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Binding of synthetic lactose operator DNAs to lactose repressors*

(nonoperator DNA/salt effects/QX86 repressor/SQ repressor)

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ABSTRACT **The nitrocellulose filter assay was used to study the interactions of wild-type (SQ) and tight-binding (QX86) *lac* repressors with synthetic *lac* operators 21 and 26 base pairs long. The repressor binding properties of both operators were very similar, indicating that both contain the same specific repressor recognition sites. The repressor–operator association rate constants (k_a) were more sensitive than dissociation rate constants (k_d) to changes in ionic strength. The responses of both k_a and k_d to ionic strength were relatively small compared to the effects previously observed with λh80*dlac* as operator DNA. These results suggest that under natural conditions there are electrostatic interactions between *lac* repressor and DNA regions outside of the 26 base pair operator sequence. Association rate constants for SQ repressor with either operator are higher than have been predicted for diffusion-limited reactions. We postulate that long-range electrostatic attractions between repressor and operator accelerate the association reaction. The presence of nonoperator DNA decreased association rate constants, the effect being more noticeable at an ionic strength of 0.05 M than at 0.20 M. Nonoperator DNA reduced k_a values for associations involving QX86 repressor to a greater extent than for those with SQ repressor. The two types of repressors also had different rate constants for interactions with synthetic operators. The values for k_a and k_d were both higher with SQ repressor than with QX86 repressor. However, the rate constants were more sensitive to ionic strength when the repressor used was QX86.**

Lactose (*lac*) repressor recognizes and binds tightly to the *lac* operator of *Escherichia coli*. This specific DNA–protein interaction prevents the transcription of genes whose product are involved in lactose metabolism. The sequences of repressor protein (1) and operator DNA (2, 3) are known, making this an ideal system for investigating the process by which proteins and DNA interact specifically to control gene expression. The binding of repressor (R) to operator (O) can be described as follows:

$$R + O \underset{k_d}{\overset{k_a}{\rightleftharpoons}} RO; K_d = \frac{k_d}{k_a} = \frac{[R]_{free}[O]_{free}}{[RO]} \quad [1]$$

k_a and k_d are the rate constants for association and dissociation, respectively; K_d is the equilibrium dissociation constant. The nitrocellulose filter assay of Riggs and coworkers (4, 5) provides a method for measuring these binding constants. This paper describes the binding of wild-type (SQ) and tight-binding (QX86) *lac* repressors to synthetic operator DNAs 21 and 26 base pairs in length.

MATERIALS AND METHODS

The synthesis of 26 base pair (duplex I) and 21 base pair (duplex II) *lac* operator DNA has been described (6–8). [γ-^{32}P]ATP at a specific activity of 1500 Ci/mmol was prepared by a published procedure (9). The DNA duplexes were quantitatively phosphorylated as described (8). Wild-type (SQ) repressor was purified by a published procedure (8). The iSQ strain was obtained from W. Gilbert. Tight-binding (QX86) repressor was provided by J. Sadler. λh80*dlac* DNA was prepared as described (10). The strain RV/80 used for this preparation was obtained from J. Sadler. Nitrocellulose filters (BA-85, 27 mm) were obtained from Schleicher and Scheull.

The membrane filter assays were performed basically as described by Riggs *et al.* (4, 5). The binding buffer contained 10 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% dimethylsulfoxide, and 50 μg of bovine serum albumin per ml. The ionic strength (I) of the binding buffer was adjusted by addition of KCl. Low (I = 0.05 M), medium (I = 0.12 M), and high (I = 0.20 M) ionic strength binding buffers contained 10 mM, 80 mM, and 160 mM KCl, respectively. Washing buffers were the same as binding buffers except that bovine serum albumin and dithiothreitol were omitted. Filters were soaked in washing buffer for at least 1 hr before use. In all binding experiments, 100-μl samples were filtered in approximately 4–5 sec and immediately washed with 200 μl of washing buffer. Experiments were carried out at room temperature.

The procedure used for measuring dissociation kinetics has been described (8). Rates of association were determined by adding repressor to solutions of operator in binding buffer. Immediately after addition of repressor (zero time), the solutions were mixed on a Vortex mixer for 3 sec. The reactions were then terminated by rapid filtering, timed at the point of sample application. In each experiment triplicate samples were filtered. The filter-retainable radioactivity before repressor addition was substracted from each time point.

