Comparison of the DNA Association Kinetics of the Lac Repressor Tetramer, Its Dimeric Mutant LacI\textsubscript{adi}, and the Native Dimeric Gal Repressor*

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The rates of association of the tetrameric Lac repressor (LacI), dimeric LacI\textsubscript{adi} (a deletion mutant of LacI), and the native dimeric Gal repressor (GalR) to DNA restriction fragments containing a single specific site were investigated using a quench-flow DNase I “footprinting” technique. The dimeric proteins, LacI\textsubscript{adi} and GalR, and tetrameric LacI possess one and two DNA binding sites, respectively. The nanomolar protein concentrations used in these studies ensured that the state of oligomerization of each protein was predominantly either dimeric or tetrameric, respectively. The bimolecular association rate constants ($k_a$) determined for the LacI tetramer exceed those of the dimeric proteins. The values of $k_a$ obtained for LacI, LacI\textsubscript{adi}, and GalR display different dependences on [KCl]. For LacI\textsubscript{adi} and GalR, they diminish as [KCl] increases from 25 mM to 200 mM, approaching rates predicted for three-dimensional diffusion. In contrast, the $k_a$ values determined for the tetrameric LacI remain constant up to 300 mM [KCl], the highest salt concentration that could be investigated by quench-flow footprinting. The enhanced rate of association of the tetramer relative to the dimeric proteins can be modeled by enhanced “sliding” (Berg, O. G., Winter, R. B., and von Hippel, P. H. (1981) Biochemistry 20, 6929–6948) of the LacI tetramer relative to the LacI\textsubscript{adi} dimer or a combination of enhanced sliding and the superimposition of “direct transfer” mediated by the bidentate DNA interactions of the tetramer.

It is well established that the binding of a protein to a specific sequence of DNA can, under some experimental conditions, proceed at rates significantly faster than those predicted by three-dimensional diffusion. This phenomenon is referred to as “facilitated diffusion”. A well studied example is the association of the Escherichia coli Lac repressor (LacI)\textsuperscript{1} with its operators (1–4). Models have been proposed and tested whereby an initial nonspecific binding to a DNA molecule is followed by one or more mechanisms by which a protein translocates along the DNA molecule to the specific binding sequence (2, 5–9).

The term “sliding” describes the one-dimensional diffusion of nonspecifically bound proteins along DNA. Characteristics of this mechanism are its sensitivity to monovalent ion concentration and its dependence on the length of the DNA molecule. Its generality is indicated by results obtained for Cro repressor (10), the restriction enzyme EcoRI (11, 12), and E. coli RNA polymerase (13). “Direct transfer” is a mechanism of facilitated diffusion whereby a DNA-bound ligand or protein transiently binds to two DNA segments simultaneously (14, 15). The binding of the bidentate protein, such as LacI, to two sites on a single DNA molecule can result in the formation of a DNA loop, allowing the protein to sample distant DNA sequences simultaneously (16–18).

Investigations into the relative contributions of the sliding and direct transfer mechanisms using LacI and a dimeric mutant protein, LacI\textsubscript{adi}, have been conducted (19, 20). However, linked self-association reactions may have complicated these comparisons. To circumvent this issue, a quench-flow DNase I “footprinting” technique (21) has been utilized to conduct kinetic studies of LacI and LacI\textsubscript{adi} at concentrations sufficient to ensure that the proteins were predominantly tetrameric and dimeric, respectively. The E. coli Gal repressor (GalR) was used as an additional model of a protein whose native form is a monodentate dimer (22). Primary sequence alignments, molecular modeling, and x-ray diffraction studies strongly suggest that GalR and LacI share similar tertiary structures (22–26).

These studies demonstrate that the LacI tetramer binds operator more rapidly than the LacI\textsubscript{adi} dimer. Simulations conducted using the model of Berg et al. (2) suggest that direct transfer alone cannot account for the salt dependence of the LacI tetramer rate enhancement. Rather, an increase in the sliding rate for the LacI tetramer relative to the LacI\textsubscript{adi} and GalR dimers is required to account fully for the differences in association rates of the proteins.

EXPERIMENTAL PROCEDURES

Proteins—The GalR used in these studies was prepared following published protocols (22). The LacI\textsubscript{adi} preparation was a gift from Dr. Sankar Adhya. The LacI used in these studies was obtained by expression in E. coli of plasmid pMB1\textsuperscript{2} and purified as described elsewhere (27). The sequence-specific DNA binding activities of the proteins were determined from binding experiments conducted under conditions where the DNA concentration exceeds the equilibrium dissociation constant.

