are similar, presumably contained within a single NH₂-terminus and not the result of binding with one NH₂-terminus/DNA molecule with additional contacts of other NH₂-termini with the same DNA molecule. These data strongly suggest the presence of four independent nonspecific DNA sites on the repressor tetramer.

**Effect of Nonspecific DNA on Operator Binding Activity of Hybrid Tetramers.** Competition assays were performed for each tetramer
Figure 23. Saturation curves for binding of hybrid tetrarmers to nonspecific DNA. Protein in the concentrations indicated was used to titrate λplac DNA (1.4 x 10^-7 M sites) in the presence of 10^-4 M IPTG. Binding observed under these conditions is not sequence specific (Chapter II). The solutions were filtered, and the data collected and analyzed as in Figure 20. These curves have been normalized to percent saturation to allow averaging of five experiments. Background was determined with no protein present. -○-, untreated repressor; -●-, 4:0; -▲-, 3:1; -■-, 2:2; -△-, 1:3.
Figure 24. Scatchard treatment of hybrid tetramer-nonspecific DNA binding data. The low saturation data shown were used to generate Scatchard plots (Bailey, 1979) by assuming the counts retained on the filters were directly proportional to the repressor bound. Symbols used are identical to those in Figure 23.
### TABLE IX

Binding Parameters for Hybrid Tetramers and Nonspecific DNA

<table>
<thead>
<tr>
<th>Tetramer</th>
<th>Binding Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native:Core</td>
<td>$K_d$ (M)$^a$</td>
</tr>
<tr>
<td>[R]$_{1/2}$ - 1/2[0]$^b$</td>
<td>Scatchard$^c$</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------</td>
</tr>
<tr>
<td>0:4</td>
<td>&lt;1 x 10$^{-4}$$^d$</td>
</tr>
<tr>
<td>1:3</td>
<td>4 x 10$^{-7}$</td>
</tr>
<tr>
<td>2:2</td>
<td>2 x 10$^{-7}$</td>
</tr>
<tr>
<td>3:1</td>
<td>8 x 10$^{-8}$</td>
</tr>
<tr>
<td>4:0</td>
<td>4 x 10$^{-8}$</td>
</tr>
</tbody>
</table>

---

$^a$ The dissociation constants are expressed in M sites as described by O'Gorman et al. (1980a).

$^b$ The dissociation constants were calculated from the concentration of repressor at the half-height minus half the concentration of nonspecific DNA sites.

$^c$ The dissociation constants were calculated from the slope of Scatchard plots of low saturation data (Bailey, 1979).

$^d$ The dissociation constant was determined by boundary sedimentation.
using the method of Lin and Riggs (1972) with the modification that the operator and protein concentrations were at least ten-fold above the dissociation constant for their interaction. Protein was added in amounts sufficient to achieve 50-80% saturation. The results of these experiments are shown in Figure 25. Because there are only small differences among the tetramers 4:0, 3:1, and 2:2 in the ratios between their respective dissociation constants for operator and dissociation constants for nonspecific DNA (Table VII and Table IX), the interaction of these tetramers with operator is affected similarly by excess nonspecific DNA (>1-fold above the $K_d$) as evidenced by the competition curves. Although the ratio between the dissociation constants for operator and nonspecific DNA would predict that poly[d(A-T)] should compete more effectively for the 1:3 tetramer than for the other tetramers, minimal competition is observed experimentally. The obvious interpretation for the data is that poly[d(A-T)] and operator do not bind to the same site in the 1:3 tetramer (see Discussion).

**DISCUSSION**

Hybrid tetramers of the lac repressor protein and its trypsin resistant core have been prepared by limited proteolysis followed by separation on phosphocellulose. This method of preparation does not perturb the protein tertiary/quaternary structure and thus is a more gentle method of preparation of these species than denaturation/re-naturation. The tetramers isolated using this method have native inducer binding activity. The 4:0 tetramer from the separation has un-
Figure 25. Competition curves for hybrid tetramer binding to operator DNA in the presence of increasing quantities of poly-[d(A-T)]. Hybrid tetramers were added to λlac DNA under conditions in which the concentration of each was ten-fold above the dissociation constant for their respective interactions (see Table VII). Poly[d(A-T)] was added in the amounts indicated and the solutions were allowed to come to equilibrium. The samples were filtered and counted as in the other filter binding assays, and the data analyzed as in Figure 22. -○-, 4:0; -▲-, 3:1; -■-, 2:2; -△-, 1:3.
altered nonspecific DNA binding activity and operator dissociation constants for \(\lambda\)plac DNA and operator fragments consistent with those for control. The only significant activity loss was observed in the stoichiometric operator assay. Under stoichiometric binding conditions (i.e., concentrations of protein and DNA 100-fold greater than the dissociation constant), loss of activity suggests either a gross change in affinity or injury to the protein population such that a portion of the tetramers are totally unable to bind operator normally. However, in comparing two samples with different proportions of active protein, the difference should be reflected in the dissociation constants. In this case the determination of the dissociation constants for the 4:0 tetramer and control are limited by the sensitivity of the assay. Because of the difficulty in determination of the dissociation constant for the 4:0 tetramer and native repressor, differences in the affinities of the 4:0 tetramer and control for the operator cannot be strictly compared. Therefore the observed activity loss may be due to the process of preparing the tetramers rendering some portion of the population unable to bind operator normally. In contrast, there is no detectable change in the nonspecific DNA binding activity. Although these measurements are under equilibrium conditions, this assay is sufficiently sensitive to detect a change in dissociation constant. Apparently, the protein contacts responsible for operator and nonspecific DNA are not identical. When taken with direct evidence that the core protein binds to operator these results suggest possible mechanisms for the loss of operator binding activity frequently observed in repressor purification. While the secondary and tertiary structures
of both the exposed NH$_2$-terminus and the core domains of the protein are preserved, the spatial relationship of these two regions may become permanently altered so that they are unable to bind together effectively, or core-operator contacts may be damaged in some way during the separation. There is a great deal of evidence to suggest that the NH$_2$-terminus is an independent, but freely moving structure (see Chapter V), and thus it seems unlikely that the tertiary structure of the protein could be permanently altered. However, the operator-active core protein is isolated by a procedure similar to that for isolation of the hybrid tetramers. To further complicate the question, we have observed that repressor that appears to 100% active when assayed with 29 base pair operator fragment under stoichiometric conditions may only appear to be 50% active in the stoichiometric assay with λplac DNA. Because of these considerations it is possible that the loss of activity in the stoichiometric operator assay is an artifact of this assay.