The concentrations of repressor active with respect to operator binding were determined by titrating the repressor solutions against known quantities of both operator duplexes I and II.

RESULTS

Purity of Synthetic Operator DNA. We have previously described the synthesis of both 26 and 21 base pair *lac* operator DNAs (6–8). The nucleotide sequences of these two DNAs, which we term duplex I and duplex II, respectively, are shown in Fig. 1. To obtain meaningful results in binding experiments, these duplexes should be essentially free of contaminating compounds. Fig. 2 shows the patterns obtained when the duplexes were electrophoresed on polyacrylamide gels under both native and denaturing conditions. Both compounds appear homogeneous and migrate as expected for their respective lengths. Duplex I is clearly resolved into its two component strands under denaturing conditions. As expected, the two single

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* This is paper 5 in a series "Studies on gene control regions." Paper 4 is Kawashima, E., Gadek, T. & Caruthers, M. H. (1977) *Biochemistry*, in press.

TGTGGAATTGTGAGCGGATAACAATT
ACACCTTAACACTCGCCTATTGTTAA
DUPLEX I

AATTGTGAGCGGATAACAATT
TTAACACTCGCCTATTGTTAA
DUPLEX II

FIG. 1. Synthetic *lac* operator DNAs. The syntheses have been described (6–8). Regions related by 2-fold symmetry are boxed.

strands of duplex II, which are nearly equivalent in base composition, are more poorly resolved.

Another check for purity was made by titrating both duplexes with *lac* repressor and measuring the percentage cpm retained on cellulose nitrate filters at plateau. The retention efficiency of λ*plac* DNA carrying the wild-type operator is about 50% (12, 13). For both duplexes we obtain retention efficiencies o approximately 55% using wild-type SQ repressor and 65% with tight-binding QX86 repressor. These values are additional evidence for the homogeneity of the synthetic duplexes.

Kinetics of Dissociation. The membrane filter assay offers a convenient method for measuring the stability of preformed repressor–operator complexes (5). We have determined the half-lives ($t_{1/2} = 0.693/k_d$) of complexes formed between synthetic operator DNAs (duplexes I and II) and repressor proteins (wild-type SQ and tight-binding QX86) at several ionic strengths. The results (Fig. 3 and Table 1), are quite reproducible. Each dissociation experiment was repeated at least three times. In all cases the half-lives measured were within ±8% of the values given in Table 1. Two interesting observations can be made concerning these results:

(*i*) Duplexes I and II are very similar in their abilities to bind repressor. The five extra base pairs in duplex I do not have a pronounced effect on the measured values for $t_{1/2}$. With wild-type repressor at $I = 0.05$ M, the difference in half-lives is only 9 sec (47 compared to 38 sec). The greatest difference between duplexes I and II is observed with QX86 repressor at $I = 0.12$ M (18 compared to 9 min). However, this 2-fold reduction in $t_{1/2}$ is quite small when compared to the reduction observed with most single site O^c mutations (12).

(*ii*) Repressor–operator half-lives are only slightly changed by varying the ionic strength. With duplex I and SQ repressor, increasing the ionic strength from $I = 0.05$ M to $I = 0.20$ M results in only a slight (47 to 41 sec) reduction in $t_{1/2}$. Similar results are observed with duplex II and SQ repressor (38 to 29 sec), duplex I and QX86 repressor (21 to 16 min), and duplex II and QX86 repressor (13 to 9 min). Previous experiments carried out with high-molecular-weight λh80*dlac* DNA have demonstrated a decrease in $t_{1/2}$ to approximately 25% of the original value when the ionic strength is raised from 0.05 M to 0.20 M (5, 12).

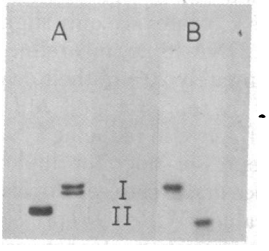

FIG. 2. Polyacrylamide gel electrophoresis of 5′-^{32}P-labeled operator duplexes. The denaturing gel (*A*) was 20% acrylamide/1% *N,N*-methylenebisacrylamide in 89 mM Tris-borate (pH 8.3)/2.2 mM EDTA containing 7 M urea. The native gel (*B*) was 15% acrylamide/0.75% *N,N*-methylenebisacrylamide in Tris-borate/EDTA. Gels were run at 200 V (10 V/cm) at room temperature. Migration was from top to bottom. Sample preparation and autoradiography were as described (11).

