Richardson, J. P. (1975) *J. Mol. Biol. 91*, 477-487.
Roe, J. H., Burgess, R. R., & Record, T. R. (1984) *J. Mol. Biol. 174*, 495-521.
Rosenberg, M., & Court, D. (1979) *Annu. Rev. Genet. 13*, 319-353.
Sandström, J. (1982) *Dynamic NMR Spectroscopy*, Academic, London.
Siebenlist, U. (1979) *Nature (London) 279*, 651-652.
Suzycki, S. J., Suzycki, J. A., & Gussin, G. N. (1976) *Mol. Gen. Genet. 143*, 167-175.
Wagner, G., & Wüthrich, K. (1979) *J. Magn. Reson. 33*, 675-678.
Wilbur, D. W., DeFries, T., & Jonas, J. (1976) *J. Chem. Phys. 65*, 1783-1789.
Wilson, W. D., Zuo, E., Jones, R. L., Zon, G. L., & Baumstark, B. R. (1987) *Nucleic Acids Res. 15*, 105-118.

Dynamics of Repressor–Operator Recognition: The Tn*10*-Encoded Tetracycline Resistance Control†

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Received April 1, 1987; Revised Manuscript Received October 14, 1987

ABSTRACT: Binding of the Tet repressor to nonspecific and specific DNA leads to quenching of the Tet fluorescence by ~22% and ~35%, respectively. This effect is used for a direct, quantitative characterization of the binding equilibria and dynamics involved in the recognition of the operator by its repressor. From the dependence of the nonspecific binding constant on the ion concentration, it is concluded that nonspecific binding is almost completely driven by the entropy change resulting from the release of three to four Na^+ ions from the double helix upon protein binding. Formation of the specific complex is driven by a higher entropy term resulting from the release of seven to eight Na^+ ions and in addition by a free energy term of −33 kJ/mol from nonelectrostatic interactions, which are attributed to the specific contacts. The dynamics of the repressor–operator recognition are resolved by stopped-flow measurements at various salt concentrations and for different DNA chain lengths into two separate steps. The first step follows a second-order mechanism and results in an intermediate complex associated with formation of about three to four electrostatic contacts between protein and DNA; apparently, this complex is equivalent to the nonspecific complex. The existence of an intermediate is also indicated by experiments in mixed Na^+–Mg^{2+} buffers, which can be described with high accuracy by competition of Mg^{2+} and protein. The intermediate complex is formed at a rate of 3×10^8 M^{-1} s^{-1} and is converted in the second reaction step to the specific complex with a rate constant of 6×10^4 s^{-1}, which is almost independent of the salt concentration. Our interpretation and the parameters obtained from our model are confirmed by competition of nonspecific DNA with operator DNA for repressor binding. The observed maximal rate constant of 3×10^8 M^{-1} s^{-1} is very close to theoretical predictions for the association without a sliding mechanism. The very small dependence of the observed rate constants on the chain length shows that the Tet repressor is not able to slide over any substantial distance even at low salt concentrations. The question of a potential contribution from sliding under our experimental conditions is critically discussed. The absence of sliding in the case of the Tet repressor under physiological conditions is compared with the high sliding efficiency of the *lac* repressor and is discussed with respect to possible molecular mechanisms of sliding in relation to biological function.

The recognition of DNA operators by their repressors is a key process in the regulation of gene activity and is well-known for its unusually high specificity. The *lac* repressor–operator system has been the main object for investigations of the specificity and dynamics of this reaction. As first demonstrated by Riggs et al. (1970a,b), the *lac* repressor not only shows a high degree of specificity but also has an unusually high rate of reaction, which appeared to be at first glance beyond the physical limits of diffusion-controlled reactions. A quantitative theoretical analysis (Adam & Delbrück, 1968; Richter & Eigen, 1974; Berg & Blomberg, 1976, 1977, 1978; Schranner & Richter, 1978; Berg et al., 1981) revealed that the high rate is due to an increase of the operator target size resulting from binding of the repressor to nonspecific DNA and sliding of the protein along the nonspecific sites. This effect leads to a reduction of the diffusion dimension from three to one and thus to a considerable increase of the association rate.

The rate parameters of the *lac* system had to be measured by the filter binding technique, which is rather indirect and subject to systematic errors. Because of the general importance of the repressor–operator reactions, it should be useful to study this type of reaction more directly, for example, by some spectroscopic technique. Thus, an initial observation of fluorescence quenching upon binding of the Tet repressor to DNA served as a basis for a systematic investigation of the specific and nonspecific binding reactions of this protein to DNA. The Tn*10*-encoded Tet repressor regulates the expression of tetracycline resistance in gram-negative bacteria

† This work was presented to the Technische Universität Braunschweig by C.K. in partial fulfillment of the requirements for the Ph.D. degree. W.H. was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der chemischen Industrie.
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0006-2960/88/0427-1094$01.50/0

(Beck et al., 1982; Hillen et al., 1982; Yang et al., 1976). It is a dimer with a molecular weight of 2 × 23 500 and binds in the absence of the inducer tetracycline to two palindromic operator sequences in the Tn*10 Tet* operon regulatory region (Wray & Reznikoff, 1983; Hillen et al., 1984). Upon addition of tetracycline, the drug is complexed with repressor and causes the protein to fall off the DNA (Hillen et al., 1984).

The Tet repressor proved to be suitable for our investigation not only because of its spectroscopic signal but also because of its rather high stability and the availability of overproducers for the protein and of plasmid constructs for large-scale preparation of specific DNA (Oehmichen et al., 1984; Hillen et al., 1982). The binding reactions were mainly studied by the stopped-flow technique. The data obtained for different salt concentrations and for DNA fragments of different length can be explained by a simple three-dimensional diffusional search without substantial contribution from sliding. Our results also provide new information on the mechanism of nonspecific versus specific binding. Finally, we have been able to characterize the dynamics of the transition from the nonspecific to the specific complex, which corresponds to the real recognition event.

Theoretical Section

The theoretical problems associated with the recognition of a specific DNA sequence by a cognate protein have been discussed and evaluated in considerable detail [for a summary, see, e.g., Berg and von Hippel (1985)]. Thus, the interpretation of our data has been based on the theoretical models described in the literature. For convenience and intelligibility, we present the relations used in our evaluation together with a brief account of their theoretical foundation.

General Formalism. We denote by the capital letters R, O, D, RO, and RD the repressor, operator, nonspecific (i.e., nonoperator) DNA, the repressor–operator complex, and the complex of repressor with nonspecific DNA, respectively. Accordingly, [R], [O], etc. are the free concentrations of a particular species, whereas $[R]^0$, $[O]^0$, and $[D]^0$ stand for the total concentration of repressor, operator, and nonspecific DNA, respectively. Repressor concentration is always given in dimer units and specific DNA in single operator units, and for nonspecific DNA the base pair (bp) was chosen as the appropriate unit. The strength of repressor–operator binding is characterized by the specific binding constant

$$K_{RO}^{obsd} = [RO]/[R][O] \quad (1)$$

The index "obsd" indicates that this is an observable quantity which is defined independently of the detailed reaction mechanism. Actually, the equilibrium which is described by K_{RO}^{obsd} could involve other chemical species, e.g., nonspecific DNA or small ions, and thus be dependent on the concentration of these species.

Accordingly, the observed nonspecific binding constant is defined by

$$K_{RD}^{obsd} = [RD]/[R][D] \quad (2)$$

Analogously, the kinetic rate constants are defined as "observed" quantities. The specific (s) association rate constant is given by

$$d[RO]/dt = k_{s,a}^{obsd}[R][O] \quad (3)$$

Thus, the specific association is here assumed to be irreversible. Again, the constant is of a merely operative nature and does not consider the complexity of the reactions proceeding in solution; for example, any intermediate step (which is of course necessary for sliding) is neglected. The nonspecific (ns) association is considered to be reversible and is described by

$$d[RD]/dt = k_{ns,a}^{obsd}[R][D] - k_{ns,d}^{obsd}[RD] \quad (4)$$

One-Dimensional Diffusion (Sliding). Schranner and Richter (1978) derived an equation for the specific association constant with one-dimensional diffusion along the DNA helix axis by a steady-state solution of the diffusion equation with coupled three- and one-dimensional diffusional flux. Their approach is based on the treatment of Richter and Eigen (1974) and considers DNA as a long, stiff molecule of ellipsoidal geometry. Thus, the range of validity of their result

$$k_{s,a}^{obsd} = 4\pi D_p \frac{\lambda}{\ln(2L/b)} \tanh \frac{L}{\lambda} \frac{N_A}{1000} \quad (5)$$

is confined to short rodlike DNA fragments. In this equation, D_p is the sum of the diffusion coefficients for the protein and the DNA fragment, $2L$ the DNA fragment contour length, λ the average distance covered by sliding of the protein, and b the sum of the DNA helix radius and the mean radius of the protein; N_A is Avogadro's number. A single operator is assumed to be located in the middle of the fragment. The sliding distance λ is given by

$$\lambda \approx \sqrt{D_1/k_{ns,d}^{obsd}} \quad (6)$$

where D_1 is the one-dimensional diffusion constant of the protein along the DNA helix.

