The N-Terminal B-Domain of T4 Gene 32 Protein Modulates the Lifetime of Cooperatively Bound Gp32−ss Nucleic Acid Complexes†

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Received June 20, 1996; Revised Manuscript Received August 27, 1996⊗

ABSTRACT: The N-terminal basic or B-domain (residues 1−21) of bacteriophage T4 gene 32 protein (gp32) provides a major determinant for highly cooperative binding by gp32 to single-stranded (ss) nucleic acids at equilibrium. In order to gain mechanistic insight into N-terminal domain function, the kinetics of dissociation of wild-type and previously characterized B-domain substitution mutant gp32s (R4K, R4Q, and K3A) from the model ribohomopolymer, poly(A), have been investigated under solution conditions identical to those used for equilibrium studies [Villemain, J. L., & Giedroc, D. P. (1993) *Biochemistry 32*, 11235−11246; Villemain, J. L., & Giedroc, D. P. (1996) *J. Biol. Chem. 271*, 27623−27629]. The dissociation of cooperatively bound gp32−poly(A) complexes was induced by sodium chloride concentration jumps and monitored by an increase in tryptophan fluorescence upon dissociation of the protein from poly(A) using stopped-flow techniques. The apparent dissociation rate constant, k_d(app), for all mutant proteins studied was found to depend strongly on the initial fractional saturation of poly(A) just as was found previously for wild-type gp32. This permitted application of Lohman's model for the irreversible dissociation of cooperatively bound gp32−nucleic acid complexes [Lohman, T. M. (1983) *Biopolymers 22*, 1697−1713] from which the molecular rate constant, k_e, the rate of dissociation of a protein monomer from the end of a gp32−ss nucleic acid complex or protein cluster, could be determined. From the [NaCl]-dependence of k_d(app), k_e determined at 0.45 M NaCl, pH 8.1, 20 °C, was found to be 62 ± 23, 78 ± 8, 328 ± 36, and 384 ± 34 s^{-1} for wild-type, R4K, K3A, and R4Q gp32s, respectively. With the exception of R4K gp32, we find a striking correlation between the relative magnitudes of k_e and K_{app}, suggesting that the molecular defect in the equilibrium binding properties of the N-terminal domain mutants resides in the increased rate at which gp32 monomers dissociate from singly contiguous binding sites at the ends of clusters. The bimolecular association rate constant measured for wild-type gp32 and a weakly binding B-domain mutant, R4T gp32, to poly(dT) was found to be nearly identical, further evidence that the primary defect is in the dissociation reaction. We conclude that the N-terminal domain strongly modulates the lifetime of cooperatively bound gp32−polynucleotide complexes. The mechanistic and functional implications of these findings are discussed.

Proteins classified as single-stranded DNA binding or helix-destabilizing proteins (SSBs)[1] are essential for biological processes such as replication, recombination, and repair (Chase & Williams, 1986; Ruvolo et al., 1991; Lohman & Ferrari, 1994). SSBs function as accessory proteins by protecting transiently formed single-stranded regions of DNA from nuclease attack and by removing secondary structure potentially inhibitory to the DNA polymerase or recombinase. Bacteriophage T4 gene 32 protein was the first-discovered (Alberts & Frey, 1970) and prototype of this class of proteins [for a review, see Karpel (1990)]. Other replicative SSBs include *Escherichia coli* single-strand binding protein (SSB) (Sigal et al., 1972), prokaryotic, conjugative plasmid-encoded, or mitochondrial SSBs evolutionarily related to *E. coli* SSB (Van Dyck et al., 1992; Jarosik & Hansen, 1994), bacteriophage-encoded SSBs (Kim et al., 1992; Lindberg et al., 1989), animal virus SSBs encoded by adenovirus (Kuil et al., 1989) and herpes virus (Bortner et al., 1993), and eukaryotic heterotrimeric SSBs, of which human replication protein A is representative (Kim et al., 1994; Heyer et al., 1990). Although not a replicative SSB, gene V protein encoded by bacteriophage fd (M13) is also considered a member of this class of proteins, since it binds preferentially and without sequence-specificity to single-stranded (ss)[1] nucleic acids (Pörschke & Rauh, 1983).