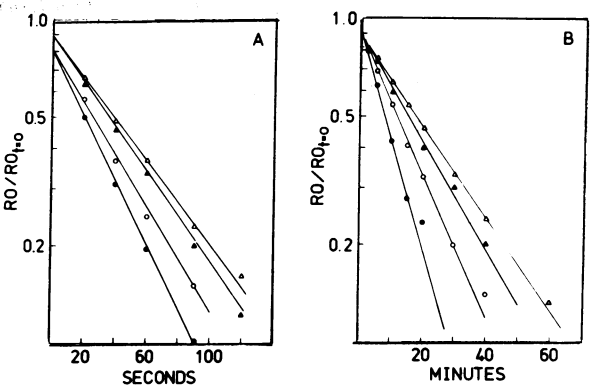

FIG. 3. Rates of dissociation of repressor–operator complexes (*RO*). Retention of 5′-^{32}P-labeled complexes on nitrocellulose filters was determined as described (8). For all experiments the operator concentration was approximately 20 pM. Each point was the average of three filters. The cpm retained in the presence of 1 mM isopropyl-β-D-thiogalactoside have been subtracted throughout. (*A*) Dissociation of SQ repressor–operator duplexes. (*B*) Dissociation of QX86 repressor–operator duplexes. The operator duplexes and ionic strengths used were: △, duplex I at $I = 0.05$ M; ▲, duplex I at $I = 0.12$ M; ○, duplex II at $I = 0.05$ M; ●, duplex II at $I = 0.12$ M.

Kinetics of Association. We have shown that the rate of dissociation of repressor–operator complexes (*RO*) is not strongly dependent on ionic strength. The value for k_d is also not affected by the presence of nonoperator DNA. Therefore, if the affinity of repressor for operator is significantly affected by nonoperator DNA or ionic strength, it should be reflected by changes in association rate constants. The rate of formation of repressor–operator complex can be described by:

$$d[RO]/dt = k_a([R]_{total} - [RO])([O]_{total} - [RO]) - k_d[RO] \quad [2]$$

During the first 120 sec of association experiments performed with QX86 repressor the dissociation term in this equation is negligible. Under these conditions the integrated form of Eq. 2 is:

$$k_a t = \frac{1}{[R]_{total} - [O]_{total}} \ln \frac{[O]_{total}\,[R]_{total} - [RO]}{[R]_{total}\,[O]_{total} - [RO]} \quad [3]$$

In Fig. 4 the association data for duplex I and QX86 repressor are displayed in this manner. The constant slopes of the lines drawn through the data points are indicative of bimolecular reactions and give a k_a of 3.3×10^8 M^{-1} sec^{-1} at $I = 0.05$ M and

Table 1. Dissociation data for repressor–operator complexes

Duplex	Ionic strength, M	$t_{1/2}$ (sec) SQ repressor	$t_{1/2}$ (min) QX86 repressor
I	0.03*	49	—
I	0.05	47	21
I	0.12	42	18
I	0.20	41	16
II	0.05	38	13
II	0.12	30	9
II	0.20	29	9

Experimental details are described in *Materials and Methods*.
* $I = 0.03$ M binding buffer contained 5 mM Tris·HCl (pH 7.4), 8 mM $MgCl_2$, 1 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% dimethyl sulfoxide, 50 μg of bovine serum albumin per ml, and 8 μg of salmon sperm DNA per ml.

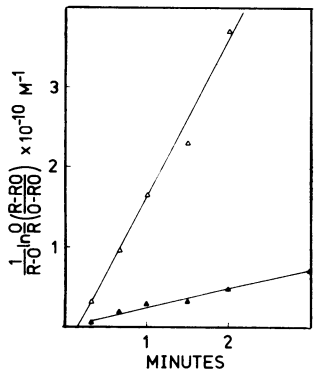

FIG. 4. Rates of association of duplex I with QX86 repressor. Experiments were performed as described in *Materials and Methods*. Each point was the average value for three experiments. The average cpm retained before addition of repressor have been subtracted from all points. Data are presented as described for a second-order reaction (see *text*). △, $I = 0.05$ M, $[O] = 1.3 \times 10^{-11}$ M, $[R] = 2.8 \times 10^{-11}$ M. ▲, $I = 0.20$ M, $[O] = 2.5 \times 10^{-11}$ M, $[R] = 4.2 \times 10^{-11}$ M.