As we will see later, $k_{ns,d}^{obsd}$ is dependent on the salt concentration, thus opening the possibility to vary the potential sliding distance by adjusting the experimental conditions.

For practical purposes, Berg et al. (1981) provide another form of eq 5, expressing λ by eq 6, $k_{ns,d}^{obsd}$ by $k_{ns,a}^{obsd}/K_{RD}^{obsd}$, and substituting the theoretical expression for $k_{ns,a}^{obsd}$ from Richter and Eigen (1974):

$$k_{s,a}^{obsd} \approx 4\pi D_p \sqrt{\frac{D_1 K_{RD} \times 1000}{4\pi D_p (1.7\ \text{Å}) \ln(2L/b) N_A}} \tanh \sqrt{\frac{4\pi D_p (1.7\ \text{Å}) L^2 N_A}{D_1 K_{RD} \ln(2L/b) \times 1000}} \frac{N_A}{1000} \quad (7)$$

A slightly different situation arises if, as in the case of this work, two operators are situated quite close together on each DNA fragment. If sliding is restricted to a special pathway on the double helix as, for example, the large groove, repressors may find their operators via sliding only from one side. In this case, the effective dimensions of the operator target resulting from sliding are reduced by a factor of 2 with respect to operators, which are accessible via (sufficiently long) segments of unspecific DNA from both sides. If all the "elementary" rate constants of, e.g., unspecific association and sliding remain unchanged, the facilitating effect due to sliding is expected to be smaller for a tandem operator than for a corresponding single operator (by a factor of approximately 2 according to a simple, idealized model). Of course, the equilibrium constants are not affected by the location of the operators, unless there is some interaction. Since we did not detect any anti- or cooperativity between the Tet operator sites, we used the simple definition of the equilibrium constant given by eq 1.

Hopping. In order to obtain the specific association rate in the absence of a sliding mechanism, a first simple approach starts from the nonspecific association rate and calculates the number of "wrong" (i.e., nonspecific) binding sites which have to be tested by the repressor before the "correct" binding site

(the operator) is found. According to this procedure (Berg et al., 1981), the apparent reaction radius is on the order of the length of a base pair, which is obviously too small. A more realistic calculation accounts for the strong spatial correlation of the base pairs as the possible binding sites and extends the reaction radius to a larger value, which should be approximately the radius of the DNA chain. In a model calculation describing the associating protein as point particle, Berg et al. (1981) find for the specific association rate without sliding

$$k_{s,a}^{obsd} \approx 1.5\pi D_p b(N_A/1000) \quad (8)$$

According to this result, the rate does not depend on the nonspecific binding affinity. The same equation has been derived for the limit case, where a nonspecific interaction is not present at all and the nonspecific DNA just acts as an excluded volume (Berg & Ehrenberg, 1982). When the repressor approaches the DNA chain, it may "test" several adjacent base pairs for the correct binding site. In a simple visualization of this process, the protein performs during each "macroscopic" association event a number of "microscopic" dissociations followed by a reassociation to the same or to a neighboring site. On the basis of this picture, the whole phenomenon is called "hopping" by Berg et al. (1981). However, it must be stressed that hopping does not describe anything else than the three-dimensional diffusional motions of the protein. Thus, hopping is not a facilitating mechanism like sliding and is present in every protein–DNA interaction. It just serves as an explanation why the effective target size is larger than the length of one base pair.

Competition with Nonspecific DNA. If the solution contains a large quantity of nonspecific DNA in addition to the operator DNA, the former may act as a competitor for repressor protein, thus reducing the observed specific association rate constant. When the nonspecific DNA is present in excess, we may write $[D] \approx [D]^0$. This condition is always fulfilled in the competition experiments described below. The evaluation may be further simplified by the realistic assumption that the nonspecific binding is always in equilibrium. In this case, the observed specific association constant is reduced by a factor

$$\frac{1}{1 + K_{RD}[D]^0}$$

Equations 5, 7, and 8 have to be multiplied by this factor to account for the competitive effect of nonspecific DNA. However, if the nonspecific interaction is not allowed to reach equilibrium, the competition effect can be hidden by some other rate-limiting step. For example, in the case of fast sliding on small fragments (the low-salt limit of eq 7), the time for the repressor to find the whole fragment may be much longer than the following transfer time to the final binding site. Consequently, competition is not effective and the correction factor has to be omitted.

Salt Dependence of Equilibrium and Rate Constants. According to polyelectrolyte theory (Manning, 1978; Record et al., 1978), the dependence of the binding constant, K^{obsd}, of the repressor protein to DNA on the salt concentration, $[Na^+]$, is given by

$$\frac{\partial \log K^{obsd}}{\partial \log [Na^+]} = -m\Psi \quad (9)$$

where m denotes the number of ion pairs in the complex and Ψ the fraction of polyelectrolyte charges compensated by counterions, which is $\Psi = 0.88$ for B-DNA in the presence of monovalent counterions. This equation is valid both for specific (K_{RO}^{obsd}, m_{RO}) and for nonspecific (K_{RD}^{obsd}, m_{RD}) binding. The factor $\Psi = 0.88$ has been calculated on the basis of polyelectrolyte theory (Manning, 1978; Record et al., 1978) for the density of negative phosphate charges of B-DNA. It has been demonstrated that the results of this polyelectrolyte theory are in excellent agreement with most experimental data obtained for DNA (Manning, 1978). Some results obtained for protein binding to DNA indicate the existence of special anion effects. We have no evidence yet for the existence of anion effects in the case of Tet repressor.

The salt dependence of the kinetic constants is evaluated as described by Lohman et al. (1978). Two possible binding mechanisms are distinguished: (1) a one-step association without salt-dependent intermediates and (2) a mechanism involving a preequilibrium with electrostatic contacts different from those of the final complex.

In the first case, the association rate is mainly influenced by the screening effect resulting from the "Debye–Hückel" ion atmosphere. This effect reduces the charge density of the DNA by a factor $\Psi_s = 0.12$ and leads to a salt dependence of the rate constant for specific association given by

$$\frac{\partial \log k_{s,a}^{obsd}}{\partial \log [Na^+]} = -m_{RO}\Psi_s = -0.12m_{RO} \quad (10)$$

A corresponding relation for the dissociation rate constant may be obtained by combination of eq 9 and 10:

$$\frac{\partial \log k_{s,d}^{obsd}}{\partial \log [Na^+]} = \Psi_c m_{RO} = 0.76m_{RO} \quad (11)$$

where Ψ_c describes the screening of phosphate charges by counterion condensation [with $\Psi = \Psi_c + \Psi_s$; cf. Record et al. (1978)]. Due to the relatively small value of Ψ_s, the salt dependence of the association rate should be relatively low. Since $\Psi_c/\Psi_s = 0.76/0.12 \sim 6$, the dissociation step should contribute 6 times more to the salt effects on K_{RO} than the association step.

In the second case, the protein has to pass through an electrostatic preequilibrium according to

$$R + O \overset{K_I^{obsd}}{\rightleftharpoons} I_n \underset{k_d}{\overset{k_f}{\rightleftharpoons}} RO \quad (12)$$

The intermediate complex I_n is characterized by the integer n, which stands for the number of ion contacts closed in the preequilibrium. Again, the first part of reaction 12 must be treated according to the molecular approach outlined above. This yields

$$\frac{\partial \log K_I^{obsd}}{\partial \log [Na^+]} = -n\Psi \quad (13)$$

Now the rate of formation of the final complex is

$$d[RO]/dt = k_f[I_n] = k_f K_I^{obsd}[R][O] \quad (14)$$

so that (again for the special case dealt with here and only for specific binding)

$$k_{s,a}^{obsd} = k_f K_I^{obsd} \quad (15)$$

Assuming k_f to be salt independent, eq 15 gives

$$\frac{\partial \log k_{s,a}^{obsd}}{\partial \log [Na^+]} = -n\Psi = -0.88n \quad (16)$$

using also eq 13 and with eq 9

$$\frac{\partial \log k_{s,d}^{obsd}}{\partial \log [Na^+]} = 0.88(m_{RO} - n) \quad (17)$$

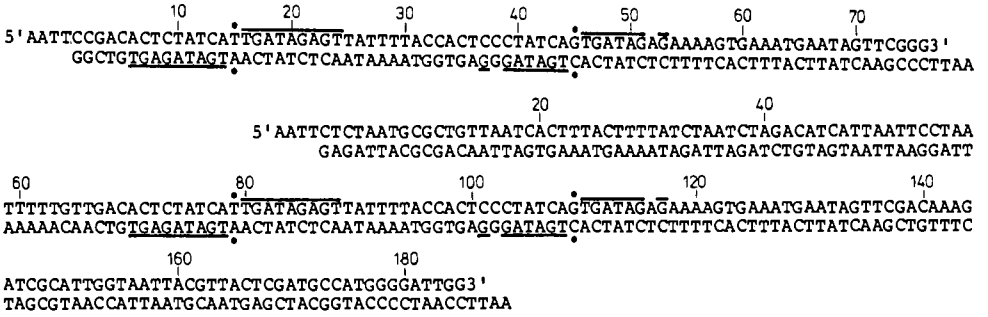

FIGURE 1: Sequences of the 76 bp and the 185 bp fragment. The palindromic operator sites are marked.