The purity of each of the hybrid tetramers has been substantiated by several methods. The discontinuous gel electrophoresis system resolved only one band for each hybrid tetramer. The detection of any repressor present is limited only by the amount of protein needed for Coomassie Blue staining. In the case of the 3:1 and 2:2 tetramers, the difference in their respective dissociation constants for operator compared to native repressor indicates that if the observed binding were due to repressor contamination, the repressor should be easily visible in disc gel electrophoresis. However, if the operator DNA fragment binding observed for the 1:3 tetramer were due to repressor contami-
nation, the necessary quantity of repressor would constitute only 1% of the total protein. This contamination would be at lower limits of detection for disc gels; no such contamination was observed.

Binding assays can also be used to establish purity. The half-time of dissociation measured for each tetramer is unique to that tetramer and is not concentration dependent. If the sample preparations were mixtures of repressor and various hybrid tetramers, the measured rate would be composed of multiple rates, each one corresponding to one species of tetramer. These rates would be apparent in the experimental curves. No biphasic behavior was evident in the measured dissociation rates. In the case of the 1:3 tetramer it is more difficult to assess purity by binding assays alone due to the wide difference in dissociation constants for operator DNA between this tetramer and native repressor. However, the competition assay with poly[d(A-T)] indicates that this tetramer has operator binding characteristics which cannot be attributed to repressor. Under the conditions of the assay, if the operator binding of this species were due solely to repressor contamination, significant competition should occur. As shown in Figure 25, minimal competition is observed; this result demonstrates that the 1:3 tetramer is a separate and distinct species.

O'Gorman et al (1980 a) have reported that the repressor tetramer is capable of accommodating two operator fragments of 29 base pair length. Further, only models with two operator binding sites per tetramer adequately simulate the equilibrium binding of inducer to repressor-operator fragment complex (O'Gorman et al, 1980 b). That only one operator in the form of λplac DNA is able to bind to repressor is
presumably due to electrostatic repulsion between the two DNA molecules. Geisler and Weber (1977) have shown that isolated, intact NH$_2$-termini (either amino acids 1-51 or 1-59) bind to DNA, and Ogata and Gilbert (1977) have demonstrated specificity of these peptides for the operator sequence. We have assessed the contributions of the NH$_2$-termini to the DNA binding activity of the protein by examining the nonspecific DNA binding activity of all the hybrid tetramers. If each monomer with an intact NH$_2$-terminus could bind DNA independently and without interaction with the other subunits, the expected behavior would be that of an increasingly concentrated solution of monomers as NH$_2$-termini are added to the hybrid tetramer. This pattern approximates the behavior observed, within the limits of precision of the assay. To test this interpretation, the nonspecific DNA binding affinity of hybrid tetramer 1:3 as a function of salt concentration was measured. This determination showed that the interaction of the 1:3 tetramer with nonspecific DNA displayed the same dependence of ionic strength as native repressor. Since the slope of a log-log plot of the dissociation constant versus the Na$^+$ concentration is related to the number of ion pairs involved in the protein-DNA interaction (de Haseth et al, 1977), repressor (4:0) and the 1:3 hybrid must utilize the same number of ion pairs in binding to nonspecific DNA under the conditions of this assay. These results suggest that the NH$_2$-termini function as independent, noninteracting binding sites for nonspecific DNA.

Examination of the dissociation constants of the hybrid tetramers for λplac DNA and the 29 base pair operator fragment, Table VII, indicates that all four monomers interact with operator. These data are
consistent with the results of Geisler and Weber (1976) who showed that although operator binding for the hybrid tetramers 4:0 and 3:1 appear to be identical under stoichiometric conditions, their respective rates of dissociation from operator differ, presumably reflecting a difference in dissociation constant. However, the differences in the operator dissociation constants with subtraction of each NH$_2$-terminus are not equivalent, and thus the contribution to the free energy of binding by each amino-terminus to operator is not equivalent. The addition of one NH$_2$-terminus to the core $K_d$ ($2 \times 10^{-7}$ M) contributed 50% of the difference in the free energy of binding between core and native repressor, as calculated from the dissociation constants of the hybrid tetramers for both λplac and operator fragment. The second amino terminus contributes approximately 25% of the difference in free energy of binding to λplac or operator fragment, while the final two NH$_2$-termini each contribute 10–15% of this difference in free energy of binding between core and intact tetramer. As indicated by the dissociation constants of the hybrid tetramers for operator, the contributions of each NH$_2$-terminus to the operator affinity are less than would be expected if every protein-DNA contact implicated by nonspecific DNA binding affinity were involved in the repressor-operator DNA interaction. This conclusion assumes that the core-operator contacts are not altered by the addition of the NH$_2$-termini, a likely possibility (see Chapter V). Specifically, addition of one NH$_2$-terminus to the core protein increases the affinity of the protein for λplac operator DNA by a factor of approximately 1000. If every contact available for interaction with DNA which is measured by the nonspecific DNA binding of the 1:3 hybrid tetramer were used in
operator DNA binding and all core contacts were maintained, we would expect the observed increase in affinity to be $10^6$, corresponding to a dissociation constant of $10^{-13} \text{ M}$ for the 1:3 hybrid. This is, in fact, the affinity of native repressor for operator. However, the observed dissociation constant of the 1:3 hybrid is $2 \times 10^{-10} \text{ M}$; from this result, the apparent affinity of the NH$_2$-terminus for operator is $10^{-3} \text{ M}$. The addition of each subsequent NH$_2$-terminus contributes progressively less to the free energy of binding between the repressor and operator.

