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Dissociation of the Lactose Repressor–Operator DNA Complex: Effects of Size and Sequence Context of Operator-Containing DNA†

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ABSTRACT: The dissociation kinetics for repressor–^{32}P-labeled operator DNA have been examined by adding unlabeled operator DNA to trap released repressor or by adding a small volume of concentrated salt solution to shift the K_d of repressor–operator interaction. The dissociation rate constant for pLA 322-8, an operator-containing derivative of pBR 322, was 2.4×10^{-3} s^{-1} in 0.15 M KCl. The dissociation rate constant at 0.15 M KCl for both λp*lac* and pIQ, each of which contain two pseudooperator sequences, was $\sim 6 \times 10^{-4}$ s^{-1}. Elimination of flanking nonspecific DNA sequences by use of a 40 base pair operator-containing DNA fragment yielded a dissociation rate constant of 9.3×10^{-3} s^{-1}. The size and salt dependences of the rate constants suggest that dissociation occurs as a multistep process. The data for all the DNAs examined are consistent with a sliding mechanism of facilitated diffusion to/from the operator site. The ability to form a ternary complex of two operators per repressor, determined by stoichiometry measurements, and the diminished dissociation rates in the presence of intramolecular nonspecific and pseudooperator DNA sites suggest the formation of an intramolecular ternary complex. The salt dependence of the dissociation rate constant for pLA 322-8 at high salt concentrations converges with that for a 40 base pair operator. The similarity in dissociation rate constants for pLA 322-8 and a 40 base pair operator fragment under these conditions indicates a common dissociation mechanism from a primary operator site on the repressor.

Transcriptional control of protein synthesis, in which a group of functionally related structural genes is coordinately regulated, was first proposed by Jacob and Monod (1961). The lactose repressor serves as a negative control unit for the *lac* enzymes by binding to the lactose operator site of the *Escherichia coli* genome and thereby physically blocking RNA polymerase transcription of the lactose metabolizing enzymes (Miller & Reznikoff, 1980). In the presence of inducer sugars, the repressor undergoes a conformational change, which results in decreased affinity for operator DNA. The excess of nonspecific DNA binding sites in the genome can then compete effectively with the operator for repressor binding, and transcription of the *lac* enzymes can be initiated.

The specific, tight binding of the lactose repressor to the operator DNA was originally characterized by using the nitrocellulose filter binding technique (Riggs et al., 1970a,b). Studies of the repressor–operator DNA complex as a function of monovalent salt concentration have indicated participation of 6–7 ionic interactions, whereas ~11 ionic interactions appear to be involved in the repressor–nonoperator DNA complex (deHaseth et al., 1977; Record et al., 1977; Revzin & von Hippel, 1977; Barkley et al., 1981; Winter & von Hippel, 1981). Studies on the salt dependence of the *lac* repressor–operator DNA complex dissociation have been reviewed by Lohman (1985). In general, the binding of proteins to DNA varies with the type of monovalent or divalent salt; the salt dependence is specific for the particular counterion on the basis of the valence and relative affinity of the cation for the anionic DNA. In the absence of divalent cations, plots of log k_d vs. log [M$^+$] for the repressor–operator DNA complex exhibit curvature thought to reflect multiple dissociation steps or an alteration in the rate-limiting step (Barkley, 1981; Winter et al., 1981; Lohman, 1985). In addition, a decreased repressor affinity for operator observed with DNA fragments less than 170 base pairs (bp)[1] suggests "long-range" effects for operator binding (Winter & von Hippel, 1981; O'Gorman et al., 1980a). The stoichiometry of the repressor–operator DNA interaction has been investigated by using the nitrocellulose filter assay (O'Gorman et al., 1980a), gel filtration, and circular dichroism

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[1] Abbreviations: bp, base pair; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactoside; ONPF, *o*-nitrophenyl β-D-fucoside; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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(Culard & Maurizot, 1981); a stoichiometry of two 29 bp operator fragments per repressor has been demonstrated. More recently, Barbier et al. (1984) found two nonspecific DNA binding sites on the lactose repressor by cross-linking small DNA fragments to the protein.

The dissociation of the repressor–operator complex has traditionally been measured by adding an excess of unlabeled operator DNA to a preequilibrated sample of repressor and labeled operator DNA (Riggs et al., 1970b). The excess unlabeled DNA "traps" any protein that dissociates from labeled operator DNA, making dissociation of the repressor-labeled operator complex essentially irreversible. The rate of dissociation is then measured as a decrease in labeled operator–repressor complex bound to nitrocellulose filters over time. Inducers and antiinducers can alter the dissociation rate of the complex; for example, IPTG, an inducer, increases the rate of dissociation of the repressor–operator complex by several orders of magnitude, while ONPF, an antiinducer, decreases the dissociation rate by approximately 8-fold (Riggs et al., 1970c; Barkley et al., 1975).

We have examined the effects of operator DNA size, the presence of pseudooperators, and varying salt on the *lac* repressor–operator dissociation rate. In addition, we have measured the stoichiometry for repressor binding to pLA 322-8, an operator-containing pBR 322 derivative; the results indicate formation of a ternary complex of two operators per repressor, an observation not previously reported for DNA of greater than 29 bp in length.

Materials and Methods

Isolation of Repressor. The lactose repressor was purified from *Escherichia coli* CSH 46 by the method of Rosenberg et al. (1977) as modified by O'Gorman et al. (1980a). The purity of the repressor (>95%) was assessed by sodium dodecyl sulfate gel electrophoresis.