Thus, by introduction of an electrostatic preequilibrium, $k_{s,a}^{obsd}$ is strongly salt dependent, the strength of this dependence being dependent on the number n. If $n > m_{RO}/2$, the association step contributes even stronger to the salt dependence of K_{RO}^{obsd} than the dissociation step.

However, an upper limit on the association rate constant is imposed by the diffusional limit of the formation of the intermediate complex. This case arises when the rate of final complex formation (k_f) is much faster than the decay of the intermediate complex toward the left side. Then the association rate $k_{s,a}^{obsd}$ is given by the rate of diffusional encounter of protein and polynucleotide. It is also no longer justified to speak of a "preequilibrium" in this case.

Finally, we have to describe the influence of divalent ions like Mg^{2+} on the preequilibrium. In this case, part of the condensed Na^+ ions is replaced by Mg^{2+} ions. Upon binding of the protein, both Na^+ and Mg^{2+} ions will be released. Hence, less Na^+ ions are released than in the Mg^{2+}-free case. Since the quantity

$$\frac{\partial \log k_{s,a}^{obsd}}{\partial \log [Na^+]}$$

is a measure of the Na^+ release, its magnitude will decrease with respect to the value without Mg^{2+}. Moreover, this quantity will no longer be independent of the Na^+ concentration, since the extent of Mg^{2+} binding to the DNA is also a function of $[Na^+]$. The theoretical treatment of these features gives the association rate constant for the preequilibrium case in the presence of Mg^{2+} and Na^+ (with the restriction $[Mg^{2+}] \ll [Na^+]$) (Lohman et al., 1978):

$$\log k_{s,a}^{obsd} = \log k_0 - n\Psi \log [Na^+] - n \log S \quad (18)$$

Here k_0 comprises the salt-independent terms, and S is given by

$$S = (1/2)(1 + \sqrt{1 + 4K_{Mg^{2+}}^{obsd}[Mg^{2+}]}) \quad (19)$$

where $K_{Mg^{2+}}^{obsd}$ is defined through

$$K_{Mg^{2+}}^{obsd} = [Mg^{2+}\cdot D]/[Mg^{2+}][D] \quad (20)$$

and exhibits the usual salt dependence

$$\frac{\partial \log K_{Mg^{2+}}^{obsd}}{\partial \log [Na^+]} = -r\Psi \quad (21)$$

r is the number of ion contacts for binding of Mg^{2+} to the polyelectrolyte and is expected to be about 2.

Materials and Methods

All chemicals used were of analytical grade. Restriction enzymes were purchased from Boehringer, Mannheim and from Renner, Dannstadt. All experiments were performed in the following buffer: 5 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, and 0.1 mM dithioerythritol (DTE). The concentrations of Na^+ and Mg^{2+} present in the solution are indicated in each case. In experiments without magnesium, 0.1 mM ethylenediaminetetraacetic acid (EDTA) was added. All experiments were conducted at 20 °C.

The Tet repressor was prepared by using an overproducing strain as described (Oehmichen et al., 1984). For the experiments, a small amount of repressor [stored as an $(NH_4)_2SO_4$ precipitate] was diluted in the desired buffer and dialyzed 3 times for 8 h against this buffer. The repressor concentration was determined spectrophotometrically by using an extinction coefficient of 30.6×10^3 cm^2/mol at 280 nm for the dimer (Altschmied & Hillen, 1984).

Two operator-containing fragments were prepared as described: a 185 bp fragment from pWH106 (Hillen et al., 1982) and a 76 bp fragment from pWH923 and purified by high-performance liquid chromatography (HPLC) on Nucleogen 4000 (Macherey & Nagel, Düren; Colpan & Riesner, 1984). Figure 1 shows the location and size of the operators within the sequences of the fragments. pWH923 was constructed by eluting the *Hin*cII–*Taq*I Tet fragment from a respective digest of pWH106 from a polyacrylamide gel, filling in the 5′-protruding ends, ligating *Eco*RI linkers (5′GGAATTCC3′) to the ends, and inserting this DNA into pWH802 (Unger et al., 1984) cut with *Eco*RI to obtain a triple insertion (details will be published elsewhere). Both fragments contained two operators located approximately in the middle and separated by 30 bp between the centers of palindromic symmetry. They were both cut from the plasmids by *Eco*RI. With the exception of the *Eco*RI linker, the smaller fragment was completely contained in the larger one, and the sequences were identical with those in vivo.

Unspecific DNA for the competition experiments was prepared by sonification (5 × 5 min at ice-bath temperature with 5-min intervals of bubbling with nitrogen) of calf thymus DNA (Boehringer Mannheim). By this procedure, we obtained DNA fragments with lengths between 100 and 800 bp as established by electrophoresis. Concentrations of both specific and nonspecific DNA were determined spectrophotometrically using an extinction coefficient of 6.55×10^3 cm^2/mol (mononucleotides) at 260 nm. Operator concentrations were calculated on the basis of the known numbers of operators per fragment and nucleotides per fragment.

Emission spectra were recorded on an Aminco SPF 500 fluorometer. The fluorescence was excited at 280 nm and the emission intensity measured with a bandwidth of 2 nm using a WG305 cutoff filter. For each sample, the absorbance at the excitation wavelength was determined on a Zeiss PMQ II spectrophotometer. With these values, the inner filter effect was corrected by using the procedure given by Porschke and Rauh (1983). Kinetic experiments were performed on a stopped-flow apparatus constructed in the MPI. The excitation

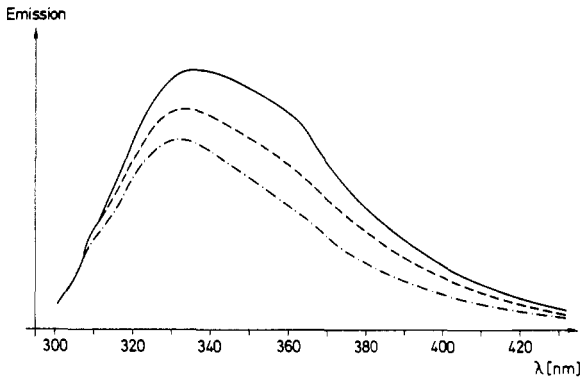

FIGURE 2: Emission spectra of free repressor [(—) 0.5 μM], repressor with *nonspecific* DNA [(---) 0.5 and 62.5 μM, respectively], and repressor bound to the 76 bp operator fragment [(-·-) 0.5 μM repressor and 0.5 μM operator, respectively] in 5 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.1 mM DTE, and 0.1 mM EDTA; excitation at 280 nm; 20 °C. All curves are corrected for the inner filter effect and dilution. The fluorescence quenching (wavelength integrated between 320 and 380 nm) amounts to 22% ● 3% for nonspecific binding (extrapolated to complete complexation) and 35% ± 3% for specific binding.

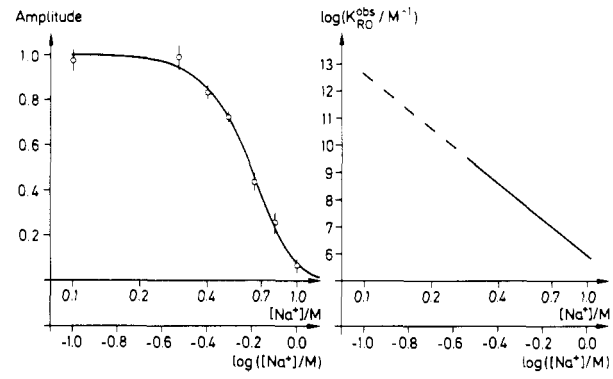

FIGURE 3: Relative fluorescence amplitude (left) observed upon mixing of equal volumes of 0.2 μM repressor to 0.2 μM 76 bp operator fragment as a function of the Na^+ concentration. The continuous line represents a fit of the data according to eq 9 with the K_{RO}^{obsd} values shown in the right panel. The dashed part of the line in the right panel is an (not measurable) extrapolation to lower salt concentrations.

wavelength was 280 nm, and emission was collected without a monochromator but with a WG305 cutoff filter. The kinetic curves were first stored on a Biomation 1010 transient recorder and subsequently transferred to the Univac 1108 of the Gesellschaft für wissenschaftliche Datenverarbeitung, Göttingen.