DNA—The 32P-labeled DNA restriction fragments used in these studies (28, 29) were present at concentrations of 10–20 ps in the reaction mixture. DNA restriction fragments of three lengths (185, 635, and 2,900 bp) containing the gal promoter region were excised from the parent plasmids (Fig. 1 and Refs. 29 and 30). For the GalR binding kinetics experiments, the restriction fragments excised from plasmid pDW001 contain the native gal operator at $O_E$ ($O_E^{G}$), $O_R$, and $O_R^{G}$.

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\footnote{These abbreviations used are: LacI, Lac repressor; GalR, Gal repressor; bp, base pair(s); bis, tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.}

\footnote{E. Jamison and M. Brenowitz, unpublished data.}
GTGTAAAC(GATTCCAC). The sequence of the second operator, \( O_2 \), whose center of symmetry is located 11 helical turns downstream from that of \( O_1 \), is not capable of binding GalR by virtue of its having been replaced with a sequence recognized with high affinity by LacI at \( O_1 \). The plasmid was cleaved with EcoRI and the restriction site filled with \(^{32}\)P-nucleotides using the Klenow fragment of DNA polymerase. The 185-bp fragment was excised from the plasmid using HindIII. The 2,900-bp fragment was generated by cleavage with XhoI instead of HindIII. The 635-bp fragment containing \( O^*_1 \) was obtained from plasmid pLJ002 by cleavage with EcoRI, end labeling with \(^{32}\)P, and excised with HindIII.

**RESULTS**

The kinetics of binding the LacI tetramer and the GalR dimers to DNA restriction fragments containing a single operator site were followed using the quench-flow footprinting technique. An example of an autoradiogram of a kinetic footprinting experiment is shown in Fig. 2A. Time-dependent changes in the protection of bases outside the specific binding sites of the repressors were not observed in any of the experiments (data not shown). The increase in site-specific protection with time is quantitated to produce progress curves for each reaction (Fig. 2B). All of the progress curves determined in these studies are adequately described by a single exponential function; no evidence of additional kinetic phases was present under any of the experimental conditions (Fig. 2B).

Extensive thermodynamic data exists for the site-specific binding of these proteins to DNA (3, 28, 36–42). LacI has been shown to form a bidentate “looped complex,” bridging \( O^*_1 \) and \( O^*_2 \), on DNA restriction fragments of length identical to those used in the present study which contain two operators competent to bind LacI (28, 30, 37). Thus, LacI can form stable bidentate interactions on even the shortest (185 bp) restriction fragment used in these studies. The values of \( k_d \) determined as a function of [KCl] for the 185-bp restriction fragment are shown in Fig. 3. The values of \( k_d \) obtained for LacI exceed those obtained for LacI\(^{\text{ad}} \) and GalR over the entire [KCl] range. The values of \( k_d \) determined are significantly faster than that predicted by three-dimensional diffusion except above 200 mM KCl for LacI\(^{\text{ad}} \) and GalR where \( k_d \) is reduced to the range of diffusion-limited reactions (\( 10^{-7} - 10^8 \text{ M}^{-1} \text{ s}^{-1} \)). In contrast, the values of \( k_d \) determined for LacI exhibit no dependence upon [KCl] from 25 to 300 mM KCl within experimental error. The differences in \( k_d \) between the dimeric and tetrameric proteins are also present in binding reactions conducted with 635- and 2,900-bp DNA restriction fragments at 25 and 100 mM KCl (Fig. 4).

**DISCUSSION**

The faster-than-diffusion association rates of LacI to operator-containing DNA have long been appreciated (1–9, 34). A two-step model of association has been proposed and tested,

\[
R + D \ldots O \quad \quad \quad R D \ldots O \quad \quad \quad R O \ldots D
\]

\[ k_1 \quad k_2 \quad k_3 \quad k_4 \]

**Model 1**

where \( R \) is the repressor, \( D \) is nonspecific DNA, and \( O \) is the operator (2, 9). In this model, the initial step, \( k_1 \), is the diffusion-limited nonspecific association of the repressor to the DNA molecule. The second step, \( k_2 \), is the facilitated translocation of
the repressor from the nonspecific binding site to the operator. One mechanism of this translocation is sliding in which the repressor is postulated to diffuse along the DNA to the operator. This mechanism has been inferred from observation of salt and DNA fragment length dependences of the rate constants (1, 3, 4, 10–12, 18, 34, 43). The physical nature of sliding is uncertain. It has been demonstrated that EcoRI translocates along the helical pitch of the DNA (44). In contrast, linear tracking of RNA polymerase along straight DNA “brushes” has been observed by fluorescence microscopy (13), suggesting that the polymerase does not follow the helical contour of the affixed DNA.