Assay of Repressor. Isopropyl β-D-thiogalactoside (IPTG) binding activity was determined by the nitrocellulose filter and ammonium sulfate precipitation methods described by Bourgeois (1971). Inducer binding was assayed in 0.01 M Tris-HCl, pH 7.4, 0.01 M $MgCl_2$, 0.2 M KCl, 10^{-3} M EDTA, and 10^{-4} M DTT. DNA binding assays using the nitrocellulose filter method were performed as described previously (Riggs & Bourgeois, 1968; Hsieh & Matthews, 1981) with some modification. One inch square filters were cut from Schleicher & Schuell nitrocellulose paper (0.45 μm). The buffer for DNA binding experiments was 0.01 M Tris-HCl, pH 7.4, 10^{-4} M DTT, 10^{-4} M EDTA, 5% dimethyl sulfoxide, and KCl at the indicated concentration (TFB). Bovine serum albumin (50 μg/mL) was added to this buffer to stabilize diluted protein (TBB). All assays were carried out at 25 °C, unless stated otherwise. Repressor concentrations represent total protein concentrations. The protein exhibited 80–100% DNA binding activity under stoichiometric binding conditions.

Preparation of pLA 322-8, pOE 101, and pIQ. The plasmid pLA 322-8 was constructed by inserting a 95 bp *Alu*I fragment (including the *lac* promoter–operator region, −57 to +38) with a blunt-end ligation into pBR 322 previously cut and filled in at the *Bam*HI and *Eco*RI sites (gift to G. Bennett from J. Sninsky, Stanford). Neither of the pseudooperator regions of the *lac* operon is present in the inserted fragment. *E. coli* C600 cells were transformed with the plasmid DNA by the procedure of Kushner (1978); those cells containing pLA 322-8 were selected on nutrient agar plates with 20 μg/mL ampicillin. The plasmid pOE 101, containing 10–12 repeats of the 40 bp operator region with flanking *Eco*RI sites, was isolated from *E. coli* HB101 cells (Sadler et al., 1980). Cells containing pOE 101 were selected on nutrient agar plates with 15 μg/mL tetracycline. The plasmid pIQ, a pBR 322 derivative containing both pseudooperators in addition to the primary operator, was isolated (by A. E. Chakerian) from *E. coli* DH9 cells (gift from J. Betz). DH9 cells containing pIQ were selected on nutrient agar plates containing 20 μg/mL ampicillin and 15 μg/mL tetracycline. Cells, either C600, HB101, or DH9, were grown in M9 minimal media and amplified overnight with chloramphenicol (Norgard et al., 1979). Cell lysis was accomplished by Brij 58–deoxycholate treatment (Clewell & Helinski, 1970). Plasmid DNA was isolated by precipitation with poly(ethylene glycol) 8000 (Humphreys et al., 1975), RNase A treatment, extraction with phenol, precipitation by ethanol, and passage over an agarose A-50m column. The product plasmid DNA was >90% pure in the supercoiled form characteristic of naturally occurring DNAs; no contaminating DNA was detectable on 0.8% agarose gels stained with ethidium bromide.

Isolation of λplac Phage DNA. A thymine-requiring *E. coli* strain, MBC 5, which carried a temperature-inducible *lac* prophage (λ*plac* 5) was obtained from Mary Barkley, University of Kentucky, and isolated essentially by the procedure of Wang et al. (1974). Overnight cultures (5 mL) were used to inoculate 200 mL of minimal media (M9 with thiamine, proline, and thymidine), and the cells were grown to an absorbance of 1.0 at 590 nm. For labeled DNA [^{3}H]thymidine was added prior to duplicate 20-min inductions at 45 °C and separated by a 10-min incubation at 32 °C; the bacteria were then grown for an additional 5 h at 32 °C. Chloroform was used to lyse the cells, cell debris was removed by centrifugation, and a CsCl gradient was performed to isolate the phage.

Preparation of 40 bp Operator Fragment. The reaction buffer for the *Eco*RI restriction digestion of pOE 101 was 6 mM Tris-HCl, pH 7.4, 6 mM DTT, and 50 mM $MgCl_2$. Plasmid pOE 101 (20 mg) was digested (1 unit of *Eco*RI/2 μg of DNA) for 24 h at 37 °C. The digested sample was passed over an agarose A-50m column equilibrated in STE (0.01 M Tris-HCl, pH 8.0, 0.1 M NaCl, and 10^{-3} M EDTA). Operator fragment was labeled at the 3′-end by treatment with DNA polymerase I (Klenow fragment) and α-^{32}P-labeled dATP and dTTP and subsequently isolated from a 7% polyacrylamide separation gel by elution with buffer X [0.5 M NH_4OAc, 0.1 M $Mg(OAc)_2$, 0.1% SDS, and 0.1 mM EDTA]; the eluate was extracted with phenol, and the fragment was precipitated with ethanol.

Nick Translation of pLA 322-8 and pIQ. Linear plasmid DNAs were produced by cleavage with appropriate restriction enzymes: pLA 322-8, *Pvu*II; pIQ, *Pst*I. The operator region on these linear plasmids was located approximately 2000 bp from either end of the DNA. Digestion was monitored on a 0.8% agarose minigel. The procedure followed for nick translation of linearized pLA 322-8, and pIQ was essentially that of Maniatis et al. (1982). A mixture of DNA (2 μg) and unlabeled and α-^{32}P-labeled dNTPs in nick translation buffer (0.05 M Tris-HCl, pH 7.2, 0.01 M $MgSO_4$, 10^{-4} M dithiothreitol, and 50 μg/mL bovine serum albumin) was chilled; DNase I (0.5 ng) and 5 units of *E. coli* DNA polymerase I were added. The reaction mixture was incubated at 12 °C for 30 min, and the reaction was stopped by adding EDTA. Nick-translated DNA was separated from unincorporated dNTPs by repeated precipitation with ammonium acetate/ethanol.