The evaluation of the kinetic data could not be simplified by approximations valid for quasi-equilibrium conditions or for pseudo-first-order reactions. Thus, the data were not evaluated in terms of exponentials, but by numerical integration using a program developed by M. Jung. The program can be used for integration of a system of differential equations together with least-squares fitting of free parameters in the equations to the experimental data. The differential equations are solved by a modified Runge Kutta procedure of fifth order; the parameters are fitted by a quasi-Newton method. Standard deviations of our fitted parameters have been estimated on the basis of the inverted Hesse matrix [cf. Nelder and Mead (1964/65)].

Results and Discussion

Equilibrium Parameters from Stopped-Flow Amplitudes. The Tet repressor protein contains two tryptophan residues per monomer unit, which emit the usual tryptophan fluorescence upon excitation at, e.g., 280 nm (cf. Figure 2). When double-helical DNA is added to the Tet repressor, the fluorescence is partially quenched. As shown in Figure 2, the extent of quenching induced by specific DNA is higher (~35%) than that observed upon addition of nonspecific DNA (~22%).

The change in the fluorescence intensity may be used to determine equilibrium parameters by titration experiments. However, the specific DNA was not available in sufficient quantities for standard titration procedures, and, thus, the required equilibrium parameters were determined by a special procedure using the stopped-flow technique. The repressor was mixed in a given amount with operator DNA, and the fluorescence change corresponding to the reaction from free repressor to the equilibrium concentration of bound repressor was recorded. Measurements at different salt concentrations show a maximal value of the fluorescence amplitude at low salt corresponding to complete complexation, whereas a decrease of the amplitude observed at high salt indicates a decrease of the binding affinity (Figure 3). The observed fluorescence amplitude is given in relative units with respect to the limit value observed at low salt and reflects the degree of binding, provided that the fluorescence intensities of the free repressor and of the repressor–operator complex are not strongly salt dependent. An analysis of the free repressor demonstrated that its fluorescence does not depend on the NaCl concentration at all. A corresponding analysis of the complex also showed the absence of any salt dependence of the fluorescence up to 0.3 M NaCl. Although these measurements could not be extended to higher salt because of complex dissociation, it is rather unlikely that the fluorescence intensity of the complex is salt dependent in this range, where the protein alone does not show any dependence at all. Thus, we may conclude that the relative amplitudes given in Figure 3 reflect the degree of binding and can be analyzed according to the law of mass action using a stability constant, which is salt dependent according to eq 9. Our amplitudes for specific binding are not affected by nonspecific quenching, because all repressor molecules finally arrive at operator sites. The unknown parameters have been determined by least-squares fitting of the experimental data. As shown in Figure 3, the data can be represented by a salt-dependent stability constant according to

$$\log K_{RO}^{obsd} = (5.9 \pm 0.2) - (6.7 \pm 0.8) \log [Na^+] \quad (22)$$

Thus, the binding constant of the repressor to the operator at 1 M NaCl is 8×10^5 M^{-1}, and the number of ions released upon complex formation is 7.6 ± 0.9 (cf. eq 9).

The parameters for repressor binding to nonspecific DNA have been determined by a similar procedure. In this case, an *equilibrated* solution containing 0.2 μM repressor *and* 500 μM (bp) DNA was mixed in the stopped-flow apparatus with 0.2 μM operator. Since the Na^+ concentration did not exceed 150 mM, both free repressor and the repressor bound to nonspecific DNA were trapped completely by the operator. The amplitude observed for this reaction increases with the salt concentration (cf. Figure 4), because the portion of free repressor in the preequilibrium mixture with nonspecific DNA increases. The quantitative description of the nonspecific preequilibrium is simplified by the fact that excluded site phenomena are avoided owing to the large excess of nonspecific DNA in the preequilibrium mixture. The salt dependence of the nonspecific binding constant is described according to eq 9, and the parameters were evaluated by least-squares fitting to the experimental data:

$$\log K_{RD}^{obsd} = (0.1 \pm 1.0) - (2.9 \pm 0.9) \log [Na^+] \quad (23)$$

Thus, at 1 M NaCl, the binding constant of the repressor to

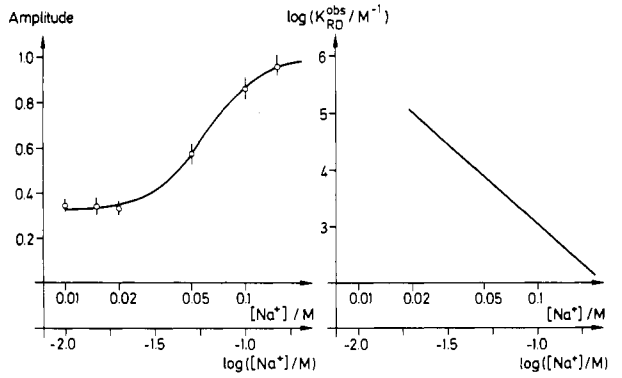

FIGURE 4: Relative fluorescence amplitude (left) observed upon mixing of equal volumes of 0.2 μM repressor + 500 μM nonspecific DNA with 0.2 μM 76 bp operator fragment as a function of the Na^+ concentration. The continuous line represents a fit (cf. text) with the K_{RD}^{obsd} values shown in the right panel.

nonspecific DNA is very low, and the number of ions released upon formation of nonspecific complexes is 3.3 ± 1.0. These results for the nonspecific binding, which can be obtained from the measured amplitudes only at a relatively low accuracy, are confirmed by independent competition experiments described below.

Two-Step Mechanism of Operator Recognition. The kinetics of repressor binding were also studied by stopped-flow measurements. Stopped-flow experiments are usually conducted under quasi-first-order conditions in the excess of one reactant, in order to simplify the evaluation of data. In the present case, this procedure is not practicable, because an excess of repressor decreases the relative fluorescence signal and thus the signal to noise ratio, whereas the amount of operator required for sufficiently high concentrations was not available. For these reasons, repressor and operator were always mixed in a 1:1 ratio.

A complication of the experiments and their evaluation resulted from a small, but clearly detectable, photoreaction of the protein under UV irradiation. Even at the lowest light intensities associated with a reasonable signal to noise ratio for the binding reaction, the photoreaction was still reflected by a decrease of the fluorescence intensity. Separate test experiments with the repressor, the repressor–operator complex, and the complex of the repressor with nonspecific DNA revealed in all cases a linear decrease of the fluorescence intensity with the irradiation time. However, the loss of reactable repressor remains very small and may be neglected. Thus, the data were evaluated according to the following differential equation for a usual second-order reaction:

$$d[RO]/dt = k_{s,a}^{obsd}[R][O] - k_{s,d}^{obsd}[RO] \qquad (24)$$

The fluorescence signal, $F(t)$, resulting from this reaction was described by

$$F(t) = F(0) - \alpha[RO] - pt \qquad (25)$$

where $F(0)$ is the fluorescence intensity at time zero, α is an empirical coefficient to describe the fluorescence change upon formation of the complex RO, and pt reflects the photoreaction with a linear dependence on the irradiation time t. The optical parameters $F(0)$, α, and p are free parameters, which are fitted together with the rate constant by a least-squares procedure. A contribution to fluorescence quenching may result also from nonspecific binding (cf. Figure 2). However, the extent of nonspecific binding under the conditions of the stopped-flow experiments with the operator DNA remains very low (except for very low salt concentration), and thus, nonspecific quenching can be neglected.