The rate of dissociation of the repressor-operator complex increases with the loss of each NH$_2$-terminus by approximately a factor of 2, while the rate of association calculated from the dissociation rate and the equilibrium dissociation constant for each hybrid tetramer decreases by a factor of 5. Goeddel et al. (1977) demonstrated that the changes in the dissociation constant of repressor for operator fragments of various lengths is reflected only in the rate of dissociation; they suggest that the repressor probably does not make a one-dimensional search down the DNA to the operator nor participate in intradomain transfer. These authors attribute the anomalously fast association rate for repressor-operator to electrostatic interaction between the protein and DNA. This view is supported by the sensitivity of the association rate to ionic strength (Riggs et al., 1970 b). O'Gorman et al. (1980 a) have shown that the electrostatic contacts of the repressor-operator interaction reside primarily in the NH$_2$-terminus. Our data suggest that the NH$_2$-termini, and thus the protein-DNA ionic contacts, are critical in the association reaction. von Hippel et al. (1975) have postulated the existence of two DNA binding sites on the
protein and have suggested that the mechanism of association of repressor to operator involves the transfer of the protein between DNA molecules. The data presented here are also consistent with this view of the association process and further suggest that both the acceleration due to electronic forces and the freedom of motion of the NH$_2$-termini are important for association of the repressor to operator. It would be predicted from these observations that the association rate of repressor to nonspecific DNA would be the same order of magnitude, if not faster than, the association rate of repressor to operator; in contrast, the association rate of core which has been stripped of its NH$_2$-termini and thus many of its basic amino acids to operator would be anticipated to be much slower than the rate of repressor-operator.

The results of poly[d(A-T)] competition with operator DNA for binding to repressor suggest two operator DNA binding sites on the protein and dissimilar contributions of the NH$_2$-terminus to these sites. In contrast to the expected results, the poly[d(A-T)] displayed minimal competition in binding to the operator DNA binding site in the 1:3 hybrid. Apparently, the operator DNA which is displaced is bound to the site to which the single NH$_2$-terminus present makes major contributions. This displacement is possibly due to the high affinity of a single NH$_2$-terminus for nonspecific DNA. However, the displaced operator can easily reassociate with the protein at the second operator DNA binding site (to which the remaining NH$_2$-terminus contributes significantly fewer contacts) and thus will not be subject to competition with the poly[d(A-T)]. The minimal competition observed for the 1:3 tetramer is due to reassociation of the operator at this second
operator binding site. These results demonstrate that the single NH$_2$-terminus has DNA contacts in both sites such that the affinity of each site is higher than the affinity of core for operator (see the model in Chapter V), but these contacts are asymmetric with regard to these two binding sites. These results have bearing on the geometry and composition of the operator binding site (Chapter V).

Studies by Kania and Müller-Hill (1977) on hybrid proteins of β-galactosidase and a repressor-β-galactosidase chimaera have addressed the requirements for an intact tetrameric structure for operator DNA binding. Crosslinking studies on the hybrids have shown that the repressor domain in the chimaera crosslinked only to other repressor domains, and the product structures were limited to dimers. These workers found that the 1:3 hybrid, which contains only one subunit of the repressor-β-galactosidase chimaera, had no detectable operator DNA binding activity, while the 2:2 hybrid exhibited a dissociation constant for λplac DNA of $\sim 10^{-9}$ M according to our calculations from their data. This binding is an order of magnitude less than the affinity we measure for our 1:3 hybrid tetramer, and only two orders of magnitude greater than core binding. Thus, while the dimeric species display some operator activity, the full binding activity seems to require all four core subunits as well as all four NH$_2$-termini.

The characteristics of the hybrid tetramers of the lactose repressor protein and its trypsin resistant core protein are consistent with a model in which all four subunits of the repressor interact with operator DNA. All four NH$_2$-termini appear to contact the operator. Although each NH$_2$-terminus interacts with operator DNA, the free energy
contribution of the individual NH$_2$-termini to operator binding is not uniform and is of lower magnitude than anticipated from the free energy of binding of isolated NH$_2$-termini to nonspecific DNA. While the precise number of core domains involved in binding to operator DNA cannot be determined from these studies, comparison of the affinity of dimeric repressor attached to β-galactosidase with the affinities measured here indicates that more than two of the core domains are required. All four NH$_2$-terminal regions are capable of binding independently to nonspecific DNA; this feature of the protein coupled with the estimated association rates of the hybrid tetraceres provides evidence to support the role of this region in the fast association reaction of repressor to operator. These studies provide valuable information for understanding the role of the different domains of the repressor protein in binding to its ligands and thus in its genetic control function.
CHAPTER V

A Model for the Lac Repressor Protein and its Interaction with Ligands
The results from the hybrid tetramer experiments coupled with other studies on the NH$_2$-terminal and core domains of the repressor (Burgum and Matthews, 1978; Matthews, 1979; O'Gorman and Matthews, 1980 a) prompted reconsideration of how this protein functions. It was clear that current abstractions on the NH$_2$-terminal region as both the operator and nonspecific DNA binding site did not adequately explain all of the existing experimental data. In addition, in the absence of any evidence that the conformational change elicited by inducer affects the NH$_2$-terminal region, it became increasingly difficult to explain how the inducer associated conformational change could affect operator but not nonspecific DNA binding and thus, how induction might occur. Attempts to answer these questions led to the proposal of a model consistent with most, if not all, of the available data on the lac repressor and its interaction with its ligands.

THE MODEL

The proposed model is illustrated in Figures 26, 27 and 29, and has the following features. The repressor has a plane rectangular structure (140 Å by 60 Å by 45 Å) with three 2-fold axes of symmetry. The protein has two potential operator DNA binding sites; these sites are identical and appear as grooves parallel to the long axis of the protein. There are four inducer binding sites, one on each subunit. Both NH$_2$-terminal and core regions of each subunit contact each operator DNA. The NH$_2$-termini bind to the operator DNA sequence in each site in a symmetrical fashion, making contact at the outer ends of the specific operator sequence and utilizing largely ionic forces. In contrast, the
core domains of the subunits bind asymmetrically and primarily by nonionic contacts. The major effect of inducer binding and the subsequent conformational change is to decrease the nonionic interactions between the core region and the operator DNA sequence, while leaving the NH$_2$-terminal contacts intact. This maintenance of NH$_2$-terminal contacts is allowed by the separate secondary structure and freedom of movement of each NH$_2$-terminus. Also, due to the flexibility available, each NH$_2$-terminus is potentially able to bind independently to nonspecific DNA.