Another mechanism, direct transfer, is postulated to occur when a bidentate protein molecule, such as LacI, mediates the formation of a transient “DNA loop.” Utilization of this kinetic mechanism by LacI has not been demonstrated unequivocally, although stable protein-mediated DNA loops occur with LacI bound to DNA containing two operators (16–18, 28, 37, 45, 46). The ability of excess operator-containing DNA to enhance dissociation by direct competition suggests that the direct transfer reaction might play a role in the binding of LacI to operator (15, 19). Thus, a plausible hypothesis is that dissociation of the LacI tetramer to dimers would eliminate the direct transfer mechanism while not affecting sliding.

Studies of LacI were conducted to compare directly the kinetic properties of this dimeric form of LacI with the tetramer (19, 20). A possible complication in these studies is the uncertain oligomeric states of the proteins at the extremely low protein concentrations necessary for manual mixing protocols. The pioneering nitrocellulose filter binding studies of the kinetics of LacI DNA binding (1–4, 34) used LacI concentrations

FIG. 2. A, autoradiogram of a quench-flow kinetics footprinting experiment of the association of 6.3 nM LacI to a 185-bp restriction fragment at 50 mM KCl as described under “Experimental Procedures.” The time scale of the experiment is 0–120 s. B, kinetic progress curve determined for the pictured autoradiogram. The solid line depicts the best fit to a single exponential function (Equations 1 and 2).

FIG. 3. The second-order association rate constants ($k_a$) determined as a function of KCl concentration for the binding of LacI (●), LacI (○), and GalR (△) to the 185-bp DNA restriction fragment containing a single operator. Each data point represents the results obtained from two or more independent determinations. For the simulations of both LacI and LacI, $D = 5 \times 10^{-7}$ cm$^2$ s$^{-1}$, [DNA] = $2 \times 10^{-11}$ M, $a = 500$ Å (persistence length), $b = 15$ Å (DNA radius), $l = 3.4$ Å (base stack height), and $M$ (DNA length) = 185, 635, or 2,900 bp (2). The lines denote the results of simulations for the model of Berg et al. (2). The simulation for LacI (—–) was generated using $D = 3 \times 10^{-14}$ cm$^2$ s$^{-1}$ and $\nu = 0$. Simulations for LacI were generated using $D = 3.3 \times 10^{-14}$ cm$^2$ s$^{-1}$ and $\nu = 10^3$ (−) or 1,000 s$^{-1}$ (–––) and using $D = 9 \times 10^{-10}$ cm$^2$ s$^{-1}$ and $\nu = 0$ (——).
on the order of $10^{-12}$ M. The more recent comparisons of DNA binding by LacI and LacI\textsuperscript{adi} utilized protein concentrations as high as $10^{-3}$ M (19, 20), well below indirect estimates of $10^{-5}$–$10^{-12}$ M for the $K_d$ of the LacI dimer-tetramer equilibrium (20, 37, 47). However, a recent analysis of dimer-tetramer linkage with sequence-specific DNA binding suggests that LacI dimers are not present even at concentrations of $10^{-12}$ M (48).

In addition to eliminating dimer-to-tetramer association, the deletion of the COOH-terminal residues of LacI which create the LacI\textsuperscript{adi} mutant also weakens the monomer-dimer association reaction (38). A $K_d$ of $7.7 \times 10^{-8}$ M was determined for this equilibrium (39). Thus, the previous studies (19, 20) may have been influenced by the coupled monomer-dimer association reaction of this protein.

The use of a quench-flow apparatus allowed experiments to be conducted at higher LacI and LacI\textsuperscript{adi} concentrations where their oligomeric states were well defined. For LacI, concentrations in the range of $6.3\text{–}25 \times 10^{-9}$ M were shown to be optimal for the formation of stable LacI-mediated looped complexes using the DNA restriction fragments and under the experimental conditions used in these studies (28, 37). LacI\textsuperscript{adi} is dimeric in the concentration range of $25\text{–}100 \times 10^{-9}$ M employed in these studies. In addition, high protein concentrations were required to saturate the operator with LacI\textsuperscript{adi} since the linked monomer-dimer equilibrium results in diminished apparent binding affinity (38, 39).

The data presented in Figs. 3 and 4 confirm the differences in $k_a$ and reveal different salt dependences for the dimeric LacI\textsuperscript{adi} and tetrameric LacI. These data also confirm the faster-than-diffusion-limited rates obtained using other methodologies, although the $k_a$ values for LacI determined are slightly lower than those determined using a 203-bp fragment containing a natural lac operator (34). A comparison of rates between the past (34) and present studies must consider the different experimental conditions and methodologies employed to assay binding and the protocols used to produce and purify the LacI protein. Technical considerations prevented obtaining quench-flow footprinting data at either the picomolar protein concentrations used in the filter binding studies or at [KCl] > 300 mM. The latter limitation was the result of the excessive DNase I concentrations required for cleavage at [KCl] > 300 mM on the millisecond time scale (data not shown). Thus, the direct comparison of the dimeric and tetrameric proteins within the present data set is essential to the interpretation of the data.