Replicative SSBs are required in stoichiometric amounts and bind nonspecifically and preferentially to ss nucleic acids with moderate to high cooperativity (Ruvolo et al., 1991). It is unknown however, to what extent highly cooperative binding is functionally important in any SSB. Gp32 binds highly cooperatively to ss nucleic acids with a head-to-tail "unlimited" type of cooperativity (Lohman et al., 1988) which gives rise to the formation of long clusters of protein monomers at equilibrium readily visualized by electron microscopy (Delius et al., 1972; Keating et al., 1987). The

† This work was supported by NIH Grant GM42569 (to D.P.G.). This work is in partial fulfillment of the requirements of the Ph.D. degree at Texas A&M University (to J.L.V.).

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⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1996.

[1] Abbreviations: gp32, T4 gene 32 protein; gp32-(A+B), gene 32 protein residues 22−253 (lacking the A and B domains); ss, single-stranded; SSB, single-strand binding protein.

S0006-2960(96)01482-1 CCC: $12.00

primary structure of gp32 (301 amino acids) (Williams et al., 1980) consists of three domains as deduced by limited proteolysis experiments (Spicer et al., 1979). From studies of the binding of wild-type gp32 and gp32 truncation mutants (Kowalczykowski et al., 1981; Newport et al., 1981; Lonberg et al., 1981; Spicer et al., 1979; Giedroc et al., 1990), the N-terminal "B" or basic domain (residues 1−21) has been proposed to contain major determinants for cooperativity of binding, while the C-terminal "A" or acidic domain (residues 254−301) participates in heterologous protein−protein interactions essential for function of the multiprotein replication (Nossal, 1992; Hurley et al., 1993) and recombination complexes (Jiang et al., 1993; Salinas & Kodadek, 1995). The remainder of the protein is the core domain or fragment (residues 22−253) and contains an intrinsic Zn^{2+} ion and major determinants for ssDNA binding. The crystal structure of the core fragment complexed to oligo$(dT)_6$ reveals that the ssDNA fits into a large cleft of the C-shaped molecule (Shamoo et al., 1995). The cleft is lined with basic as well as aromatic amino acids (Casas-Finet et al., 1992; Prigodich et al., 1986). The Zn(II) domain organizes one of the two globular domains of the molecule in forming part of the ssDNA binding groove, as anticipated from spectroscopic and biochemical experiments (Giedroc et al., 1991; Qiu et al., 1994).

The binding of gp32 to model polynucleotide lattices is well-modeled by the large-ligand overlap cooperative binding model of McGhee and von Hippel (1974). The binding site size is 7.5 (±0.5) nucleotides per cooperatively bound gp32 monomer. Most estimates of the cooperativity parameter, ω, for the binding of gp32 to homopolynucleotides cluster around ∼1000 (Kowalczykowski et al., 1981; Villemain & Giedroc, 1993). K_{app} is defined as the product of the intrinsic association constant, K_{int}, and ω. As a test of the simple model that the N-terminal B-domain, and in particular, the Lys^3-Arg^4-Lys^5 sequence, controls the magnitude of the cooperativity parameter (Casas-Finet et al., 1992; Casas-Finet & Karpel, 1993), we previously characterized a set of single amino acid substitution mutants of Arg^4 (Villemain & Giedroc, 1993) and a single mutant of Lys^3, K3A gp32 (Villemain & Giedroc, 1996). The hierarchy of binding affinities for poly(A) (pH 8.1, 20 °C) was found to be R4K > K3A ≥ R4Q ≫ R4T ≫ R4G gp32. Surprisingly, resolution of K_{int} and ω for each gp32 mutant revealed that the decrease in the magnitude of K_{app} did not directly correlate with the extent of diminution of the magnitude of ω (Villemain & Giedroc, 1993). For example, ω resolved for K3A and R4Q gp32s was indistinguishable from wild-type gp32 despite each having incurred a ∼5−8-fold decrease in K_{app}, depending on conditions. R4K and R4T gp32s were found to exhibit approximately 2- and 5-fold reductions in ω, respectively, with 2- and ∼50-fold reductions in K_{app}. R4G gp32 was found to bind ∼1000-fold less tightly to poly(A) and only small residual cooperativity ($\omega \approx 5-35$) (Villemain & Giedroc, 1993, 1996). These findings support the idea of a functional *interdependence* of the three domains of gp32, with the N-terminal domain providing one of multiple determinants of binding cooperativity. Although the structure of the core fragment complexed with a ssDNA oligonucleotide (Shamoo et al., 1995) does not include the B-domain, the proximity of the N-teminal residue (Gly^{22}) of the core fragment to the ssDNA cleft suggests that the N-terminal domain and ssDNA binding site may cooperate in enabling gp32 to form cooperatively bound clusters of monomers at equilibrium.