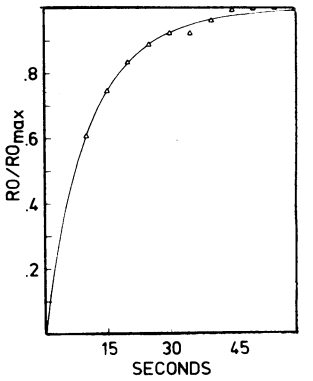

FIG. 5. Association of duplex I with SQ repressor at an ionic strength of 0.05 M. The active repressor concentration was 6.22×10^{-11} M; the operator concentration was 2.33×10^{-11} M. The filter-retainable cpm were determined at the indicated times as described in *Materials and Methods*. Each point was the average for three experiments. The cpm retained before repressor addition have been subtracted throughout. The curve drawn through the data points was calculated as described in the *text* for a value of $k_a = 2.0 \times 10^9$ M^{-1} sec^{-1}.

0.4×10^8 M^{-1} sec^{-1} at $I = 0.20$ M. Both of these experiments were done in the absence of nonoperator DNA. Table 2 gives the k_a values determined in this manner for QX86 repressor associations under a variety of conditions. In every instance the results were consistent with second-order kinetics.

Association rate constants must be determined differently for experiments utilizing SQ repressor. Because of the short half-lives of our synthetic operator–SQ repressor complexes, the dissociation term in Eq. 2 is significant throughout the time course of the association reactions. Eq. 2 must be integrated in its entirety to find the value of k_a that best fits the experimental data points. Fig. 5 shows the association of duplex I and SQ repressor at $I = 0.05$ M. The curve drawn through the figure is that calculated for $k_a = 2.0 \times 10^9$ M^{-1} sec^{-1} using the integrated form of Eq. 2. Clearly the data points fit the theoretical curve quite well and are consistent with second-order reaction kinetics. All k_a values for SQ association listed in Table 2 were obtained in this way. When k_a values for QX86 associations are calculated by this method, the results are identical to those obtained using Eq. 3. A careful examination of the data in Table 2 reveals the following pertinent points:

(*i*) Under all conditions tested, association rates are markedly higher with SQ than QX86 repressor. Previously these two repressors had been shown to have nearly identical k_a values for association with λh80*dlac* (5, 14) or with λ*plac* (15).

Table 2. Association rates of repressors with operator DNAs

Duplex	Ionic strength, M	Nonoperator DNA	$k_a \times 10^{-8}$ M^{-1} sec^{-1} SQ repressor	$k_a \times 10^{-8}$ M^{-1} sec^{-1} QX86 repressor
I	0.05	No	20	3.3
I	0.20	No	10	0.4
II	0.05	No	18	2.1
II	0.20	No	10	0.4
I	0.05	Yes*	4	0.8
I	0.12	Yes*	4	0.5
I	0.20	Yes*	3	0.3
II	0.05	Yes*	4	0.6
II	0.12	Yes*		0.4
II	0.20	Yes*	3	0.3

Experimental details are described in *Materials and Methods*. Values for k_a were determined as described in *Results*.
* 8 μg of salmon sperm DNA per ml.

(*ii*) A diffusion-limited k_a of approximately 10^8 M^{-1} sec^{-1} was originally estimated for the repressor–operator interaction (5). With wild-type repressor and synthetic operator we find association rate constants an order of magnitude greater than this. The k_a of 2.0×10^9 M^{-1} sec^{-1} we measure with duplex I at $I = 0.05$ M is nearly as high as the value of 7×10^9 M^{-1} sec^{-1} obtained by Riggs *et al.*, using 50,000 base pair λh80*dlac* DNA (5).

(*iii*) When duplex I or II is the only DNA in the association reactions, there is a noticeable dependence of k_a on ionic strength. QX86 associations appear to be more sensitive to changes in ionic strength than SQ associations. When the ionic strength is increased from 0.05 M to 0.20 M, the QX86 association rate constants of 3.3×10^8 M^{-1} sec^{-1} with duplex I and 2.1×10^8 M^{-1} sec^{-1} with duplex II are both reduced to 0.4×10^8 M^{-1} sec^{-1}. The rate constant for association of SQ repressor with either duplex I or II at $I = 0.20$ M is 1.0×10^9 M^{-1} sec^{-1}, or one-half the values obtained at $I = 0.05$ M. This salt effect is much less drastic than previously observed with λh80*dlac* operator DNA. With λh80*dlac*, increasing the ionic strength from 0.05 to 0.20 M lowered the k_a from 7×10^9 to 3×10^8 M^{-1} sec^{-1}, a decrease to less than 5% of the original value (5).