A typical stopped-flow reaction curve together with a least-squares fit is shown in Figure 5. Stopped-flow measurements at different NaCl concentrations in the range from 0.01 to 1 M demonstrated that the dissociation rate constant $k_{s,d}^{obsd}$ is virtually zero for $[Na^+] \leq 0.2$ M. The $k_{s,d}^{obsd}$ values for higher salt concentrations were not fitted as an independent parameter but were calculated according to $k_{s,a}^{obsd}/K_{RO}^{obsd}$ with K_{RO}^{obsd} given by eq 22 and $k_{s,a}^{obsd}$ as a free parameter fitted to the stopped-flow curves. The $k_{s,a}^{obsd}$ values determined by this procedure show a very special dependence upon the salt concentration (cf. Figure 6). At low Na^+ concentrations, the $k_{s,a}^{obsd}$ value approaches a plateau value of about 3×10^8 M^{-1} s^{-1},

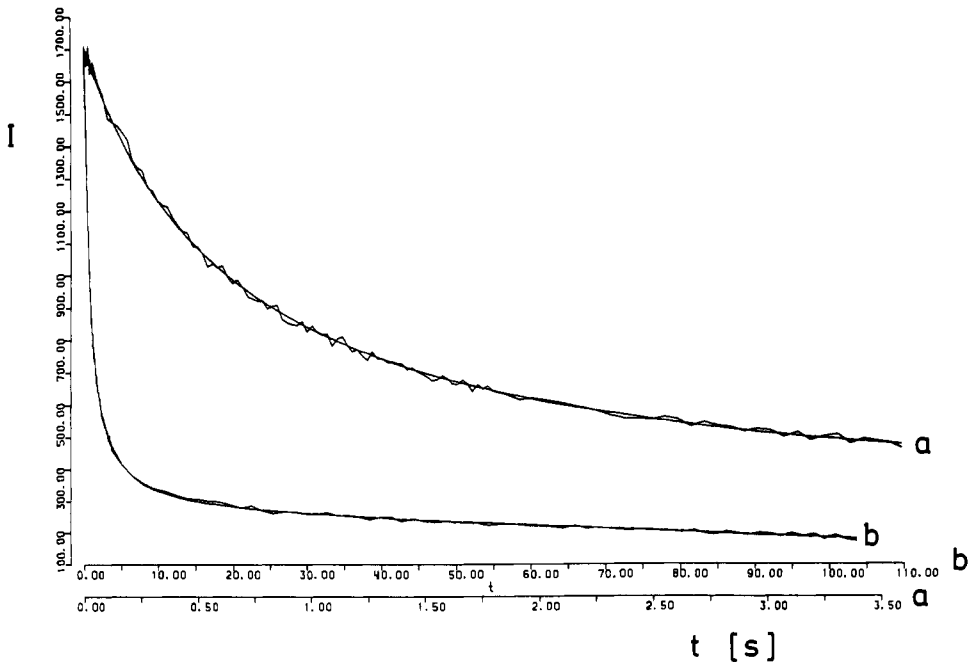

FIGURE 5: Change of fluorescence intensity I (in arbitrary units) upon mixing of equal volumes of 0.2 μM repressor with 0.2 μM 76 bp operator fragment as a function of time at 150 mM NaCl (shown in two sweep rates a and b). The line without noise represents a fit according to eq 24 and 25.

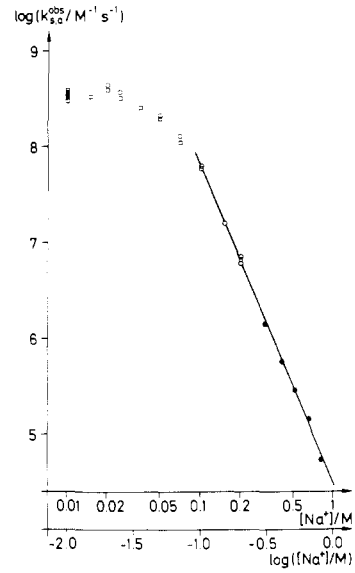

FIGURE 6: Rate constants $k_{s,a}^{obsd}$ for the association of repressor with the 76 bp operator fragment (○) and with the 185 bp operator fragment (□) as a function of the Na^+ concentration. The symbols (●) indicate results from experiments with the 76 bp fragment, where the reaction curves are influenced by a contribution from the dissociation rate, which was calculated by using K_{RO}^{obsd} (cf. Figure 3).

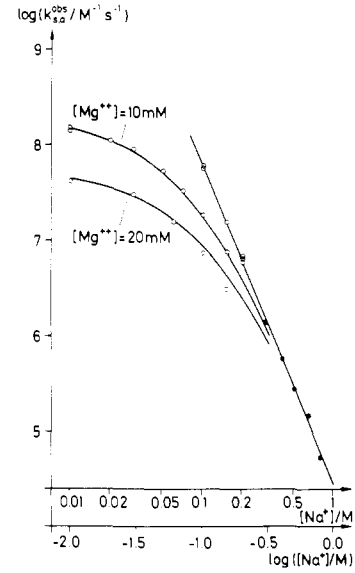

FIGURE 7: Rate constants $k_{s,a}^{obsd}$ in mixed Na^+–Mg^{2+} buffers. Symbols as defined in Figure 6. The data for $[Mg^{2+}] = 0$ are from Figure 6.

whereas a linear decrease of log $k_{s,a}^{obsd}$ with increasing log $[Na^+]$ is observed for $[Na^+] > 0.1$ M. The rate constants found in the latter range can be represented with high accuracy by

$$\log k_{s,a}^{obsd} = (4.5 \pm 0.1) - (3.3 \pm 0.1) \log [Na^+] \quad (26)$$

The decrease of the association rate constant at high salt concentration to values far below the limit of diffusion control and the unusually strong salt dependence observed in this range are not consistent with a one-step "screening" mechanism. These results demonstrate the existence of a salt-dependent intermediate on the reaction pathway to the repressor–operator complex. Our conclusion on the existence of such an intermediate is a simple consequence from the strong salt dependence of the association rates and is consistent with standard treatments of kinetic data. Since the slope $\partial \log k_{s,a}^{obsd}/\partial \log [Na^+]$ is very similar to that found for the nonspecific binding, it is likely that the intermediate complex corresponds to nonspecific binding.

An important observation is the close similarity of the rate constants for the fragments with 76 and 185 bp. If the Tet repressor would slide along these fragments, the rate constants at low salt should differ by a factor of about 2.4 according to eq 7. The limit value of the association rate 3×10^8 M^{-1} s^{-1} found at low $[Na^+]$ does not require sliding as a facilitating mechanism. It is consistent with a simple three-dimensional diffusional search including the hopping phenomenon (Berg et al., 1981). The observed decrease of $k_{s,a}^{obsd}$ at higher salt ($k_{s,a}^{obsd} \sim K_{RD}^{obsd}$) is apparently determined only by the salt-dependent intermediate in the reaction pathway. A pure sliding mechanism would lead to a dependence $k_{s,a}^{obsd} \sim (K_{RD}^{obsd})^{1/2}$ (cf. eq 7). However, from the observed salt dependence, we only can conclude that sliding is not a rate-limiting step for the conditions of high salt. A detailed discussion of the sliding problem is given below.

For a description of the final steps in the recognition of the operator, a decision on the efficiency of Tet repressor sliding is not required. Our results demonstrate the existence of a salt-dependent intermediate complex, which must be located very close to or at the specific site. This intermediate is then converted in an intramolecular reaction to the final specific complex. If the intermediate complex is identical with the nonspecific complex, we may calculate the rate of conversion to the final complex (k_f). Using the simple relation $k_{s,a}^{obsd} = k_f K_I^{obsd}$, we obtain by division

$$\log k_f = (4.3 \pm 1.0) - (0.4 \pm 0.9) \log [Na^+] \quad (27)$$

This result should be regarded as approximate, because the intermediate complex and the nonspecific complex might not be completely equivalent. The nonspecific binding constant represents an average for a large number of sites with different sequences, whereas the intermediate complex is formed at the specific site. Nevertheless, we may conclude that the transition rate constant k_f is approximately 6×10^4 s^{-1} at 0.1 M NaCl and is almost independent of the salt concentration.

Competition Experiments. Our kinetic model may be tested by experiments in mixed buffers containing ions which compete with the repressor for DNA binding more efficiently than Na^+ ions. For this purpose, the binding of Tet repressor to operator DNA has been studied in mixed Na^+–Mg^{2+} buffers. The association rate constants have been determined by the procedure described above in 10 and 20 mM Mg^{2+} as a function of the Na^+ concentration. The competition of repressor and Mg^{2+} in the intermediate complex is indicated by the curved graphs shown in Figure 7. The affinity of Mg^{2+} to DNA decreases with increasing $[Na^+]$. Thus, in the limit of high $[Na^+]$, the association constants approach the values observed for $[Mg^{2+}] = 0$.