Measurement of the Repressor–Operator Dissociation Kinetics. The buffer used for the dissociation kinetics was made from a 2 × stock of TFB, mentioned previously, to

minimize differences in buffer preparation; KCl was added to the appropriate concentration and the solution brought to twice the original volume. Lactose repressor protein was equilibrated 15 min at room temperature with labeled pLA 322-8, pIQ, λp*lac*, or 40 bp fragment in TBB. Repressor concentrations ranged from 5.4×10^{-12} to 1.1×10^{-10} M, while initial operator concentrations varied from 3.5×10^{-13} to 3.5×10^{-10} M. The repressor–operator dissociation was monitored in one of two ways: (1) An excess of unlabeled DNA was added to the equilibrated solution, as described by Riggs et al. (1970a,c), Winter et al. (1981), and Barkley (1981). (2) A salt jump (Lohman, 1980; Winter et al., 1981), performed by adding a small volume of 3 M KCl to the equilibrated mixture, resulted in an approach to a new equilibrium. Each sample was gently inverted 3 times immediately following addition of the excess DNA or 3 M KCl. Duplicate or triplicate aliquots (0.4 mL) were withdrawn from a total volume of 20 mL and filtered at the desired times on nitrocellulose filters prerinsed with the appropriate buffer. Nonspecific binding was determined by adding IPTG to 10^{-3} M.

Determination of Stoichiometry for pLA 322-8 Binding to Repressor. For the repressor titration, repressor (0 to 1×10^{-8} M) was added to preequilibrated ^{32}P-labeled pLA 322-8 in a total volume of 0.5 mL of TBB with 0.15 M KCl. The mixture was incubated for 30 min at 25 °C before filtering through nitrocellulose filters. For the DNA titration, repressor (1×10^{-9} M) was added to preequilibrated labeled DNA (7.7×10^{-11} M) and unlabeled pLA 322-8 [$(0.5–9.3) \times 10^{-9}$ M] in a total volume of 0.1–0.2 mL. These samples were further incubated for 30 min at room temperature prior to filtration through nitrocellulose filters. Bound radiolabeled DNA was measured by scintillation counting. Incubation times from 15 min to 3 h did not significantly alter results.

Quantitation of DNA. DNA was quantitated by absorbance at 260 nm ($A_{260}^{0.1\%} = 20$) or by 0.1% ethidium bromide fluorescence as described by Le Pecq and Paoletti (1966). Fluorescence measurements were made on an SLM-400 spectrofluorometer using an excitation wavelength of 546 nm and an emission wavelength of 590 nm. Calf thymus DNA was used as the standard.

Results

Repressor–Operator Dissociation Measured by "Trapping" Released Protein with Excess Unlabeled DNA. Following equilibration of repressor and ^{32}P-labeled DNA, an excess of the corresponding unlabeled DNA was added, and aliquots of the mixture were filtered at specific time intervals. The dissociation of the repressor–^{32}P-labeled operator complex was monitored as the decrease in labeled, filter-bound complex with time. The repressor–operator dissociation was monitored for 2 h to ensure equilibrium was attained. Accurate kinetic analysis required consideration of the actual equilibrium level of DNA binding rather than the IPTG-insensitive binding. At excesses of unlabeled operator DNA less than 50-fold over ^{32}P-labeled operator and repressor concentrations for plasmid or 95-fold for 40 bp operator fragment, the exponential dissociation reached an equilibrium value greater than the binding background (i.e., radiolabel retained in the presence of inducer). Equilibrium values from individual experiments varied from the level of binding calculated by using the proportions of labeled to unlabeled DNA; multiple repetitions were therefore required to obtain an average value. Absorbance at 260 nm was found to be an inaccurate measure of the DNA concentrations, particularly for the 40 bp fragment. Ethidium bromide fluorescence determinations of DNA concentration resulted in greater reproducibility in behavior from different

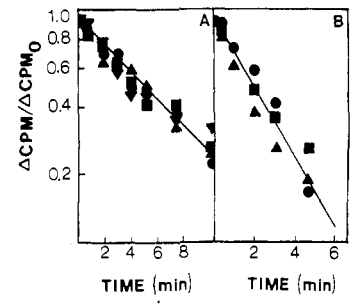

FIGURE 1: Dissociation rate analysis utilizing the observed equilibrium DNA binding. Unlabeled DNA was added to the equilibrated repressor–^{32}P-labeled operator complex in 0.15 M KCl TBB, and triplicate aliquots were filtered at the desired times. Reactions were monitored until equilibrium was obtained (2 h); this equilibrium value for each DNA excess was subtracted from each time point, and the curves were normalized relative to binding at time zero (Δcpm/Δcpm$_0$). (A) Repressor (3.6×10^{-11} M) and labeled pLA 322-8 (3.6×10^{-11} M) were equilibrated 15 min. The following excesses of unlabeled pLA 322-8 over the initital DNA concentration were added to this preequilibrated solution to "trap" any repressor that dissociates from the labeled operator: (▼) 50-fold; (■) 25-fold; (●) 12.5-fold; (▲) 2.5-fold. (B) Repressor (3.6×10^{-11} M) and labeled 40 bp fragment (3.6×10^{-11} M) were equilibrated 15 min before the addition of an excess of unlabeled 40 bp fragment over the initial DNA concentration. The following excesses of unlabeled 40 bp fragment were added at zero time: (●) 95-fold; (■) 22-fold; (▲) 5-fold.

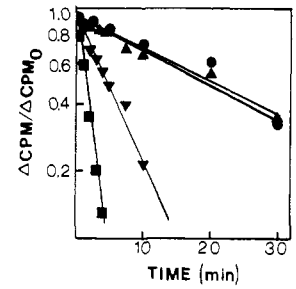

FIGURE 2: Repressor dissociation from pIQ, λp*lac*, pLA 322-8, and the 40 bp operator fragment. Concentrations of repressor and labeled operator equilibrated for pLA 322-8 and the 40 bp fragment are listed in Figure 1. A 50-fold excess and a 95-fold excess of unlabeled DNA were added to the equilibrated protein and pLA 322-8 or 40 bp fragment, respectively. For pIQ and λp*lac*, repressor (4×10^{-13} M) was equilibrated with 4×10^{-13} M labeled DNA before a 1000-fold excess of unlabeled DNA was added at time zero. (●) pIQ; (▲) λp*lac*; (▼) pLA 322-8; (■) 40 bp fragment.