(*iv*) Associations performed in binding buffer containing 8 μg of salmon sperm DNA per ml are much less responsive to changes in ionic strength than associations done without nonoperator DNA. The k_a values for synthetic operator–SQ repressor associations are nearly identical at the three ionic strengths tested when salmon sperm DNA is present. With QX86 repressor, the k_a values are only slightly affected.

(*v*) Nonoperator DNA (8 μg/ml) reduces association rate constants to approximately 20% of their original values at $I = 0.05$ M regardless of the repressor used. At $I = 0.20$ M the extra DNA is much less effective in reducing k_a values, especially in the case of QX86 repressor–operator duplex associations.

(*vi*) The difference in size between duplexes I and II has very little effect on k_a values. With SQ repressor there is no measurable difference in association rates between the two duplexes. With QX86 repressor, duplex I appears to have slightly higher k_a values than duplex II.

DISCUSSION

The synthetic *lac* operator duplexes I and II were shown to be essentially homogeneous when analyzed by polyacrylamide gel

Table 3. Repressor–operator equilibrium constants calculated from kinetic data

Duplex	Ionic strength, M	$K_d \times 10^{12}$ M	
		SQ repressor	QX86 repressor
I	0.05	7.4	1.7
I	0.20	17	18
II	0.05	10	4.2
II	0.20	24	32

K_d values were calculated from $K_d = k_d/k_a$ using k_a values obtained in the absence of nonoperator DNA.

electrophoresis under both native and denaturing conditions. When these operators were titrated with *lac* repressor protein, more than 50% of the operator DNA was retained by nitrocellulose filters, the same value that is obtained with λ*plac* as the operator source (12, 13). The purity of duplexes I and II allowed us to directly measure both repressor–operator binding constants and the effects of various parameters on these constants.

Other investigators, using a different approach, have chemically synthesized a 21 base pair *lac* operator equivalent to our duplex II (13, 16, 17). However, when this DNA was analyzed by gel electrophoresis, it was found to be highly impure (13). Additionally, variable filter retention efficiencies of 4% (13), 10% (17), and 40% (18) have been reported, presumably with the same deoxyoligonucleotides. The more recent filter retention efficiency (18) is comparable to our results with saturating amounts of active repressor.

Duplexes I and II were found to be very similar in their abilities to bind *lac* repressor. The differences in association rate constants between the two duplexes were very small. Slightly larger differences were observed in dissociation rates. Equilibrium constants obtained from the kinetic data are shown in Table 3. From these values it can be calculated that the extra five base pairs of duplex I decrease the free energy of binding to SQ repressor by only 0.2 kcal (836 J)/mol and to QX86 repressor by about 0.5 kcal (2.09 kJ)/mol. Therefore, it appears unlikely that these five base pairs are involved in specific binding to repressor.

The binding of duplexes I and II to *lac* repressor is influenced by changes in ionic strength. These effects are small when compared to those observed when the operator is present in a high-molecular-weight DNA such as λh80*dlac* (5). The overall effects on K_d values of increasing the ionic strength from 0.05 M to 0.20 M are shown in Table 3. When wild-type (SQ) repressor is used, K_d increases by about 2-fold; with tight-binding (QX86) repressor, the increase in K_d is about 10-fold. Riggs *et al.* (5) have shown that this same increase in ionic strength results in an approximate 100-fold increase in K_d for the interaction between λh80*dlac* and wild-type repressor. This implies that electrostatic interactions between operator and repressor are not as significant with duplexes I and II as they are when the operator sequence is found in large DNA fragments. Record and coworkers (19) have recently developed a method for determining the number of ion pairs involved in ligand–nucleic acid interactions. Their theory is based on measuring the dependence of the equilibrium constant on monovalent cation concentration. Since our results were obtained in the presence of the competitive Mg^{2+} ligand, determination of the number of ion pairs involved in the binding of repressor to duplex I or II cannot be directly calculated from their formula (19). However, it does appear that DNA regions outside of the 26 base pairs of the operator sequence may be involved in ion pair formation with repressor. We also find that increasing the ionic strength from 0.05 M to 0.20 M gives an increase of about 0.5 kcal (2.09 kJ)/mol in the free energy of binding of SQ repressor to either duplex I or II. This indicates that no ion pairs are formed between the extra five base pairs of duplex I and repressor.