Processes such as replication, recombination, and repair in which gp32 functions do not occur at equilibrium and may be expected to be sensitive to the kinetics of gp32 binding to ss nucleic acids. DNA synthesis by T4 DNA polymerase, for example, can occur in vitro at rates >400 nucleotides s^{-1} depending on the presence of accessory factors and solution conditions (Nossal, 1992; Capson et al., 1992; Cha & Alberts, 1989). It is possible therefore that by simply altering the rates of association and/or dissociation of gp32 to and from ss nucleic acids by mutation may render it weakly functional or nonfunctional, despite being characterized by only a relatively small defect in equilibrium binding affinity. We reasoned that modestly defective N-terminal domain mutants of gp32 might serve as useful reagents with which to probe the role of the B-domain by investigating the dissociation kinetics of cooperatively bound B-domain mutant gp32−ss nucleic acid complexes. These studies are facilitated by our previous determination of the cooperativity of binding (ω) by B-domain mutants at equilibrium (Villemain & Giedroc, 1993, 1996). In addition, a quantitative dissociation kinetic model (Lohman, 1983) is available which describes the irreversible dissociation of cooperatively bound wild-type gp32 from ss homopolynucleotides very well (Lohman, 1984a).

In this paper, the kinetics of irreversible dissociation of wild-type gp32 and three strong-binding B-domain mutant proteins, R4K, R4Q, and K3A gp32s (Villemain & Giedroc, 1993, 1996), from the model polynucleotide, poly(A), have been investigated using stopped-flow fluorescence techniques under solution conditions previously used to investigate their equilibrium binding properties. The strong dependence of the apparent rate of dissociation from poly(A), k_d(app), on the initial binding density found for each B-domain mutant protein suggests that the primary pathway of dissociation is via dissociation of singly contiguously bound proteins from the ends of protein clusters with no redistribution of the remaining protein clusters, exactly as previously found for wild-type gp32 (Lohman, 1984a). The relative magnitudes of k_d(app) for each mutant protein, however, are found to be dramatically faster over the entire binding density range. Interpretation of these data according to Lohman's model (Lohman, 1983) suggests that the entire observed deficiency in nucleic acid binding affinity exhibited by each B-domain mutant at equilibrium is largely manifest by an increase in k_e, the molecular rate at which singly contiguously bound gp32 monomers dissociate from the ends of protein clusters. These findings provide direct evidence that the N-terminal B-domain modulates the lifetime of cooperatively bound gp32−ss nucleic acid clusters in a manner which may have implications for functional efficacy of B-domain mutant proteins.[2]

MATERIALS AND METHODS

Materials

T buffer is 0.01 M Tris-HCl/0.1 mM Na_3EDTA, pH 8.1, at 20 °C (Villemain & Giedroc, 1993). Poly(A) and poly(dT) were obtained and fractionated as described (Villemain

[2] J. L. Villemain, S. Morrical, and D. P. Giedroc, manuscript in preparation.