DNA preparations. If the measured equilibrium binding was considered the end point of the reaction, a monophasic dissociation curve resulted for all excesses of pLA 322-8 DNA (Figure 1A). Similar results as a function of the unlabeled excess were reported for λΦ80d*lac* by Riggs et al. (1970a). Analysis of dissociation of the 40 bp fragment in this manner also indicated no significant effect of the DNA excess on the kinetic behavior (Figure 1B). Modeling of these experiments by numerical integration programs using the corresponding association rate constants (Whitson et al., 1986) indicated that variation of the observed dissociation rates as a function of the ratio of unlabeled excess DNA (from 2.5-fold to 100-fold over labeled DNA) were within the standard deviations of these measurements. Therefore, reassociation events would not be expected to influence this mode of analysis even at the lowest ratios of trapping DNA used in these studies. At lower ratios (<2.5-fold) of unlabeled DNA, the predicted dissociation rate constants increased; experimental confirmation of this predicted behavior was not possible due to the small difference in radiolabel bound. Half-lives for complex dissociation of the 40 bp fragment, pLA 322-8, pIQ, and λp*lac* measured under similar conditions were 1.3, 4.8, 19.1, and 20.0 min, respec-

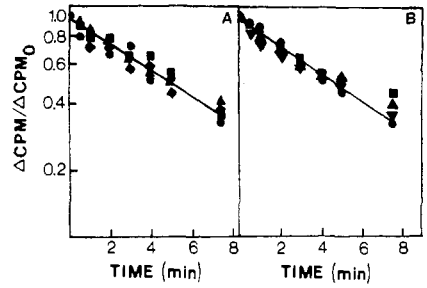

FIGURE 3: Effects of varying initial operator or repressor concentrations. (A) A 1000-fold molar excess of unlabeled pLA 322-8 over repressor was constant, while initial [^{32}P]pLA 322-8 operator concentration was varied relative to the repressor concentration (4×10^{-11} M) in the following ratios of DNA/repressor: (●) 1/10; (■) 1/1; (♦) 2/1; (▲) 10/1. (B) A [^{32}P]DNA/repressor ratio of 1/1 was maintained as the repressor concentration was varied. Excess unlabeled pLA 322-8 was 2×10^{-9} M. (●) 5.4×10^{-12} M; (▲) 1.1×10^{-11} M; (■) 4.0×10^{-11} M; (▼) 1.1×10^{-10} M.

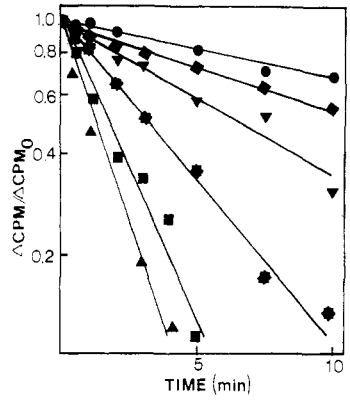

FIGURE 4: Effects of [KCl] on the dissociation of the repressor–pLA 322-8 complex. Protein and labeled pLA 322-8 concentrations were 3.6×10^{-11} M, and a 50-fold excess of unlabeled pLA 322-8 over the inititial DNA concentration was added. Dissociation of the repressor-operator complex was monitored as a function of [KCl]. (●) 0.100 M KCl; (♦) 0.125 M KCl; (▼) 0.150 M KCl; (*) 0.175 M KCl; (■) 0.20 M; (▲) 0.250 M KCl.

tively (Figure 2), corresponding to dissociation rate constants of 9.3 (±0.50) $\times 10^{-3}$ s^{-1}, 2.4 (±0.31) $\times 10^{-3}$ s^{-1}, 6.0 (±0.56) $\times 10^{-4}$ s^{-1}, and 5.8 (±0.55) $\times 10^{-4}$ s^{-1}.

For the above experiments, repressor and operator DNA concentrations were approximately equal. To determine whether relative or absolute concentrations would influence the observed rates, initial labeled operator concentration was varied relative to the repressor concentration, ranging from a ^{32}P-labeled operator DNA:repressor ratio of 1:10 to 10:1 (Figure 3A). A monophasic dissociation curve with a half-life of 4.8 min was observed for all initial pLA 322-8 concentrations. Using repressor concentrations from 5×10^{-12} to 1×10^{-10} M and maintaining a 1:1 ratio of [^{32}P]pLA 322-8 to repressor and 2×10^{-9} M unlabeled pLA 322-8 also produced monophasic dissociation curves with the same half-life (Figure 3B). Similar variations of repressor and the 40 bp operator fragment also resulted in monophasic curves with identical half-lives (data not shown). The dissociation rate of the 40 bp operator from repressor was unaffected whether the unlabeled DNA utilized was pLA 322-8 or the 40 bp fragment.