EVIDENCE FOR THE MODEL

Major Structural Features. Steitz et al. (1974) have presented powder X-ray diffraction and electron microscopic studies on microcrystals of repressor protein; their results suggest that the protein exhibits a plane rectangular structure of 222 symmetry. The molecular dimensions are 140 Å by 60 Å by 45 Å. These workers propose that the operator DNA binds to repressor with its long axis aligned with that of the repressor and with its 2-fold axis of symmetry coincident with a 2-fold axis of the repressor. While structures with other shapes have been observed using conventional electron microscopy (Ohshima et al., 1975; Abermann et al., 1976), the general instability of the repressor protein to environmental extremes leads us to favor the structure suggested by the studies on microcrystals of the protein. The presence of two DNA binding sites proposed by Steitz et al. (1974) has been confirmed directly by measurements of operator DNA fragment (29 base pairs) binding to the protein (O'Gorman et al., 1980 a), by
formulating models to predict the data obtained for inducer binding to repressor-operator fragment complex (O'Gorman et al, 1980 b), and by the DNA binding studies using hybrid tetramers of core and repressor proteins (Chapter IV). The conclusions from these results are reflected in the model for the protein which is illustrated in Figure 26. This structure is similar to that proposed by Steitz et al (1974) in its dimensions and symmetry.

The presence of separate domains in the protein has been evident from a variety of studies on the protein. Mutations which affect binding to operator and frequently also to nonspecific DNA (i) map predominantly in the NH₂-terminal 60 amino acids of the protein (Pfahl et al, 1974; Müller-Hill et al, 1975; Coulondre and Miller, 1979; Beyreuther, 1978). In contrast, mutations which affect binding to inducer and/or the conformational change in response to this ligand (iS) do not appear at all in the NH₂-terminal amino acids, but are dispersed throughout the remainder of the protein structure (the core region). The clustering of these iS mutants has been interpreted recently by Miller (1979) in terms of the sugar binding site and the secondary structure required to form this site. In addition, Schlotmann et al (1975) proposed the existence of a "hinge" or "transmitter" region which conveys information regarding sugar binding from the core region to the NH₂-terminal putative DNA binding site; Beyreuther (1975) suggested that this hinge region is amino acids 50-60, since these residues appear to be extremely susceptible to attack by proteases. Removal of the NH₂-terminal amino acids 1-51 or 1-59 by proteolytic digestion results in a core protein which does not bind normally to either operator or non-
Figure 26. Lactose repressor protein bound to operator DNA. The protein is a rectangular planar molecule 140 by 60 by 45; three different views are shown. The sequence of DNA indicated is the 29 base pair operator DNA fragment which has been used in previous studies (Chapters III and IV). indicate the NH$_2$-terminal regions. indicates the areas of contact between the core region and the operator DNA sequence. are the inducer binding sites. Dotted lines indicate boundaries which are not in direct view. + indicates ionic interactions of the NH$_2$-termini, while ▲ indicates a nonionic interaction of the NH$_2$-termini (e.g., intercalation by tyrosine, hydrogen bonding). The existence of these contacts are indicated by the literature, but the positions are only suggested and should not be considered definitive.
specific DNA (Platt et al., 1973; Lin and Riggs, 1975). The NH₂-terminus can be isolated intact (i.e., amino acids 1-51 or 1-59) and has been shown to bind to DNA (Geisler and Weber, 1977, 1978; Jovin et al., 1977) and to exhibit some specificity for the operator sequence (Ogata and Gilbert, 1978, 1979). The isolated NH₂-terminus possesses significant secondary structure (Jovin et al., 1977), and NMR studies have indicated that the amino acid side chains in this region in native repressor are highly mobile relative to those in the core region (Buck et al., 1978; Wade-Jardetzky, 1979). These results suggest a secondary structure for the NH₂-terminus which is different from the core, and further, that the attachment between the NH₂-terminus and the core region must be sufficiently flexible to allow the observed freedom of motion. Yet, binding of 8-anelino-1-napthalenesulfonate (ANS) to intact repressor is significantly greater than binding to core protein or to the isolated NH₂-terminus; thus, it appears likely that some contact between the NH₂-terminal region and the core region must exist and provide a site for ANS binding (York et al., 1978). The separate structure of the NH₂-termini and the presumed contact between the core and NH₂-terminal regions is indicated in the depiction of the model (Figures 26 and 27).

Studies on inducer binding to core protein (Friedman and Matthews, 1978) and results of chemical modification experiments (Manly and Matthews, 1979; Burgum and Matthews, 1978) led to the hypothesis that this region of the protein contained sites for interaction with operator DNA. Recent studies on isolated core protein have demonstrated specific, inducer-sensitive binding of this tetrameric species to operator-containing DNA (Matthews, 1979), while the nonspecific DNA
Figure 27. Lactose repressor protein free and bound to inducer. A single side view of the repressor is shown; A is the free repressor, while B is repressor in the presence of inducer. The symbols are defined in Figure 26. Note both the separate structure and the freedom of motion of the NH$_2$-terminal regions.
binding of the core protein was not detectable by the assay \( K_d > 10^{-5} \).
The core region has been suggested to have an asymmetric shape by low angle X-ray scattering (Wade-Jardetzky et al., 1979). Mutants which are deficient in aggregation ability map primarily in the core region of the molecule (Miller, 1979). Thus, the core regions of the monomers must interact to form the quaternary structure, while the NH\(_2\)-termini exist as separate units attached to the core regions. In the model presented, the NH\(_{2}\)-termini can fold over the end of the core region or they may exist as freely mobile, separate domains (Figures 26 and 27).
The two DNA binding sites indicated in the model are formed by grooves which parallel the long axis of the protein structure; the faces of each groove are contributed by both core and NH\(_{2}\)-terminal sections of the protein monomeric structure (Figure 26). The sugar binding site is indicated on the side of each core region; this location is arbitrary. Miller (1979) has suggested that such a site could be formed by beta pleated sheets.

Interaction of the Protein with Operator DNA. The 29 base pair operator DNA fragment used in the studies reported in the preceding chapters (4-7) binds to the repressor protein as indicated in Figure 26. These fragments consist of the central 21 base pair interior sequence which is protected from nuclease digestion by repressor protein. This sequence is bounded by Eco RI restriction sites as shown. Bahl et al. (1977) have suggested that only 17 base pairs are essential for specific interaction of the operator sequence with repressor; however, other workers have presented evidence for interaction with base pairs outside this 17 base pair sequence (Goeddel et al., 1978). Methylation studies
suggest interactions for the repressor in both the major and minor grooves of the operator DNA (Gilbert et al., 1975).