& Giedroc, 1993). The poly(A) used in these experiments was polydisperse, with the smallest fragments running along side of ~500 bp duplex DNA fragment. Wild-type and B-domain mutant gp32s were expressed and purified essentially as described (Giedroc et al., 1990) with the modifications outlined in Villemain and Giedroc (1993). R4K, R4Q, R4T, R4G, and K3A gp32s refer respectively to $Arg^4 \rightarrow Lys$, $Arg^4 \rightarrow Gln$, $Arg^4 \rightarrow Thr$, $Arg^4 \rightarrow Gly$, and $Lys^3 \rightarrow Ala$ single amino acid substitution mutants of wild-type gp32 (Villemain & Giedroc, 1993, 1996); gp32-B is an N-terminal truncation mutant which lacks residues 1−21 (Giedroc et al., 1990). In general, the proteins used in this study were freshly thawed from frozen stocks and diluted with T buffer containing the desired [NaCl] before each experiment. The concentrations of all gp32s and poly(A) were determined from $\epsilon_{280} = 4.13 \times 10^4$ M^{-1} cm^{-1} (Giedroc et al., 1990) and $\epsilon_{260} = 10\,300$ M (nucleotide)$^{-1}$ cm^{-1} (Kowalczykowski et al., 1981), respectively.

Methods

Stopped-Flow Methods. The intrinsic tryptophan fluorescence of gp32 is quenched upon complex formation with nucleic acids (Kelly et al., 1976). Therefore, association and dissociation of gp32 from ss nucleic acids can be monitored by observing either a decrease (association) or an increase (dissociation) in fluorescence signal as a function of changing solution conditions. Rapid mixing experiments (dead time = 1.8 ms) were performed on an SLM-8000C spectrofluorimeter equipped with a stopped-flow attachment. The excitation wavelength was 292 nm which minimizes the inner filter correction as well as the extent of photobleaching of the protein. Total fluorescence emission was detected by placing a cutoff filter directly in front of the photomultiplier tube to eliminate emission at wavelengths <345 nm. This setup allowed adequate signal detection for the protein concentrations typically used (0.2−0.3 μM) since the λ_{max} of gp32 is 347 nm (Kelly & von Hippel, 1976), without complications arising from protein monomer−monomer self-association (Carroll et al., 1972). All experiments were conducted at 20 °C for the purpose of comparing kinetic data obtained from these studies and equilibrium results obtained previously (Villemain & Giedroc, 1993, 1996). In each individual experiment, the time courses of association or dissociation from 10−14 original runs were averaged to reduce the random experimental noise.

Association Kinetics. Equal volumes of either wild-type or R4T gp32 were mixed with poly(A) to give the indicated final concentration of protein and poly(A) in T buffer/0.10 M NaCl, 20 °C. Averaged time courses were fit to either a single exponential decay, $F(t) - F(\infty) = \Delta F = Ae^{-t/\tau}$, or a sum of two exponential decays, $\Delta F = Ae^{(-t/\tau_f)} + Be^{(-t/\tau_s)}$ where $F(\infty)$ is the fluorescence signal at final equilibrium. Best-fit parameter values for A, τ_f, B, τ_s, and $F(\infty)$ were obtained using nonlinear least-squares analysis.