Salt and Sequence Context Effects on Repressor–Operator DNA Dissociation. Increasing the salt concentration increased the repressor–pLA 322-8 DNA dissociation rate (Figure 4), a result consistent with previously observed electrostatic effects on repressor–operator dissociation (Riggs et al., 1970a; de-Haseth et al., 1977; Record et al., 1977; Revzin & von Hippel, 1977; Barkley et al., 1981; Winter et al., 1981). A 25-fold

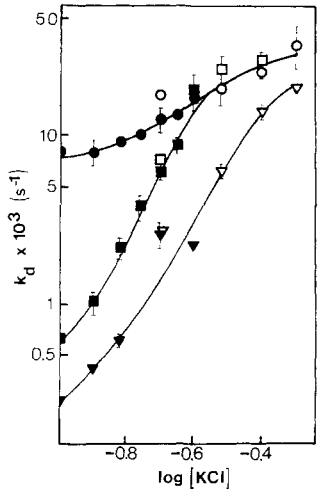

FIGURE 5: Dissociation rates of repressor–operator complexes as a function of [KCl]. Dissociation rates were determined by addition of excess unlabeled DNA (filled symbols) or use of salt jump techniques (open symbols) at the indicated [KCl], as described under Materials and Methods. Each point represents the average of two to six rate determinations. Standard deviations are indicated by error bars, except where the deviations were less than the area of the symbol. Dissociation rate constants for three types of operator DNA are illustrated: (●, ○) 40 bp operator containing fragment; (■, □) pLA 322-8, ~4600 bp operator containing fragment; (▼, ▽) pIQ, ~6200 bp operator and pseudooperator containing fragment.

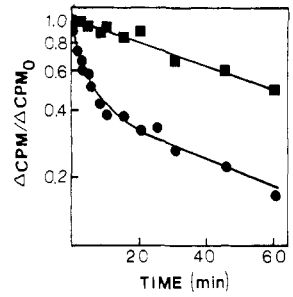

FIGURE 6: Dissociation of the repressor–pIQ complex at two repressor concentrations. A 1/1 ratio of repressor and pIQ concentrations was maintained, and unlabeled DNA concentration was the same (8.4×10^{-10}) in 0.10 M KCl TBB. (●) [repressor] = 4.0×10^{-11} M; (■) [repressor] = 4.0×10^{-13} M.

increase in the off-rate for pLA 322-8 was observed between 0.1 and 0.25 M KCl. The salt dependence of the repressor–operator interaction varied nonlinearly with the size and pseudooperator content of the operator-containing DNA (Figure 5). While the dissociation rate for the 40 bp fragment increased only 3-fold over the KCl range 0.1–0.25 M, the dissociation rate for pIQ increased 75-fold over the same concentrations.

At low KCl concentrations, biphasic dissociation of repressor and pIQ was observed at initial protein and operator concentrations $>10^{-11}$ M; when the protein and operator concentrations were lowered to $<5 \times 10^{-13}$ M or the salt concentration increased to >0.15 M KCl, this behavior was eliminated (Figure 6). Barkley (1981) reported similar biphasic behavior for λ*plac* at 2×10^{-12} M, which disappeared when the repressor concentration was lowered to 5×10^{-13} M in 0.05 M NaCl. Biphasic dissociation was not observed for pLA 322-8, which differs from pIQ primarily by the absence of the pseudooperators. Biphasic character was also observed for λ*plac* dissociation from repressor under similar conditions. Increasing the salt concentration results in an increase in the K_d for repressor–operator binding and a corresponding increase in the K_d for repressor–pseudooperator binding. Thus, the

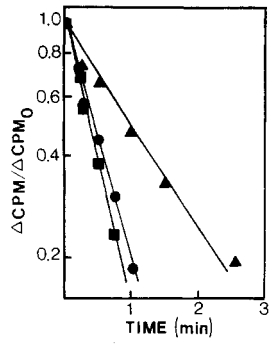

FIGURE 7: Use of salt jump to monitor dissociation of the repressor–operator complex. A small volume of 3 M KCl was added to a preequilibrated solution of repressor and pLA 322-8 (each at 4.0×10^{-11} M) in 0.10 M KCl. (▲) Salt jump from 0.10 to 0.20 M KCl; (●) salt jump from 0.10 to 0.30 M KCl; (■) salt jump from 0.10 to 0.40 M KCl.

Table I: Comparison of Dissociation Rates Determined by Salt Jump and Trapping Techniques[a]

	dissociation rate constant (s^{-1})	
	pLA 322-8	pIQ
salt jump[b]	$7.2 (\pm 0.4) \times 10^{-3}$	$2.7 (\pm 0.2) \times 10^{-3}$
excess DNA[b]	$6.1 (\pm 0.7) \times 10^{-3}$	$2.3 (\pm 0.4) \times 10^{-3}$
Guggenheim of salt jump[c]	$3.8 (\pm 1.4) \times 10^{-3}$	$4.0 (\pm 1.2) \times 10^{-3}$
Guggenheim of excess DNA[c]	$8.7 (\pm 1.2) \times 10^{-3}$	$3.1 (\pm 2.0) \times 10^{-3}$

[a] Salt jump and trapping techniques are described under Materials and Methods. The salt concentration was 0.20 M KCl. [b] Standard deviations represent the average of two to six separate rate determinations. [c] Standard deviations represent the deviations of individual points from a slope calculated from a single rate determination.

presence of the biphasic character for pIQ and λ*plac* depends on the repressor and operator concentrations relative to the K_d for pseudooperator binding.

Repressor–Operator DNA Dissociation Resulting from a Salt Jump. Repressor–operator dissociation was also monitored by abruptly increasing the salt concentration by addition of a small volume of a concentrated salt solution as described previously (Lohman, 1980; Winter et al., 1981). This increase in the salt concentration results in a corresponding increase in the K_d, eliciting an approach to a new equilibrium. Figure 7 illustrates salt jumps used to measure repressor–pLA 322-8 complex dissociation. The measured dissociation rate approached a maximum at 0.3–0.4 M KCl in TBB for both pLA 322-8 and the 40 bp operator fragment. An approach to a maximum dissociation rate was also observed for pIQ at higher salt concentrations (0.5 M KCl); however, the rate constants differed for pIQ vs. pLA 322-8 (Figure 5). Dissociation rate constants obtained from salt jumps of 0.1–0.2 M KCl are compared in Table I to the rate constants determined at 0.2 M KCl by the addition of an excess of unlabeled DNA. For each type of operator DNA, the dissociation rate constant measured was slightly faster for the salt jump data, although within the experimental error of these methods.