The fact that k_a exhibits a greater dependence on ionic strength than does k_d suggests that electrostatic attractions may be important for association reactions, but relatively unimportant after the repressor–operator complex has been formed. The fact that k_a values for duplex I or II with SQ repressor seem anomalously high suggests that long-range electrostatic forces may accelerate the rate of association. For uncharged macromolecules a diffusion-limited k_a on the order of 10^8 M^{-1} sec^{-1} was originally predicted for the repressor–operator interaction (5). Even allowing for the greater diffusion coefficient that duplexes I and II would have compared to high-molecular-weight operator DNA does not increase this upper limit to more than about 5×10^8 M^{-1} sec^{-1}. Yet we find that k_a equa6s 2.0×10^9 M^{-1} sec^{-1} for the reaction between duplex I and SQ repressor at $I = 0.05$ M. By increasing the ionic strength to 0.20 M, the association rate constant is reduced to 1.0×10^9 M^{-1} sec^{-1}. This reduction can be explained in terms of a shielding of the long-range attraction between repressor and operator. These high rates cannot, however, be explained by models of one-dimensional sliding along nonoperator DNA (20, 21) nor by the intradomain transfer model (22) since we are working with a very small DNA fragment. Richter and Eigen (21), taking into consideration electrostatic attraction between repressor and operator, have calculated a maximum diffusion-limited association rate of 2.6×10^9 M^{-1} sec^{-1}. Our measurements agree quite well with this value. The even higher k_a of 7×10^9 M^{-1} sec^{-1} measured with λh80*dlac* at $I = 0.05$ M (5) could then be considered to arise from both electrostatic attraction and an acceleration due to adjacent nonoperator DNA. At $I = 0.20$ M this is apparently not the case. λh80*dlac* has been shown to have a k_a of 3×10^8 M^{-1} sec^{-1} at this salt concentration (5), whereas duplexes I and II have higher values of 1.0×10^9 M^{-1} sec^{-1}. It seems that nonoperator DNA serves to slow association rates despite being on the same DNA chain. Berg and Blomberg (23) have reached the same conclusion from theoretical considerations. This is what one would expect if the salt concentration was sufficient to eliminate any possible intramolecular transfer mechanisms, but still low enough to retain nonoperator DNA binding capabilities. In fact, the k_a values for association of duplexes I and II with SQ repressor are both reduced to 3×10^8 M^{-1} sec^{-1} when 8 μg of salmon sperm DNA per ml is included in the reaction. Nonoperator DNA was found to be effective in reducing k_a values in all the association experiments we performed. However, the effect was much more noticeable at $I = 0.05$ M than at $I = 0.20$ M. This result is in agreement with other studies which demonstrated that the strength of repressor binding to nonoperator DNA decreases rapidly with increasing ionic strength (24, 25). We also found that association rates for QX86 repressor were affected to a greater extent than those for SQ repressor. This is probably due to a greater affinity of QX86 repressor than SQ repressor for nonoperator DNA. Pfahl (15) has noticed this same effect upon association rates with λh80*dlac* DNA.

Several other differences between wild-type (SQ) and tight-binding (QX86) repressors were brought out by these experiments. Association rate constants for reaction with operator duplexes were found to be much lower for QX86 than for SQ repressor. The difference is a factor of 25 at 0.20 M ionic strength, which results in the equilibrium constants for these

two repressors being nearly identical at this salt concentration (see Table 3). The conclusion is also supported by equilibrium binding experiments (unpublished results). (Since the QX86 and SQ repressor stocks were prepared in different laboratories, the possibility exists that differences in physical states may have influenced the association rates.) These results are in contrast to previous experiments which indicated that k_a values of association to high-molecular-weight operator were the same for wild-type and tight-binding repressors at $I = 0.05$ M (5, 14, 15). Another noticeable difference between the two repressors is in the electrostatic contribution to the free energy of binding operator. In going from $I = 0.05$ M to 0.20 M, ΔG is increased by over twice as much for QX86 repressor as it is for SQ repressor. Finally, the rate constants of association and dissociation for the two repressors behave differently in response to changes in duplex size. When the operator is reduced from 26 to 21 base pairs, both k_a and k_d are altered more significantly for QX86 than for SQ repressor, suggesting that QX86 repressor interacts to a greater extent with the first five base pairs of duplex I than does SQ repressor.

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