As shown in Figure 7, the experimental data can be described with high accuracy by eq 18, 19, and 21. Least-squares fitting provided the parameters for Mg^{2+} binding:

$$\log K_{Mg^{2+}}^{obsd} = (0.0 \pm 0.2) - (1.7 \pm 0.1) \log [Na^+] \quad (28)$$

and for the rate constant of repressor binding

$$\log k_{s,a}^{obsd} = (4.5 \pm 0.05) - (3.3 \pm 0.1) \log [Na^+] - (3.8 \pm 0.1) \log S \quad (29)$$

with S defined by eq 19. Both the limit value of the Mg^{2+} binding constant at $[Na^+] = 1$ M and the number of Na^+ ions released upon Mg^{2+} binding (2.0 ± 0.1; cf. eq 21) are in excellent agreement with the expected values and thus confirm the validity of our model.

Another series of competition experiments was conducted with nonspecific DNA. The repressor (0.2 μM) was first

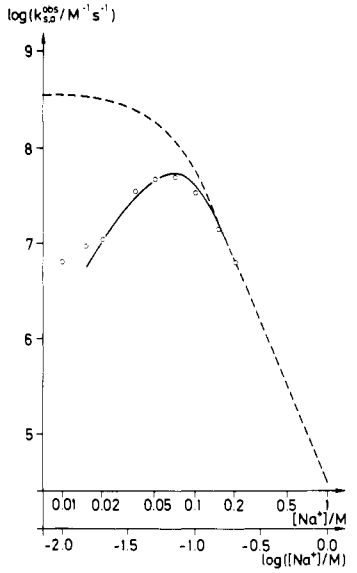

FIGURE 8: Rate constants $k^{obsd}_{s,a}$ measured in the presence of 250 μM nonspecific DNA as a function of the [Na$^+$] concentration ([R]^0 = [O]^0 = 0.1 μM). The dashed line shows the $k^{obsd}_{s,a}$ values found in the absence of added nonspecific DNA. The continuous line is calculated by using the least-squares fitting of log K^{obsd}_{RD} (eq 30).

equilibrated with a large excess of sonicated DNA (500 μM) and then mixed in the stopped-flow apparatus with a solution of *Tet* operator (0.2 μM). Under these conditions, nonspecific binding is virtually always in equilibrium, and the experimental data may be evaluated according to eq 24. Since the excess of nonspecific DNA is not directly connected with the operator by chemical bonds, a sliding reaction of the repressor along nonspecific DNA—if existent at all—would not be effective. Thus, the influence of the nonspecific DNA is restricted to the competitive effect.

The association rate constants obtained by these experiments show a maximum in their dependence on the Na$^+$ concentration (Figure 8). At low [Na$^+$], the observed increase of the rate with [Na$^+$] is mainly caused by a decrease of the competition with nonspecific DNA via a decrease of K^{obsd}_{RD}, whereas the opposite effect at high [Na$^+$] is mainly due to the preequilibrium at the operator site, in a manner corresponding to that found without addition of nonspecific DNA (cf. Figure 6).

These results may be used for an independent evaluation of K^{obsd}_{RD} by least-squares fitting according to eq 9 using the fact that the association rate constant is multiplied by the factor $1/(1 + K_{RD}[D]^0)$ in the presence of nonspecific DNA. The data obtained in the range [Na$^+$] $\geq$ 0.02 M, where eq 9 is valid without restriction, can be described with high accuracy by

$$\log K^{obsd}_{RD} = (0.7 \pm 0.4) - (2.6 \pm 0.3) \log [Na^+] \quad (30)$$

Thus, the parameters evaluated from the competition kinetics are very similar to those obtained from amplitude measurements (cf. eq 23). The data shown in Figure 8 suggest that K^{obsd}_{RD} does not follow the log–log salt dependence for [Na$^+$] < 0.02 M. Another indication for this deviation was found by fluorescence titrations at low salt concentrations, where K^{obsd}_{RD} did not exceed a value of approximately 3 $\times$ 10^5 M^{-1}. We cannot offer any simple explanation for this observation but suggest a change of the binding mode as a potential reason for the unusual salt dependence.

Our results demonstrate that the effect of added nonspecific DNA is completely as expected for a simple competition mechanism. Thus, the competition is not modulated by a contribution from a transfer mechanism, as proposed by Berg et al. (1981). According to this mechanism, the repressor could be passed from fragment to fragment without full dissociation, which should accelerate the reaction by a reduction of the activation barrier. However, an acceleration effect could not be detected.

Table I: Association of Tet Repressor to Plasmid pWH923 Linearized by *BalI*[a]

expt	[R]^0 (M)	[O]^0 (M)	[D]^0 (M)	$k^{obsd}_{s,a}$ (M^{-1} s^{-1})	K^{obsd}_{RD} (M^{-1})
1	1 $\times$ 10^{-7}	1 $\times$ 10^{-7}	6.63 $\times$ 10^{-5}	2.86 $\times$ 10^7	1.4 $\times$ 10^5
2	1 $\times$ 10^{-7}	3 $\times$ 10^{-7}	1.99 $\times$ 10^{-4}	1.05 $\times$ 10^7	1.4 $\times$ 10^5

[a] Conditions: 5 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.1 mM EDTA, and 0.1 mM DTE.

Finally, our results on the competition by nonspecific DNA and on the problem of sliding have been examined once more by stopped-flow measurements of the repressor association to the complete plasmid pWH923 DNA. The plasmid, which contains 3979 bp with six operator sites, was linearized by incubation with *BalI*. Since the six operators are rather close to each other and only two of them are connected with a long sequence of nonspecific DNA, one experiment was performed with a 3-fold excess of operator with respect to repressor; in this case, each repressor has a chance to arrive at an "external" operator, guided by a long stretch of nonspecific DNA. Since the experimental conditions were selected for fast equilibration of nonspecific binding, the experiments could again be evaluated according to eq 24. The results compiled in Table I demonstrate a decrease of $k^{obsd}_{s,a}$ due to the presence of the nonspecific plasmid DNA. An evaluation of K^{obsd}_{RD} using $k^{obsd}_{s,a}$ (maximum value) = 3 $\times$ 10^8 M^{-1} s^{-1} provides values which are in close agreement with that derived from the respective point of Figure 8 (at 10 mM Na$^+$, K^{obsd}_{RD} = 1.8 $\times$ 10^5 M^{-1}). Thus, association of repressor to the plasmid, if corrected for the competition effect, is not faster than to the short fragments described above.

Interpretation of the Data with Respect to a Possible Sliding Step. According to eq 7, sliding on small fragments at low salt should lead to an association rate which is proportional to the length of the fragment. In the case of the fragments used in this work, one should observe a 185/76 $\approx$ 2.4-fold difference in the limit association rate for the two fragments, which clearly is not observed. However, in the absence of sliding, a noticeable competition effect of unspecific DNA would be expected: with K_{RD} = 1.8 $\times$ 10^5 M^{-1} at 10 mM NaCl and [O]^0 = 1 $\times$ 10^{-7} M, the competition factor $1/(1 + K_{RD}[D]^0)$ is 0.59 for the 76 bp fragment and 0.38 for the 185 bp fragment, yielding a 1.6-fold faster association of the smaller fragment. Thus, the measured equivalence of the rate constants appears to indicate some contribution of a facilitating mechanism. However, the theory is not really designed to treat very short fragments appropriately, since end effects of the DNA chain as well as the special shape and charge distribution of the protein become important.

Another puzzling fact is the magnitude of the nonspecific association rate constant, which was measured independently with nonoperator containing DNA pieces at low salt (Kleinschmidt, 1986). We found $k^{obsd}_{a,ns} \approx 3 \times 10^6$ M^{-1} s^{-1} (number given on the basis of base pair units) for the association to a DNA chain of approximately 100 bp. Due to experimental difficulties, this value is not very accurate. If we convert the units to concentrations of whole fragments, we get an association rate of about 3 $\times$ 10^8 M^{-1} s^{-1}, which is equivalent to the specific association rate in the low-salt limit for both operator-containing fragments. Thus, the entire length

of the fragments (or at least a considerable part of it) appears to be a trap for repressor, which guides the protein immediately to the specific site. There are reasonable doubts that an association mechanism without sliding can achieve this: the theory gives only a number of ~10 bp screened by "hopping" during a macroscopic binding event. On the other hand, the detailed structure of, e.g., the 76 bp fragment shows (cf. Figure 1) that almost every nonspecific binding site is in the immediate neighborhood of an operator site (within a distance of 5–10 bp). Moreover, the largest possible sliding path amounts to only 22 bp for the 76 bp fragment, so the fragment data alone do not allow an estimate of an upper limit for the sliding efficiency.