Guggenheim Analysis of the Data. The Guggenheim method of analysis can be used for first-order reactions in which the original concentration and the end point of reaction are unknown. This method of analysis is useful when the amount of radiolabel bound at $t = \infty$ is difficult to determine. We have used it in these studies to verify that the reaction did not involve a slower rate as equilibrium was approached and that the equilibrium value observed was accurate. A sample Guggenheim plot for pLA 322-8 in 0.2 M KCl is shown in Figure 8, where the slope of the line represents the first-order dissociation rate (Gutfreund, 1977). Comparisons to values obtained directly using salt jump or the addition of excess

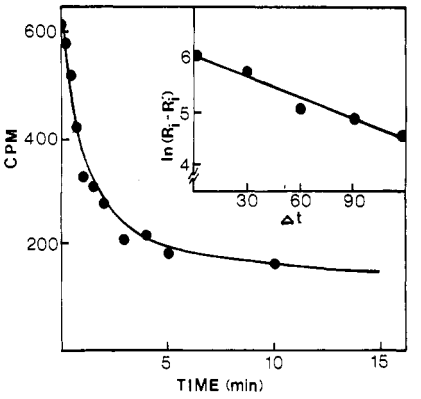

FIGURE 8: Guggenheim analysis of repressor–pLA 322-8 complex dissociation. Procedures were as described in Figure 1 and under Materials and Methods, except that the buffer is 0.20 M KCl TBB. Repressor and pLA 322-8 concentrations were 3.6×10^{-11} M, and a 50-fold excess of unlabeled pLA 322-8 over the initital DNA concentration was added. Background counts were determined by adding IPTG (2×10^{-3} M) and were subtracted from each point. The Guggenheim plot (inset) is determined from the real time plot as the ln of the change in cpm at 2 times vs. the change in time (Gutfreund, 1977). The slope of the line from the plot is equivalent to the first-order rate constant.

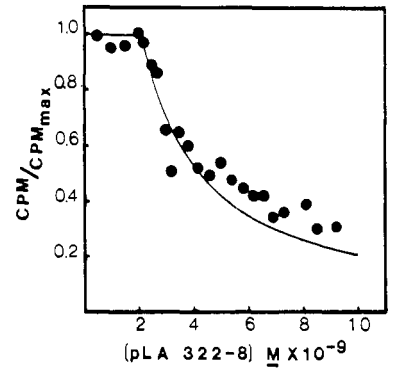

FIGURE 9: Stoichiometry determination of repressor and pLA 322-8. Repressor (1.0×10^{-9} M) was added to preequilibrated [^{32}P]DNA (7.7×10^{-11} M) and unlabeled pLA 322-8 [$(0.5–9.2) \times 10^{-9}$ M] and incubated for 30 min. Background counts were determined by adding IPTG (2×10^{-3} M) and subtracted from each point. The resulting bound cpm values were normalized to the cpm with no addition of unlabeled DNA (cpm_{max}) to enable averaging of four separate experiments. This averaging was essential due to deviations from the predicted values for equilibrium binding; this variation was also observed in the kinetic experiments. The line represents the theoretical fraction of labeled DNA bound assuming two binding sites. See Results for further description of the theoretical line.

unlabeled DNA are listed in Table I. Standard deviations were greater for the Guggenheim analysis since a single rate determination was used.

Stoichiometry Determination for Repressor Binding to pLA 322-8. To determine the stoichiometry of operator binding, two variations of the nitrocellulose filter-binding technique were used; a constant concentration of repressor was titrated with pLA 322-8, or a constant concentration of pLA 322-8 was titrated with repressor. For the repressor titration, pLA 322-8 (1.1×10^{-9} M) was preequilibrated in 0.15 M KCl TBB, and repressor ($0–5.0 \times 10^{-9}$ M) was added and incubated for 30 min before filtering. The results indicated saturation of repressor binding at 6.6×10^{-10} M when operator was 1.1×10^{-9} M, which corresponded to an operator DNA/repressor molecule ratio of 1.7 (data not shown). In the DNA titration, repressor concentration was constant at 1.0×10^{-9} M, the pLA 322-8 concentration was varied ($0–5.0 \times 10^{-9}$ M), and the labeled operator was constant (7.7×10^{-11} M). The results

Table II: Salt Dependence of the Dissociation Rate Constants

DNA	$\partial \log K_{RD}/\partial \log [M^+]^a$	$\partial \log K_{RO}/\partial \log [M^+]^b$	$(\partial \log k_d/\partial \log [M^+])_{calcd}{}^c$	$(\partial \log k_d/\partial \log [M^+])_{obsd}$
40 bp fragment	−10	−5.6	0.6	0.69
pLA 322-8	−10	−7.3	2.3	3.4
pIQ	−10	−7.0	2.0	2.9

[a] $\log K_{RD} = -10 \log [M^+] - 2.5$, calculated by Winter et al. (1981) for the buffer used in these experiments on the basis of values from Revzin and von Hippel (1977), deHaseth et al. (1977), and Lohman et al. (1980). [b] From the accompanying paper (Whitson et al., 1986). [c] Calculated from $\partial \log k_d/\partial \log [M^+] = {}^1/_2(\partial \log K_{RD}/\partial \log [M^+]) - \partial \log K_{RO}/\partial \log [M^+]$ (Lohman, 1985).

shown in Figure 9 demonstrate saturation of available operator sites on the protein at 2×10^{-9} M total DNA when the repressor was 1×10^{-9} M. That is, at a ratio of [DNA]/[protein] of 2, more DNA cannot be bound, and additional operator DNA resulted in dilution of the bound radiolabeled DNA. Thus, the stoichiometry of operator binding under these conditions was 2. The solid curve drawn in Figure 9 represents the theoretical binding calculated as described below. At conditions well above the K_d, stoichiometric binding occurs, and any unlabeled DNA added will compete with the labeled DNA. The fraction of labeled operator bound to repressor with increasing unlabeled operator would be constant until all protein binding sites become saturated. Following saturation, further addition of unlabeled operator will result in decreased label bound. The fractional retention may be calculated by assuming a stoichiometry of 2 operators/repressor; nonspecific DNA binding was not considered in these calculations.