Dissociation Kinetics. One sample containing gp32 and sufficient poly(A) to give the desired fractional saturation in T/0.05 M NaCl and a second sample containing [NaCl] in T buffer were prepared at 2× their final concentrations. Over the entire fractional saturation range, [gp32] was fixed at either 0.2 or 0.3 μM. Equal volumes of two samples were subjected to rapid mixing as described above, and the time course of fluorescence was recorded. [NaCl] jumps were made from an initial salt concentration, $[NaCl]_{init} = 0.05$ M, to final salt concentrations of $[NaCl]_{final} \geq 0.31$ M for wild-type gp32 (Lohman, 1984a) and ≥0.275 M for R4K, R4Q, and K3A gp32s. Since the magnitude of the signal change between initial and final values did not change at [NaCl] higher than those indicated, complete irreversible dissociation of the gp32−polynucleotide complex occurred in each experiment. The concentration of NaCl for which this is true for wild-type gp32 was determined previously (Lohman, 1984b). Since the binding is stoichiometric, the fractional saturation of the lattice (f_{sat}) was calculated from final input concentrations of protein and poly(A) (Villemain & Giedroc, 1993, 1996); this is true for R4K, K3A, and R4Q gp32s used in this study. The averaged data were fit to a single exponential decay, $F(t) = Ae^{(-kt)} + F(\infty)$, to obtain the apparent dissociation rate constant, k_d(app), where k is k_d(app) and $F(\infty)$ is the fluorescence signal at equilibrium. The nonlinear least-squares fitting routine, NONLIN, running on a Macintosh IIci platform was used for analysis of the data to obtain the amplitude A, k_d(app), and $F(\infty)$ with associated 67% confidence intervals.

RESULTS

Measurement of the Bimolecular Association Rate Constant, k_a(app), for the Binding of R4T and Wild-Type gp32 to Poly(dT) in 0.10 M NaCl

The mechanism proposed by Lohman and Kowalczykowski (1981) for the formation of cooperatively bound gp32 protein clusters consists of multiple steps which can be fit by the sum of two exponential decays where the time constants τ_f^{-1} and τ_s^{-1} designate fast and slow exponential decays, respectively. The magnitude of the fast relaxation time constant, τ_f, is strongly dependent on base composition of the nucleic acid lattice and solution conditions including salt concentration (Lohman & Kowalczykowski, 1981). With poly(dT) at 0.10 M NaCl or so-called strong-binding conditions (Lohman & Kowalczykowski, 1981), τ_f^{-1} is approximately equal to the product of the bimolecular association rate constant and [nucleic acid], with the assumption that dissociation of gp32 from the nucleic acid is negligible. If the nucleic acid concentration is kept in vast excess over total gp32 as required for pseudo-first-order conditions, then τ_f^{-1} is directly proportional to [nucleic acid]. Thus, a linear plot of τ_f^{-1} versus [nucleic acid] is expected to be obtained for wild-type gp32 under these conditions with a slope equal to k_a(app).

Figure 1 shows that a linear dependence of τ_f^{-1} on [poly(dT)] in 0.10 M NaCl is observed for both wild-type and R4T gp32s at a constant [gp32] (0.3 μM). From the slopes of these plots, k_a(app) = 6.8 (±0.4) × 10^6 and 6.2 (±0.2) × 10^6 M^{-1} s^{-1} for wild-type and R4T gp32s, respectively. These data suggest that the bimolecular association rate constants for these two proteins are not significantly different from one another. Since the R4T substitution results in a substantial ~50-fold reduction in K_{app} over a wide range of [NaCl], relative to smaller reductions in R4K, K3A, and R4Q gp32s (Villemain & Giedroc, 1993, 1996), we anticipate that this conclusion can be extended to these stronger binding B-domain mutant gp32s as well.

Additional association kinetic experiments were carried out with wild-type, R4K and R4Q gp32s under the same