A strong argument against efficient sliding is the data of the association to the whole plasmid, which show that even a frame of 2000 unspecific bp on each side of the operator does not accelerate the binding process. A possible difficulty for a simple interpretation could arise from the fact that the diffusional mobility of the small fragments is certainly higher than that of the whole plasmid with roughly 4000 bp. However, the association rate is determined by the sum of the diffusion coefficients of the protein and the DNA. The contribution of the protein to the translational as well as to the rotational diffusion coefficient considerably exceeds that of the DNA fragments used in our experiments (Mandelkern et al., 1981; D. Porschke et al., unpublished results). Since this contribution is constant in all experiments, we estimate a maximum sliding distance of not more than 40–60 bp at 10 mM NaCl.

In summary, the complete set of our experimental data suggests the interpretation that Tet repressor can slide over a short distance of approximately 50 bp under low salt conditions. This interpretation explains the equivalence of the association rates for specific and nonspecific association as well as the absence of a competition effect and finally is also consistent with the data obtained for the plasmid. However, it should be emphasized again that the present theories do not describe all the special features of our system adequately. Thus, a potential contribution of sliding cannot be identified for the Tet system under all conditions quantitatively. However, we may conclude definitely that sliding does not contribute under physiological conditions (~160 mM NaCl). In addition, at this salt concentration, the time course of the association reaction is determined by the salt-dependent intermediate, which decreases the association rate constant to about 10^7 M^{-1} s^{-1}.

Conclusions

The quenching of the tryptophan fluorescence upon binding of the Tet repressor to DNA provides a convenient spectroscopic signal to study the interactions of this protein with DNA. We have measured the fluorescence quenching mainly by the stopped-flow technique to characterize both thermodynamics and kinetics of specific and nonspecific interactions of the Tet repressor with double-helical DNA. From our experimental results, we have been able to derive a complete and consistent description of the reactions involved in the recognition of the *Tet* operator by the Tet repressor. Similar data have been collected previously for the recognition of the *lac* operator by the *lac* repressor (Winter et al., 1981; Barkley, 1981). A comparison given below reveals clear differences of the results obtained for the Tet and the *lac* system. Some of these differences may be attributed to the different experimental techniques used for investigation. The filter binding technique applied in the case of the *lac* system has often been qualified as indirect and subject to systematic errors. However, it seems to be more likely that the differences discussed below are due to a different mode of protein–nucleic acid interactions in the *lac* and the Tet system.

The binding parameters of both *lac* and Tet repressors are strongly dependent on the salt concentration, indicating a large number of ion contacts between the protein and the DNA. The number of DNA counterions released upon complex formation between *lac* repressor and *lac* operator is eight to nine (at pH 8.0; Record et al., 1977; Barkley et al., 1981); the corresponding number for the Tet system is between seven and eight. For a comparison of these numbers, it should be considered that the *lac* repressor is a tetramer with a total molecular weight of 150000, whereas the Tet repressor is a dimer with a total molecular weight of 47000 (Hillen et al., 1983). The DNA binding domain of the *lac* repressor is located at the N-terminus (denoted as "headpiece") and has about seven lysine and arginine residues per monomer. By analogy with other DNA binding proteins, it has been suggested that the headpiece domain is organized in the form of a "helix turn helix motif" (Matthews et al., 1982), which is believed to be a general element of protein structure designed for recognition of DNA. Analysis of the Tet repressor sequence also revealed a segment (Isackson & Bertrand, 1985) which is a good candidate for folding into a "helix turn helix motif". This segment has six arginine and lysine residues.

The stability constant K_{RO}^{obsd} extrapolated to 1 M NaCl reflects the nonionic interactions between the repressor and the operator. In the case of the *lac* repressor, this value is 7.9×10^6 M^{-1} (Record et al., 1977), and the corresponding number for the Tet repressor is 8×10^5 M^{-1}. Thus, the free energy changes resulting from nonionic contacts are of similar magnitude. Since both the ionic and the nonionic contributions to the free energies of binding are relatively close to each other for the *lac* and the Tet repressor–operator complex, the specific binding constants should be similar over the whole range of salt concentrations.

In contrast to the close analogy for the specific binding, a clear difference is observed with respect to nonspecific binding. The number of ions released upon nonspecific binding of the *lac* repressor is about 11 to 12 (Revzin & von Hippel, 1977; de Haseth et al., 1977) and thus is higher than the corresponding number for specific binding. The Tet repressor replaces only three to four ions upon nonspecific binding, which is about half as much as found for specific binding. For both repressors, nonspecific binding appears to be mainly driven by the entropy associated with ion release. However, the affinity resulting from this ion release (at $[Na^+] < 1$ M^{-1}) is much higher for the *lac* than for the Tet repressor; for example, the binding constant for the *lac* repressor arrives at a value $K_{RD}^{obsd} = 10^6$ M^{-1} already at 0.1 M NaCl, whereas the K_{RD}^{obsd} value for the Tet repressor does not exceed 3×10^5 M^{-1} even at millimolar salt concentrations.

Another difference between the *lac* and the Tet system is found with respect to sliding. According to the experimental results first obtained by Riggs et al. (1970b) and later extended by Barkley (1981) and Winter et al. (1981) together with the theoretical models (Richter & Eigen, 1974; Schranner & Richter, 1978; Berg et al., 1981), *lac* repressor slides along the DNA double helix for a considerable number of base pairs. This has been demonstrated for the *lac* repressor by the expected dependence of the association rate on the DNA chain length and by a dependence of the rate on the nonspecific binding constant ($k_{s,a}^{obsd} \sim K_{RD}^{1/2}$), which is consistent with the theory of sliding. Neither of these dependences has been found for the Tet repressor, and, thus, we have to conclude

Table II: Number of Ionic Contacts for *lac* and Tet Repressors in Different Complexes[a]

	lac	Tet
operator site	8–9	7–8
nonspecific sites	11–12	3–4
preequilibrium	5–6	3–4

[a] The data for the *lac* repressor are from Record et al. (1977), Revzin and von Hippel (1977), de Haseth et al. (1977), and Lohman et al. (1978).

that sliding does not contribute to any large extent to the recognition of the Tet operator by its repressor. The definite absence of sliding in the Tet system at physiological salt concentrations is quite in contrast to the rather high efficiency of sliding in the *lac* system, where one-dimensional diffusion constants of 4.5×10^{-10} cm^2 s^{-1} in NaCl or 1.5×10^{-9} cm^2 s^{-1} in the presence of Mg^{2+} lead to an increase of the operator target size to about 300 or 1000 bp, respectively.

Since the main requirement for any sliding effect is nonspecific binding, it is very likely that the difference in the sliding efficiency results from some difference in the nonspecific binding. We have discussed already the equilibrium constants for nonspecific binding and the numbers of nonspecific ion contacts, which are much higher for the *lac* than the Tet repressor. Probably the ion contacts formed by the *lac* repressor are adapted for optimal sliding: the positive charges of the repressor may be arranged in a special pattern resulting in a minimal potential difference upon movement of the protein from one nonspecific binding site to the next one. If the positions of the positive charges are optimized in this manner, the DNA appears to be a smooth cylinder for the protein. From this point of view, the absence of detectable sliding for the Tet repressor may be explained by an unfavorable arrangement of the positive charges at the DNA binding site. An optimal arrangement of these charges appears to be more difficult for the Tet repressor, simply because of their relatively low number; furthermore, other functions like specific binding and protein stability (which are at least as important) have to be optimized too.

According to these considerations, the Tet repressor would not be able to slide along DNA, because the number of ion contacts is too small. Effective sliding may require a relatively large molecule. Probably the Tet repressor is a representative of DNA binding proteins where evolutionary pressure led to a reduction of size rather than an increase of association rate. In the latter case, an increasing size may be (partly) compensated by a reduction of the copy number. An alternative explanation for the large difference in the sliding efficiency may be based on the fact that the *lac* repressor is a tetramer with four DNA binding domains, whereas the Tet repressor is a dimer with only two equivalent sites. The DNA binding domains of the *lac* repressor are known to be rather flexible. Thus, it is conceivable that this repressor slides via formation of contacts to adjacent nonspecific DNA sites by different binding domains one after another. Since the protein may slide by this mechanism with at least one binding domain (probably even two) keeping contact at any time to the double helix, the activation energy for individual steps of motion remains low.

The association of both repressors has been analyzed by a kinetic model with an intermediate complex. According to this model, the number of ion contacts formed in the intermediate complex is five to six for the *lac* repressor (Barkley, 1981; Lohman et al., 1978) and three to four for the Tet repressor. The number of ion contacts formed for the different types of complexes is compiled in Table II. It is remarkable that the number of ion contacts found for the *lac* repressor in the intermediate n and in the nonspecific complex m_{RD} are not equivalent. Thus, an essential feature of a *simple* model with an intermediate (transition rate from preequilibrium to final complex should not depend on salt) is not valid for the *lac* repressor because of sliding. In the case of the *lac* repressor, the relation $k_{s,a}^{obsd} \sim K_{RD}^{1/2}$ implies a value of n which is half as large as m_{RD}, and thus the n value for the *lac* repressor is without direct physical meaning. For the Tet repressor, n and m_{RD} are identical, and thus the intermediate complex is equivalent to the nonspecific complex.