Discussion

The dissociation of the repressor from operator has traditionally been measured as the time-dependent decrease in labeled operator found in the complex upon addition of excess unlabeled DNA (Riggs et al., 1970a; Winter et al., 1981; Barkley, 1981). In our experiments, the dissociation curves contained a single phase when the observed equilibrium DNA binding was considered in the analysis. Although using the observed equilibrium in our analysis was not critical when equilibrium binding and nonspecific binding were equivalent, measuring the actual equilibrium value precluded dependence on other factors. There were no measurable effects of repressor or operator concentrations or the ratio between the two when the data were analyzed by using observed equilibrium binding.

Although salt dependence of a reaction is typically considered an ionic strength effect, this situation does not hold true for protein–DNA binding. In solutions containing monovalent salts, double-stranded DNA behaves thermodynamically as though 88% of the negative charge along its length were neutralized by monovalent cations including those "condensed" along the DNA and those involved in "screening" (Record et al., 1976, 1978; Manning, 1978). Each cation interacts with DNA with its own relative affinity and may be displaced when repressor binds to the DNA (deHaseth et al., 1977). The dissociation process is affected by the counterion concentration, with an increase in rate as the counterion concentration increases. Variable salt dependence for dissociation of the repressor–operator DNA complex using DNAs of different sizes was observed in our studies; this behavior suggests a multistep dissociation process (Lohman et al., 1978).

The dissociation mechanism of the repressor–operator may include either or both of the facilitative mechanisms that have previously been proposed, sliding and/or direct transfer (Riggs et al., 1970a; Richter & Eigen, 1974; Berg & Blomberg, 1978; Berg et al., 1981; von Hippel et al., 1975). Sliding is defined as facilitated translocation along the DNA by maintaining nonspecific contacts in a one-dimensional random walk. Intersegment transfer is described as a ring-closure event in which the repressor is transiently bound to the DNA at two sites. The rate constant for sliding is predicted to be independent of salt concentration, since the process involves no net displacement of counterions (Berg et al., 1981). Increasing the salt concentration would have a greater effect on the nonspecific vs. specific DNA dissociation rate constant, since the nonspecific interaction involves three to five more ionic contacts than does the operator-specific interaction (de Haseth et al., 1977; Record et al., 1977; Revzin & von Hippel, 1977; Barkley et al., 1981; Winter & von Hippel, 1981). The sliding model predicts that the dissociation rate will be a function of $K_{RD}{}^{1/2}/K_{RO}$ (Berg & Blomberg, 1978; Barkley, 1981; Berg et al., 1981), and $\partial \log k_d/\partial \log [K^+] = {}^1/_2\, \partial \log K_{RD}/\partial \log [K^+] - \partial \log K_{RO}/\partial \log [K^+]$ (Lohman, 1985), where K_{RD} is the nonspecific DNA equilibrium constant and K_{RO} is the operator binding constant. Values calculated from this equation are compared to the measured salt dependence in Table II; agreement for the 40 bp operator is excellent, while the values for the plasmid DNA are more divergent but appear to be consistent with the sliding mechanism.

A second possible method of dissociation is intersegment transfer, in which repressor is transiently bound to the DNA at two sites. The existence of such a mechanism is substantiated by stoichiometry determinations. O'Gorman et al. (1980a,b) and Culard and Maurizot (1981) have previously reported a stoichiometry of two 29 bp operator fragments/repressor. Daly and Matthews (1986) required a stoichiometry of two 40 bp operator fragments/repressor in order to balance a thermodynamic circuit for inducer and operator binding. In addition, Barbier et al. (1984) observed a similar stoichiometry for nonspecific DNA fragments, and Fried and Crothers (1984) obtained evidence from electrophoretic data for the direct transfer of repressor between DNA molecules. The stoichiometry of 2 pLA 322-8 molecules/respressor molecule is consistent with these data and indicates that charge repulsion between two segments of DNA does not prevent double occupancy of the repressor by large DNAs. These data indicate that direct transfer, involving a transition state with both DNA binding sites of the repressor filled, is feasible. It is possible that the divergence between calculated and observed values for salt dependence of the dissociation rate constant for the plasmid DNAs (Table II) may be due to the participation of direct transfer processes. In fact, Mazur (1984) has suggested that quantitative differences between experimental values for association rates and those predicted for a sliding mechanism may be due to a contribution of direct transfer to this process.

Once the repressor is bound to the operator, we postulate that an intramolecular ternary complex of operator–repressor–pseudooperator or nonspecific DNA may form. Formation of this ternary complex would stabilize binding and influence repressor dissociation from the DNA as observed. Assuming DNA is a flexible coil, it is possible to determine the effective local concentration of a pseudooperator or nonspecific DNA when repressor is bound to the primary operator (Shore & Baldwin, 1983). For the "secondary operator", located 407 bp from the primary operator in the β-galactosidase coding

region, the effective operator concentration is $\sim 10^{-7}$ M, on the basis of the assumption that the DNA is a random coil. Considering the weaker affinity of repressor for this pseudooperator ($\sim$10-fold), the relative concentrations would still allow repressor binding. In addition, the persistence length (150 bp), which is a measure of the ability of DNA segments to bend into a loop, would allow formation of a ternary complex at the pseudooperator site located in the *lacZ* gene. The decreased dissociation rate observed for pIQ and λp*lac*, which contain pseudooperators, even at high salt concentrations suggests a role for these sequences in further slowing repressor–operator DNA dissociation. The persistence length of the DNA and lower affinity of the pseudooperator located in the I gene would preclude ternary complex formation involving this pseudooperator. Although the only difference between pLA 322-8 and pIQ is the presence of pseudooperators in the latter, the dissociation rate was reduced at all salt concentrations for pIQ.