The binding of the operator fragments to repressor protein is significantly less salt dependent than repressor binding to \( \lambda \)plac DNA (O'Gorman et al., 1980 a; Goeddel et al., 1977); this result suggests that ionic contacts made on the outer regions of the operator DNA sequence in \( \lambda \)plac DNA are missing in repressor binding to operator DNA fragments (Figure 26). The binding of fragments to the repressor-inducer complex displays essentially the same salt dependence as the repressor-operator fragment interaction (O'Gorman et al., 1980 a); this similarity implies that the ionic contacts are similar for free and induced repressor. Such contacts must involve \( \text{NH}_2 \)-terminal regions since the core-operator contacts are nonionic in nature. These observations are reflected in the model. In addition, studies utilizing hybrid tetrarmers containing differing ratios of core protein:native repressor indicate that each of the \( \text{NH}_2 \)-termini make contact with both \( \lambda \)plac DNA and operator DNA fragments (Chapter IV). These interactions appeared to be major for two of the \( \text{NH}_2 \)-termini and minor for the additional two \( \text{NH}_2 \)-termini. This differential in contact between the four \( \text{NH}_2 \)-termini and operator sequence is indicated in Figure 26.

Core protein binding to both \( \lambda \)plac and operator fragment DNA's displays identical association constants and essentially no salt dependence (O'Gorman et al., 1980 a); thus, the affinity of this region of the protein for both operator DNA's involves primarily, if not exclusively, nonionic forces. Goeddel et al. (1977) report that synthetic operator DNA's less than 26 base pairs in length bind less well
than λplac DNA and suggest that this is due to electrostatic interactions between repressor and the DNA sequence outside of this region. In addition, these workers find that when binding affinity is decreased by substitution of thymine with BrdUrd, less ionic strength dependence of the binding is observed (Goeddel et al, 1978). This decrease suggests that the remaining affinity is more nonionic in nature and attributable to core-operator interaction according to our model.

Studies of operator DNA binding to repressor utilizing either DNA methylation (Ogata and Gilbert, 1977, 1978, 1979), ethylation (von Hippel, 1979), or cross-linking of BrdUrd-substituted DNA (Gilbert et al, 1975 a) have indicated sites in the operator sequence in which these reactions are modified by the presence of repressor protein. It is of interest to note that there is significant symmetry in the outer regions of the operator sequence (i.e., positions 6-10 and 22-26) in the effects observed upon both modification and base substitution (Figure 28). The bases in positions 6, 7, 25 and 26 have been identified as points of contact for the repressor (Goeddel et al, 1978). Base substitution at symmetrical points in these areas results in identical loss in free energy of binding for the synthetic operator sequences. According to our model the interaction in these outer regions would be made by the NH₂-termini. Lin and Riggs (1975) found that repressor binding to poly [dTGG:dCCA] (positions 8-10 and 22-24) was tighter than to poly [d(A-T)] but was not inducer sensitive; this finding is consistent with the model presented, since the increased affinity would involve only the NH₂-termini, which are essentially unaffected by inducer binding. However, in the inner region of the sequence, where the core (nonionic) contacts would
Figure 28. Operator DNA sequence. The wild-type sequence of the operator is shown (Gilbert et al., 1975) and the positions numbered for reference in the text. The arrow indicates the central base pair of the symmetric region of the operator, and the bars show the symmetric sequences. The negative and positive signs near the bases indicate either protection (−) or enhancement (+) of methylation in the presence of repressor (Ogata and Gilbert, 1979). Operator constitutive mutations are indicated (Gilbert et al., 1975) as well as the thymines which can be cross-linked to the repressor protein (Ogata and Gilbert, 1977). Phosphate linkages which exhibit altered reactivity in the presence of inducer are indicated by asterisks between the base pairs (von Hippel, 1979). The outer regions of the sequence which we are proposing to be involved in interactions with the NH₂-terminal region are boxed by dotted lines. The interior of the sequence is proposed to bind specifically with the core domain of the protein.
be made according to the model we are proposing, significant asymmetry in the chemical effects is observed. The asymmetry of contacts in this central region of the operator sequence is further suggested by the change in the methylation pattern for induced repressor (Ogata and Gilbert, 1979). This asymmetry was first noted in the prevalence of $^{32}C$ mutation placement to the promoter proximal side of the symmetry axis of the central operator sequence (Gilbert et al, 1975 b). In addition, by inference from the homology between the operator region and the "pseudooperator" present in the Z gene, it appears that the promoter proximal half of the central region contributes significantly to recognition of repressor and/or maintenance of high affinity (Sobell, 1979). Goeddel et al (1978) found that base substitutions in the symmetry-equivalent base pairs in this central region result in dissimilar changes in the measured binding free energies. The central base pair in the symmetrical operator sequence has been placed in the model so that of the two adjacent core subunits make greater contact with the central operator sequence than the remaining two. This specific juxtaposition of the two macromolecules accounts for the asymmetry observed in the effects of repressor on the chemical modification of the central region of the operator DNA and the effects of base substitution.

It is unclear whether operator bound to the repressor maintains the B structure of DNA; studies using supercoiled DNA have shown that the repressor binding unwinds the DNA by 40-90° (Wang et al, 1974), and kinks and/or intercalation of amino acid side chains would also alter the structure of the DNA. If present, all of these potential per-
turbations could introduce additional asymmetry into the interaction of the protein with the operator. Several models have been postulated for the role of specific amino acids and/or base pairs in the interaction of repressor and operator (Adler et al, 1972; Gursky et al, 1977; Jones and Olson, 1977). Our model is not derived from data which would allow speculation regarding the specific amino acid residues involved in defined contacts with the DNA, only the postulation that certain types of contact do exist (e.g., ionic, nonionic, etc.).