Finally, our results should be discussed with respect to the conditions in vivo. The salt concentration in a bacterial cell is estimated to be 160 mM NaCl and 3 mM $MgCl_2$. When the contribution of Mg^{2+} is neglected, the binding constants for the Tet repressor are

$$K_{RO}^{obsd}(160 \text{ mM NaCl}) \approx 2 \times 10^{11} \text{ M}^{-1}$$

$$K_{RD}^{obsd}(160 \text{ mM NaCl}) \approx 3 \times 10^{2} \text{ M}^{-1}$$

The ratio of the specific to the nonspecific binding constant (7×10^8) is even slightly higher than the corresponding ratio obtained for the *lac* repressor (3×10^8). These specificity factors should be compared with the excess of nonspecific to specific sites in *Escherichia coli* cells of 4×10^6 to 1 or 2, respectively. Thus, repressors are preferentially attached to the operator by a factor of 100–1000. From the concentration of DNA in *E. coli*, $[D]^0 \sim 2.5$ mM, we may estimate that competition leads to a reduction of the association rate by a factor of not more than $1 + K_{RD}^{obsd}[D]^0 = 1 + (3 \times 10^2)(2.5 \times 10^{-3}) = 1.75$. Thus, the nonspecific DNA does not seriously compete with the operator under usual conditions. When the inducer tetracycline is present, however, nonspecific binding may be important. In the case of the *lac* repressor, for example, binding of the inducer does not affect nonspecific binding (von Hippel et al., 1975) and reduces operator binding by a factor of not more than 100–1000. Thus, inducer binding to the *lac* repressor leads to a shift of DNA binding from the operator site to nonspecific sites. By a similar mechanism, nonspecific binding may have an important biological function also for the Tet repressor.

ACKNOWLEDGMENTS

We thank Dr. Otto G. Berg for critical comments on the manuscript.

Registry No. Na, 7440-23-5; tetracycline, 60-54-8.

REFERENCES

Adam, G., & Delbrück, M. (1968) in *Structural Chemistry and Molecular Biology* (Rich, A., & Davidson, N., Eds.) pp 198–215, W. H. Freeman, San Francisco, CA.

Altschmied, L., & Hillen, W. (1984) *Nucleic Acids Res. 12*, 2171–2180.

Barkley, M. D. (1981) *Biochemistry 20*, 3833–3842.

Barkley, M. D., Lewis, P. A., & Sullivan, G. E. (1981) *Biochemistry 20*, 3842–3851.

Beck, C. F., Mutzel, R., Barbe, J., & Muller, W. (1982) *J. Bacteriol. 150*, 633–642.

Berg, O. G., & Blomberg, C. (1976) *Biophys. Chem. 4*, 367–381.

Berg, O. G., & Blomberg, C. (1977) *Biophys. Chem. 7*, 33–39.

Berg, O. G., & Blomberg, C. (1978) *Biophys. Chem. 8*, 271–280.

Berg, O. G., & Ehrenberg, M. (1982) *Biophys. Chem. 15*, 41–51.

Berg, O. G., & von Hippel, P. H. (1985) *Annu. Rev. Biophys. Biophys. Chem. 14*, 131–160.

Berg, O. G., Winter, R. B., & von Hippel, P. H. (1981) *Biochemistry 20*, 6929-6948.
Colpan, M., & Riesner, D. (1984) *J. Chromatogr. 296*, 339-353.
de Haseth, P. L., Lohman, T. M., & Record, M. T. (1977) *Biochemistry 16*, 4783-4790.
Hillen, W., Klock, G., Kaffenberger, I., Wray, L. V., & Reznikoff, W. S. (1982) *J. Biol. Chem. 257*, 6605-6613.
Hillen, W., Gatz, C., Altschmied, L., Schollmeier, K., & Meier, I. (1983) *J. Mol. Biol. 169*, 707-722.
Hillen, W., Schollmeier, K., & Gatz, C. (1984) *J. Mol. Biol. 172*, 185-201.
Isackson, P. J., & Bertrand, K. P. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6226-6230.
Kleinschmidt, C. (1986) Thesis, Technische Universität Braunschweig.
Lohman, T. M. (1986) *CRC Crit. Rev. Biochem. 19*, 191-245.
Lohman, T. M., de Haseth, P., & Record, M. T. (1978) *Biophys. Chem. 8*, 281-294.
Mandelkern, M., Elias, J. G., Eden, D., & Crothers, D. M. (1981) *J. Mol. Biol. 152*, 153-161.
Manning, G. S. (1978) *Q. Rev. Biophys. 11*, 179-246.
Matthews, B. W., Ohlendorf, D. H., Anderson, W. F., & Takeda, Y. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1428-1432.
Nelder, J. A., & Mead, R. (1964/65) *Comput. J. 7*, 308-313.
Oehmichen, R., Klock, G., Altschmied, L., & Hillen, W. (1984) *EMBO J. 3*, 539-543.
Porschke, D., & Rauh, H. (1983) *Biochemistry 22*, 4737-4745.
Record, M. T., de Haseth, P. L., & Lohman, T. M. (1977) *Biochemistry 16*, 4791-4796.
Record, M. T., Anderson, C. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 11*, 103-178.
Revzin, A., & von Hippel, P. H. (1977) *Biochemistry 16*, 4769-4776.
Richter, P. H., & Eigen, M. (1974) *Biophys. Chem. 2*, 255-263.
Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970a) *J. Mol. Biol. 48*, 67-83.
Riggs, A. D., Bourgeois, S., & Cohn, M. (1970b) *J. Mol. Biol. 53*, 401-417.
Schranner, R., & Richter, P. H. (1978) *Biophys. Chem. 8*, 135-150.
Unger, B., Klock, G., & Hillen, W. (1984) *Nucleic Acids Res. 12*, 7693-7703.
von Hippel, P. H., Revzin, A., Gross, C. A., & Wang, A. C. (1975) in *Protein-Liquid Interactions* (Sund, H., & Blauer, G., Eds.) pp 270-288, de Gruyter, Berlin, FRG.
Winter, R. B., Berg, O. G., & von Hippel, P. H. (1981) *Biochemistry 20*, 6961-6977.
Wray, L. V., Jr., & Reznikoff, W. S. (1983) *J. Bacteriol. 156*, 1188-1191.
Yong, H.-L., Zubay, G., & Levy, S. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1509-1512.

Influence of Calmodulin on the Human Red Cell Membrane Skeleton†

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Received April 23, 1987; Revised Manuscript Received September 3, 1987

ABSTRACT: The calcium receptor calmodulin interacts with components of the human red cell membrane skeleton as well as with the membrane. Under physiological salt conditions, calmodulin has a calcium-dependent affinity for spectrin, one of the major components of the membrane skeleton. It is apparent from our results that calmodulin inhibits the ability of erythrocyte spectrin (when preincubated with filamentous actin) to create nucleation centers and thereby to seed actin polymerization. The gelation of filamentous actin induced by spectrin tetramers is also inhibited by calmodulin. The inhibition is calcium dependent and decreases with increasing pH, similar to the binding of calmodulin to spectrin. Direct binding studies using aqueous two-phase partition indicate that calmodulin interferes with the binding of actin to spectrin. Even in the presence of protein 4.1, which is believed to stabilize the ternary complex, calmodulin has an inhibitory effect. Since calmodulin also inhibits the corresponding activities of brain spectrin (fodrin), it appears likely that calmodulin may modulate the organization of cytoskeletons containing actin and spectrin or spectrin analogues.

The mechanical properties of the human red blood cell are determined by a network of proteins attached to the cytoplasmic surface of the membrane. It is also believed that the network is involved in maintaining the lipid asymmetry and unique biconcave shape of the cell. This network or membrane skeleton is composed of spectrin, actin, and proteins 4.1 and 4.9, the latter being a minor component. The cytoskeleton is attached to the membrane by ankyrin which possesses binding sites for both spectrin and the transmembrane protein band 3 (anion channel) (Cohen, 1983; Gratzer, 1983; Marchesi, 1984; Bennett, 1985). In addition, protein 4.1 appears to have a polyphosphoinositide-dependent affinity for glycophorin (Anderson & Marchesi, 1985).

† This work was supported by grants from Magn. Bergvalls Stiftelse and the Swedish Natural Science Research Council (K-KU 1517-104 and K-KU 3602-110).
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0006-2960/88/0427-1104$01.50/0