The local concentration of nonspecific DNA in pLA-322-8 is $\sim 10^{-4}$ M base pairs; thus, occupation of the presumed second site on the repressor by these sequences of the DNA will occur up to salt concentrations where the K_{RD} value is sufficiently decreased ($1/K_{RD} > 10^{-4}$ M). This value for K_{RD} is observed at $\sim$0.22–0.25 M KCl (Revzin & von Hippel, 1977; deHaseth et al., 1977; Lohman et al., 1980), which corresponds to the point at which the dissociation rate constants for the 40 bp operator and operator-containing plasmid converge. This convergence of the dissociation rate constants for the 40 bp operator fragment and pLA 322-8 plasmid DNA at these high salt concentrations ($\sim$0.25 M KCl) is consistent with a common, possibly limiting, dissociation mechanism with diminished salt dependence. Flanking nonspecific DNA no longer appears to influence the dissociation process (at >0.25 M KCl), presumably because the high salt concentrations preclude the electrostatic interactions required for the binding of nonspecific DNA at the second site on the repressor. Thus, the dissociation rates of the 40 bp fragment and pLA 322-8 under these conditions would primarily represent the release of operator-specific contacts by the protein.

In summary, the dissociation rate of the *lac* repressor–operator complex has customarily been determined by adding an excess of unlabeled operator-containing DNA to a preequilibrated repressor–labeled operator complex. We report rates analyzed using the measured equilibrium binding for a variety of conditions. While dissociation of the 40 bp operator fragment was minimally salt dependent, significant salt dependence was observed for pLA 322-8 up to 0.25 M KCl, at which point the dissociation rate constants for both plasmid and fragment converged. This convergence of the dissociation rate constants for operator-containing fragment and plasmid DNA at high salt concentrations indicates a common, possibly limiting, dissociation mechanism. The salt dependences of the dissociation rate constants for all DNAs examined are consistent with those predicted for a sliding model, although the differences for the plasmid DNAs suggest the possible influence of a direct transfer process. Evidence presented for repressor interaction with DNA at two sites and for diminished dissociation rates in the presence of intramolecular sites provides a possible function of both pseudooperator and nonspecific DNA sequences in stabilizing the repressor–operator via an intramolecular ternary complex.

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Thermodynamic Analysis of the Lactose Repressor–Operator DNA Interaction†

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ABSTRACT: Kinetic and equilibrium constants for lactose repressor–operator DNA interaction have been examined as a function of salt concentration, size and sequence context of the operator DNA, and temperature. Significant salt effects were observed on kinetic and equilibrium parameters for pLA 322-8, an operator-containing derivative of pBR 322, and pIQ, an operator and pseudooperator-containing derivative of pBR 322. The association rate constant and equilibrium constant for the 40 base pair operator fragment were also salt dependent. Data for all the DNAs were consistent with a sliding mechanism for repressor–operator association/dissociation [Berg, O. G., & Blomberg, C. (1978) *Biophys. Chem. 8*, 271–280]. Calculation of the number of ionic interactions based on salt dependence yielded a value of ~8 for repressor binding to pIQ and pLA 322-8 vs. ~6 for the repressor–40 base pair fragment. These data and the differences in binding parameters for the plasmids vs. the 40 base pair operator are consistent with the formation of an intramolecular ternary complex in the plasmid DNAs. Unusual biphasic temperature dependence was observed in the equilibrium and dissociation rate constants for pLA 322-8, pIQ, and the 40 base pair fragment. These observations coupled with a discontinuity found in the inducer association rate constant as a function of temperature suggest a structural change in the protein. The large positive entropy contributions associated with repressor binding to all the DNAs examined provide the significant driving force for the reaction and are consistent with involvement of ionic and apolar interactions in complex formation.

The lactose repressor–operator DNA association rate is more rapid than three-dimensional diffusion would predict (Berg et al., 1981; Riggs et al., 1970). Protein translocation along the DNA has, therefore, been proposed to account for this facilitated association, in which the dimensionality of the search for the specific site is diminished; this movement has been postulated to consist primarily of sliding and/or intersegment transfer (Riggs et al., 1970; Richter & Eigen, 1974; Berg & Blomberg, 1976; Berg et al., 1981; von Hippel et al., 1975). Sliding is described as the one-dimensional protein movement along the DNA while nonspecific contacts are maintained. von Hippel et al. (1975) postulated intersegment transfer in which the protein is transiently bound between DNA segments; thus, translocation occurs as a series of random steps.

DNA has been modeled thermodynamically as a linear array of univalent negative charges [for review, see Lohman (1985)]. In a solution of a single salt, monovalent counterions may interact with the DNA by direct condensation, reducing the structural charge, or with the remaining bulk ions in solution, screening the phosphates of the DNA. Although the counterions condensed to the DNA are thought to be mobile, double-stranded DNA behaves thermodynamically as though 88% of its structural charge is neutralized in monovalent salts, independent of the salt concentration. When a protein binds to the DNA, it neutralizes some of the phosphates and consequently results in the release of counterions as well as affecting those counterions involved in screening. The repressor–operator DNA association is thermodynamically favored since the entropy of the system increases, due at least in part to release of ions from the DNA and the protein (Record et al., 1978).

Studies of the temperature dependence of the *lac* repressor–operator interaction have yielded limited information. Riggs et al. (1970) reported a 1.2-fold increase in the repressor–λ*plac* dissociation rate as temperature was increased from 1 to 37 °C and a 4-fold decrease in the association rate between 1 and 24 °C. Barkley et al. (1981) observed similar

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