The model presented provides that each of the four core regions as well as each of the four NH₂-terminal regions make some contact with the operator DNA. This provision is based both on studies with hybrid tetramers which indicate that all four NH₂-termini contribute to binding operator DNA (Chapter IV) and on equilibrium binding data of inducer to repressor-operator complexes which can be simulated only by models in which all four subunits are affected by operator DNA binding (O'Gorman et al, 1980 b). In contrast, Kania and Müller-Hill (1977) have described studies on hybrid tetramers composed of β-galactosidase and repressor-β-galactosidase chimaeric monomers from which they conclude that the protein may bind to operator DNA as a dimer. However, the affinities of the 2:2 hybrids for operator which can be derived from their binding plots are significantly lower than for native repressor protein (they are in fact lower than our 1:3 hybrid tetramer of repressor and core protein, Chapter IV). Thus, the dimeric structure (at least attached to β-galactosidase) is not sufficient to provide all the contacts for native operator DNA binding. It is clear that our model predicts that a dimeric structure would have appreciable, though not native, operator and nonspecific DNA binding capacity.
Interaction of Repressor with Nonspecific DNA. The isolated NH$_2$-termini have been shown to bind to nonspecific DNA (Geisler and Weber, 1977) and the presence of the NH$_2$-terminal region alone (amino acids 1-60) attached to β-galactosidase results in the ability of that protein to bind to phosphocellulose (Müller-Hill, et al, 1977). The core protein does not have measurable affinity for nonspecific DNA (Matthews, 1979). Measurement of nonspecific DNA binding by hybrid tetramers containing 0 to 4 NH$_2$-terminal regions indicates that the NH$_2$-termini in the tetrameric structure can bind to nonspecific DNA independently (Chapter IV); this result was also suggested by Geisler and Weber from their studies on isolated NH$_2$-termini (1977). At low ratios of protein to DNA, the NH$_2$-termini apparently interact independently with nonspecific DNA; as the ratio of protein to DNA is increased, the NH$_2$-termini must occupy sites on the same DNA. At levels of protein which would saturate all available DNA sites, the protein will occupy sites on both sides of the DNA (Zingsheim et al, 1977). At low protein to DNA ratios, the DNA molecules essentially compete for binding to the NH$_2$-terminal regions; this situation results in each NH$_2$-terminus binding effectively independently, presumably by taking advantage of the mobility available to this region. A decrease in the contact between the NH$_2$-terminus and its adjacent core as a result of nonspecific DNA binding is suggested by the results of Worah et al (1978). These workers measured the binding of poly [d(A-T)] to repressor protein by monitoring the decrease in ANS fluorescence upon DNA binding. They interpreted this decrease in terms of either quenching of the ANS fluorescence or the dissociation of ANS
due to DNA binding. These workers have also suggested that ANS binds at the interface between the core protein and the \( \text{NH}_2 \)-terminus. In light of these observations, we favor the interpretation that binding of non-specific DNA to the \( \text{NH}_2 \)-terminus results in a dissociation of this region from its contact with the core protein thus resulting in release of a portion of the ANS molecules bound at that interface. Because the interaction of the native protein (and hybrid tetramer with a single \( \text{NH}_2 \)-terminus) with nonspecific DNA is highly ionic strength dependent, the binding must involve ion pairs. Record et al. (1977) have estimated the number of ion pairs involved in the interaction of repressor and nonspecific DNA. It is interesting to note that the nonspecific binding of the 1:3 hybrid tetramer (containing only one \( \text{NH}_2 \)-terminus) displays similar salt dependence to intact repressor (Chapter IV); this result implies that a similar number of ion pairs are involved in the interaction, a situation consistent with the binding of each \( \text{NH}_2 \)-terminus independently under the conditions of the measurements (about equal mixtures of protein and nonspecific DNA). The binding of non-operator DNA appears to be less specific than binding to operator, not merely in terms of the sequence of the DNA but also in terms of the spatial juxtaposition within the molecule which is required. The model presented is consistent with this aspect of repressor function.

We have not addressed the precise mechanism of interaction of the \( \text{NH}_2 \)-terminal region with nonspecific DNA. However, Richmond and Steitz (1976) have modified a poly[d(A-T)] analog to fill the major groove and thus eliminate any interactions occurring in this region of the DNA. This modified DNA binds normally to repressor; therefore, no
major groove interactions are required for nonspecific DNA binding. Further, minor groove interactions for nonspecific binding have been demonstrated by Kolchinsky et al (1976). Thus, contacts between the repressor protein and nonspecific DNA must occur along the polyphosphate backbone and in the minor groove of the molecule.

**Effects of Inducer on Repressor Protein.** The inducer binding site has been determined by genetic studies to be in the core region of the repressor protein (Pfahl et al, 1974; Miller-Hill et al, 1975; Coulondre and Miller, 1979); no iS mutants are found in the NH2-terminal amino acids (Miller, 1979). We have placed the inducer binding site on the side of the core protein, removed from the operator DNA binding site of the core and from the NH2-terminal regions. This placement is arbitrary and reflects only the demonstrated distinction between these regions. In the presence of inducer, the repressor protein assumes a new conformation which no longer binds with the same high affinity to operator DNA or is trapped into its conformation with lower affinity for operator. (O’Gorman et al, 1980 b). However, the interaction of the protein with nonspecific DNA is unchanged. The conformational change does not affect the ionic contacts between repressor and operator DNA fragments, as indicated by the similar salt dependence of the binding to fragments in the presence and absence of inducer (O’Gorman et al, 1980 a). Thus, the changes which occur must be in the nonionic components of the interaction, which are contributed primarily by the core protein according to our model. The changes envisioned in response to inducer are illustrated in Figure 29. The core protein has changed in dimensions to 120Å by 70Å by 45Å, thus be-
**Figure 29.** Repressor protein in the presence of operator DNA and inducer protein. The symbols are defined in Figure 26. In the presence of inducer the core region of the protein becomes more spherical as indicated by sedimentation and other physical studies (Ohshima et al., 1972; Kwok and Matthews, unpublished results). The extent of this change in shape has been exaggerated in the Figure (a 20 Å decrease in the length of a 10 Å increase in width); the actual alterations in the dimensions of the protein are not currently known, but the illustration reflects the significant change in the shape of the core region of the protein in response to inducer. The views of the structure are identical to those shown in Figure 26. Note that the NH₂-terminal regions make the same contacts with the operator DNA sequence as in the absence of inducer, while the core region contacts are significantly disrupted by the conformational change consequent to inducer binding. The inducer binding site is illustrated as "closing in" around the sugar molecule and thus altering the structure of the core region of the protein; the NH₂-termini are unaffected by this change because of their separate structure and mobility (see Figure 27).
coming more spherical, while the NH$_2$-termini are able to assume the same spatial arrangement as in the absence of inducer. Sedimentation studies (Ohshima et al., 1972) indicate that the repressor does assume a more spherical shape upon binding inducer; the probable magnitude of this change has been exaggerated in the schematic representation of the model for emphasis (Figure 29). The compaction of the protein on binding inducer removes some amino acid residues from solvent exposure and should increase the rotational relaxation time of the protein. Spectral and physical studies of the protein are consistent with this view (Ohshima et al., 1972; Kwok and Matthews, unpublished results). Isolated core protein binding to operator DNA's is nonionic, and the affinity of the core for operator DNA changes by a minimum of 50-fold in the presence of inducer. Thus, the core contacts with operator DNA are significantly affected by the binding of inducer, while those in the NH$_2$-terminal region remain essentially unchanged (see Figure 29).