The association rate constants obtained for tetrameric and dimeric proteins are both comparable to or greater than a diffusion-limited case, consistent with a facilitating mechanism for both the dimeric and tetrameric proteins. The values of $k_a$ obtained for the dimeric proteins demonstrate the [KCl] dependence anticipated for a binding reaction facilitated by sliding, decreasing to a diffusion-limited level ($10^{-7}$–$10^{-8}$ M\textsuperscript{-1} s\textsuperscript{-1}; 2, 7) at 200 mM KCl (Fig. 3). The independence of $k_a$ for the tetramer over this [KCl] range was unexpected. The model of Berg et al. (2) postulates the observed value of $k_a$ may be dependent upon sliding, direct transfer, or both mechanisms. The model is dependent upon the physical properties of the DNA double helix and the free diffusion constants for the proteins. The values for these parameters were taken from Ref. 2 for the simulations described below. The value for the non-specific binding constant, $K_{BD}$, was calculated from Ref. 49.

The expression for $K_{BD}$ is approximate at low salt concentrations and in the presence of Mg$^{2+}$. However, the inclusion of these effects had no effect on the simulations (data not shown).

The goal of these simulations was to determine whether the values of $k_a$ determined for LacI could be described by superimposing a direct transfer rate constant ($v$ s\textsuperscript{-1}) upon the linear diffusion rate constant ($D_1$, cm$^2$ s\textsuperscript{-1}) determined from LacI\textsuperscript{adi} data. Because of the high correlation between $v$ and $D_1$, it is not possible to determine both constants simultaneously. Thus, the assumption was made that LacI\textsuperscript{adi} binding is facilitated solely by sliding. In the absence of direct transfer ($v = 0$), the values of $k_a$ determined for LacI\textsuperscript{adi} as a function of [KCl] and DNA length are described by values of $D_1$, ranging from $3 \times 10^{-14}$ to $2 \times 10^{-13}$ cm$^2$ s\textsuperscript{-1} (Figs. 3 and 4). This range of values is less than that minimally required to describe the binding of the tetramer LacI as described below.

The dotted lines in Fig. 3 represent simulations in which increasing values of the direct transfer constant ($v = 10$ and 1,000 transfers s\textsuperscript{-1}, the latter value being theoretical upper limit for $v$, 2), are superimposed upon the value of $D_1$, $3 \times 10^{-14}$ cm$^2$ s\textsuperscript{-1} which describes the [KCl] dependence of binding of the dimeric LacI\textsuperscript{adi}. This combination of sliding and direct transfer accounts for the rate enhancement of LacI relative to LacI\textsuperscript{adi} at low salt (<75 mM KCl) but not at high salt. The simulated curve approaches an asymptotic limit that is divergent from the LacI data in the higher salt concentrations. Thus, an increase in $v$ alone does not account for the relative difference in association rates observed between LacI and LacI\textsuperscript{adi} (Fig. 3).

Simulations of the LacI data with $D_1$ ranging from $3 \times 10^{-10}$ to $7 \times 10^{-11}$ cm$^2$ s\textsuperscript{-1} and $v = 0$ (Figs. 3 and 4, solid lines) depict the increase in sliding in the absence of direct transfer which can account for the LacI data. Although the LacI data can also
be modeled adequately by combinations of $\nu$ and $D_2$, an increase in $D_2$ appears to be a necessary component of LacI rate enhancement relative to LacI$^{adi}$. Because of the high theoretical correlation between $\nu$ and $D_2$, it is not possible to determine unique values for the two parameters. Thus, although the association of the LacI tetramer is enhanced relative to the LacI$^{adi}$ and GalR dimers, the portion (if any) of this enhancement caused by direct transfer cannot be determined from these data.

The simplest conclusion is that sliding by LacI is enhanced relative to the LacI$^{adi}$ (Fig. 4), although both the direct transfer and sliding mechanisms may contribute to the rate enhancement observed for the tetramer. The LacI tetramer does not appear to be simply a dimer of LacI$^{adi}$ dimers. However, these differences do not appear to be manifested at the protein-DNA interface. Thermodynamic studies of the salt dependence of sequence-specific binding have been conducted for a dimer clearly differ. However, this difference cannot be attributed to nonspecific DNA binding, the portion (if any) of this enhancement caused by direct transfer would be expected to be minimal.

In addition, thermodynamic studies have demonstrated that the protein-DNA contacts made upon the wrapping of the DNA around LacI could facilitate sliding by the tetramer. In conclusion, the association kinetics of the LacI tetramer and the LacI$^{adi}$ dimer clearly differ. However, this difference cannot be unambiguously ascribed to the direct transfer mechanism and suggests that the interactions between LacI and DNA contributing to operator location may be more complex than originally thought.

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