The significant freedom of motion and the independence in binding to nonspecific DNA of the NH$_2$-termini are not affected by the presence of inducer nor does the sugar ligand alter the ability of the NH$_2$-termini to make the specific contacts with the operator sequence indicated by the model (Figure 29). Because of the free movement of this region (Figure 27), even if the spatial relationship between the attachments of the core regions to the NH$_2$-termini is altered by the conformational change of the core in response to inducer, the NH$_2$-termini can still assume a position to interact specifically at the indicated sites at each end of the operator sequence (Figure 29). The specificity of these NH$_2$-terminal contacts results in the higher affinity
observed for operator DNA relative to nonspecific DNA even in the presence of inducer (Bourgeois and Pfahl, 1976). Methylation studies by Ogata and Gilbert (1978, 1979) also support this view, since isolated NH$_2$-termini and induced repressor produce similar effects on operator DNA methylation protection/enhancement patterns. These effects are dissimilar from the pattern observed for intact repressor, specifically in the region of the operator DNA sequence which we are postulating interacts with the core domain of the repressor molecule (see Figures 26, 28, 29). A large body of evidence for the conformational change affecting various amino acid residues in the core region is available (Laiken et al, 1972; Matthews, 1974; O'Gorman and Matthews, 1977; Sams et al, 1977), while there is no experimental data to support any effect of inducer binding on residues in the NH$_2$-terminal region. The absence of mutants in this region which affect inducer binding may be construed as negative evidence for a relationship between the NH$_2$-termini and the inducer binding site (Miller, 1979). The view that the specificity and inducer-sensitivity of the repressor are properties attributable to the core region rather than the NH$_2$-termini is contrary to much speculation in the literature (Ogata and Gilbert, 1979; Müller-Hill et al, 1977; Wade-Jardetzky, 1979).
CHAPTER VI

Conclusion - Implications of the Model
The lac repressor protein represents one of the simplest systems of genetic control under study: the repressor binds to the operator apparently physically blocking transcription of the structural genes. The model presented in Chapter V is based on a wealth of experimental evidence and attempts to explain how the repressor seeks and locates its operator, the nature of the high affinity of the protein-operator interaction and how induction occurs. Certainly, there are still questions to answer about this protein-DNA interaction, but the model provides a framework for designing and interpreting future experiments. Indeed, it is our hope that this model will stimulate reconsideration of the mechanism of repressor function, and that new experiments will be formulated to test and modify the features of the model.

There are several obvious experiments which could be used to test the validity of this model. Undoubtedly the most complete answer would come from the three dimensional crystallographic structure of the repressor co-crystallized with operator fragment. Steitz et al (1978) have reported the crystallization of the core protein and also the hybrid tetramer composed of three core subunits and one native monomer. Unfortunately, the space group of the protein is particularly unfavorable for crystallographic analysis (two tetramers per unit cell), and portends years of hard work before the structure of the protein is solved. Even if the native protein is crystallized and its structure solved, the protein-DNA contacts may not be defined. It has been suggested that difficulties encountered in the crystallization of the repressor protein may be due to the un-
usually free-moving NH$_2$-termini (J. M. Rosenberg, personal communication). However, there are several good experiments besides the X-ray structure which can be done immediately to test this model.

It is evident from the model that the results of chemical and enzymatic treatment of the operator DNA sequence in the presence and absence of core protein would be expected to yield significantly different patterns of modification than the repressor protein. These effects would be especially apparent in the outer regions of the sequence which interact primarily with the NH$_2$-termini. According to the model, crosslinking of the protein and the operator DNA should yield amino acids in the NH$_2$-terminus attached to bases in the outer regions of the operator sequence, while amino acids in the core region would react with base residues in the inner, central sequence of the operator DNA. While the model makes no predictions for specific amino acids interacting with the DNA or inducer molecules, it is apparent that future efforts to chemically modify the repressor's active sites, either with reagents specific for a given amino acid or by affinity labelling, will yield additional information on the role of specific residues in the structure and function of the protein.

Can the model of the lac repressor give any insights into the general mechanism of protein-DNA binding? Genetic control proteins from procaryotes can be designated as either negative or positive control proteins according to their mechanism of action. Negative control proteins are termed repressors (e.g., the lac repressor, the λ repressor, the tryptophan repressor) and bind to the DNA pre-
venting transcription of some portion of the genome. The repressors responsible for regulation of anabolic or catabolic pathways are modulated by the presence of low molecular weight metabolites which cause a conformational change in the protein, affecting the affinity of the proteins for their target DNA sequence. Therefore, this class of negative control proteins can be further divided by the effect of small ligands on the protein-DNA interaction. Repressors such as \text{lac} which are removed from the operator on binding of signal molecules are inducible repressors; repressors such as \text{trp} which bind to the operator with a much higher affinity when complexed with the effecting metabolite, regulate repressible systems. Positive control proteins such as \text{CAP} enhance transcription of a portion of the genome and can also be further classified as either inducible or repressible. The possible permutations are schematically represented in Figure 30 (Hood \textit{et al.}, 1975).

Positive control proteins are generally considered to be melting proteins. The classical characteristic of melting proteins is the ability to bind to both single-stranded and duplex DNA. Both \text{CAP} and RNA polymerase bind to single-stranded and duplex DNA (Riggs \textit{et al.}, 1971; Nissley \textit{et al.}, 1972; de Haseth \textit{et al.}, 1978), while the \text{lac} repressor displays only minimal affinity for single-stranded DNA. Presumably, repressors as a class do not have this dual activity and therefore are simpler in their mechanism of function. However, the paucity of physical studies on other DNA binding proteins means even this conclusion is speculative. The \text{lac} repressor is unique among the repressors listed in Table X in its unusually high affinity for
Figure 30. Symmetry diagram of possible operon control systems involving a regulatory protein and a controlling metabolite. M represents a controlling metabolite and P represents a regulatory protein (Hood et al, 1975).
Positive
(P is an activator)

Negative
(P is a repressor)

Inducible
(M turns transcription on)

Repressible
(M turns transcription off)

P inactive → P active → genes ON
(e.g., lac, gal)

P inactive → P active → genes OFF
(e.g., trp, his)

P inactive → P active → genes OFF
(no examples)
TABLE X

Some Genetic Regulatory Proteins and Their Characteristics

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight (daltons)</th>
<th>Active Form</th>
<th>Domains</th>
<th>Action</th>
<th>$K_D^1$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lac repressor</td>
<td>37,500</td>
<td>tetramer</td>
<td>yes</td>
<td>negative</td>
<td>$10^{-13}$</td>
</tr>
<tr>
<td>trp repressor</td>
<td>58,000</td>
<td>--</td>
<td>--</td>
<td>negative</td>
<td>$10^{-10}$</td>
</tr>
<tr>
<td>λ repressor</td>
<td>27,000</td>
<td>dimer</td>
<td>yes</td>
<td>negative</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>cro protein</td>
<td>7,500</td>
<td>dimer</td>
<td>--</td>
<td>negative</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>CAP</td>
<td>22,500</td>
<td>dimer</td>
<td>yes</td>
<td>positive</td>
<td>--</td>
</tr>
</tbody>
</table>

$^{1}$ The approximate dissociation constants for each protein and its target DNA sequence are reported.

$^{2}$ The λ repressor also stimulates its own synthesis.
its operator and in its tetrameric quaternary structure. These differences caution against freewheeling generalizations about negative control proteins.

However, it does appear that many of the DNA binding proteins have domains. Mild proteolysis of CAP with subtilisin in the presence of cAMP produces a large NH₂-terminal resistant core which binds cAMP but has lost its ability to bind DNA (Krakow and Pastan, 1973). The eucaryotic histone proteins have a highly basic, variable NH₂-terminal region and a globular, conserved COOH-terminal region (Isenberg, 1979). A structure as rich in basic amino acids as the NH₂-terminus would be predicted to be used primarily in nonspecific DNA binding, a concept consistent with its variable nature and current views of histone function. The globular portion has a normal amino acid composition and appears to be involved in specific histone-histone interactions.

Other workers (Müller-Hill et al, 1977) have suggested that the λ repressor and the lac repressor are very similar in their mechanisms of binding to DNA. The λ repressor binds to an operator region on λ phage blocking transcription of the early genes and maintaining the phage in the lysogenic state. Similarities in the proteins were based on genetic evidence that in both proteins the NH₂-terminal region was responsible for DNA binding (Oppenheim and Noff, 1975; Lieb, 1976). Further studies on the λ repressor using proteolysis and physical techniques indicate that the NH₂-terminal domain is the DNA binding species, and the COOH-terminal regions of the protein are involved in subunit-subunit interactions. The two domains are approximately the same size and papain resistant, while the connecting amino acid sequence
between the two domains is much longer than in the lac repressor (40 amino acids) (Pabo et al, 1979). The model proposed by Ptashne et al (1980) for the action of λ repressor and cro protein in the regulation of this system is dependent largely on the subunit-subunit and dimer-dimer interactions between COOH-terminal regions. It should be noted that the λ repressor does not respond to the presence of a low molecular weight signal molecule, and therefore requires no conformational change.

It is likely that the relationship between the ionic and non-ionic contacts of the DNA binding proteins will be an important feature in their function. A sophisticated example of this idea is found in RNA polymerase from E. coli. The polymerase is composed of four subunits which form the core enzyme: a₂, β, β'. The σ subunit is responsible for promoter recognition and transcription initiation and must be added to the core enzyme to form the active holoenzyme. Consideration of the functions of the protein-promoter interaction is awesome. Not only must the DNA sequence specify initiation and polymerase binding, but it must specify the strand to be transcribed and the direction of transcription; Pribrnow (1975) has suggested the minimum sequence which can fulfill these requisites. The polymerase must recognize and act on these signals. Perhaps it should be briefly noted that there appear to be at least two classes of promoters: those which are initiated by RNA polymerase alone and those which require other positive control proteins for full transcriptional levels. How these proteins function together is still unknown. Genetic analysis and reconstitution experiments indicate that the β' subunit of RNA polymerase
is responsible for template binding (Khesin et al., 1969; Panny et al., 1974). This subunit is the most basic of all the subunits. The core enzyme exhibits strong affinity for both duplex and single-stranded DNA ($K_a = 2 \times 10^6 \text{ and } 8 \times 10^7 \text{ M}^{-1}$, respectively), and this binding is extremely sensitive to ionic strength. The addition of the $\sigma$ subunit to the core enzyme to make the holoenzyme lowers the association constant of the polymerase for double stranded DNA to $1 \times 10^5 \text{ M}^{-1}$ but increases the enzyme's affinity for single-stranded DNA to $2 \times 10^{11} \text{ M}^{-1}$ while also conferring specificity to the protein-DNA interaction (de Haseth et al., 1978). The holoenzyme affinity for promoters is much less salt sensitive than the core enzyme, and the $\beta'$ subunit, implicated in template binding, is also responsible for interaction with the $\sigma$ subunit. These results support the idea of ionic contacts between proteins and DNA being responsible for general or nonspecific DNA binding, but primarily nonionic contacts being involved in sequence specific binding.

Thus, it appears that many of the DNA binding proteins have a basic NH$_2$-terminal region attached to a globular protein. The NH$_2$-terminus is involved in nonspecific and specific DNA binding. The globular portion of the proteins appears to be multi-functional; in the lac repressor this region is involved in operator binding, inducer binding and tetramer formation. Its operator contacts are primarily nonionic, in keeping with its globular nature. Both the lac repressor and $\lambda$ repressor have NH$_2$-termini which contain operator-specific contacts, but in neither of these cases do the NH$_2$-termini respond to conformational changes. Whether the core-operator interaction of the lac
repressor will be unique in this class of proteins is of course unknown. But it is clear that progress is being made in understanding protein-DNA interactions, and that the relationship and multiple functions of the two domains of the lac repressor is an important concept in the study of these proteins.
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