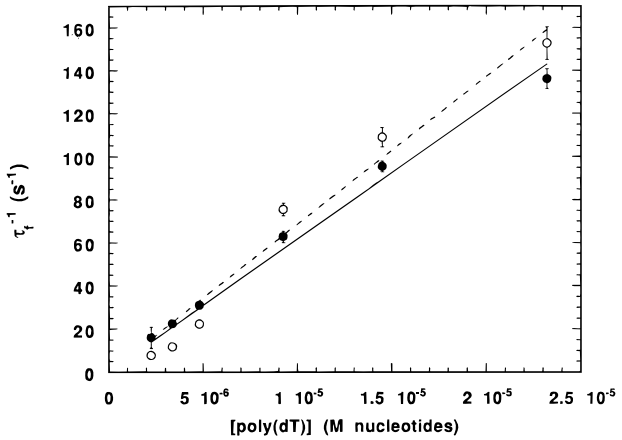

FIGURE 1: Dependence of τ_f^{-1} for wild-type and R4T gp32s on [poly(dT)] in T/0.1 M NaCl, pH 8.1, 20 °C. Wild-type and R4T gp32 concentrations were constant at 0.3 μM. The slope from a linear least-squares fit forced through the origin gives the bimolecular association rate constant, k_a(app), with k_d(app) = 0. k_a(app) = 6.8 ± 0.4 × 10^6 M^{-1} s^{-1} for wild-type gp32; k_a(app) = 6.2 ± 0.2 × 10^6 M^{-1} s^{-1} for R4T gp32.

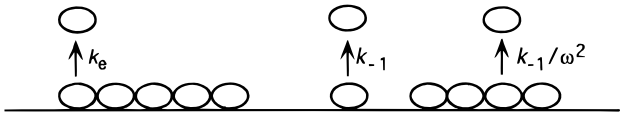

FIGURE 2: Cartoon model of the three pathways of polynucleotide lattice dissociation of a highly cooperatively binding protein. k_{-1}, k_e, and k_{-1}/ω^2 define the rates of dissociation for a protein molecule bound to an isolated, a singly contiguous, and a doubly contiguous binding site. Adapted from Lohman (1983).

solution conditions but with poly(A) as the polynucleotide lattice (data not shown). As expected, τ_f^{-1} was found *not* to be directly proportional to [poly(A)] but instead gave a nonlinear dependence on [poly(A)], consistent with so-called weak-binding conditions, in which a pre-equilibrium formation of non-cooperatively bound gp32−poly(A) complex is established (Lohman & Kowalczykowski, 1981). Unfortunately, the extent of fluorescence signal change associated with the binding of weaker binding mutants, R4Q and K3A gp32s at [NaCl] > 0.10 M NaCl, was found to be very small, as expected (Villemain & Giedroc, 1993); these experiments were therefore not carried further. Although a comprehensive analysis of the association kinetics of B-domain substitution mutants has not been carried out here, these experiments suggest that the primary deficiency in cooperative binding to ss nucleic acids (Villemain & Giedroc, 1993, 1996) does not originate in the rate of association of protein with nucleic acid, but instead may lie in the dissociation portion of the reaction.

Dependence of the Dissociation Rate Constant on Initial Fractional Saturation (Binding Density) of the Nucleic Acid for Wild-Type gp32 and Select Arg[4] and Lys[3] gp32 Mutants gp32 Mutants

Theoretical Considerations. For the highly cooperative binding of a protein to a homogeneous polynucleotide lattice, three distinct pathways of dissociation directly into solution can be considered (Figure 2). These are dissociation of protein molecules from either an isolated site, defined by k_{-1}, from a singly contiguous site at the end of a protein cluster, defined by k_e, or from a doubly contiguous site from the interior of a cluster of monomers, defined by k_{-1}/ω^2. The dissociation of isolated monomers, while expected to be fast, will make a negligible contribution to the overall rate since the concentration of protein bound to such sites will be very small in the event that the binding is highly cooperative. This will be the case even at low levels of lattice saturation (Lohman, 1983). On the other hand, dissociation of monomers from the interior of clusters is expected to be slow, given the ω^2 penalty, particularly when ω is large. As a result, the dominant mechanism of dissociation is expected to be dissociation of proteins from the ends of protein clusters, k_e, with no subsequent redistribution of the remaining protein clusters (Lohman, 1983). This mechanism of dissociation describes the high-salt-induced dissociation of wild-type gp32 very well (Lohman, 1984a,b). If this is indeed the case for the dissociation of R4K, R4Q, and K3A gp32s, a single exponential decay should describe the dissociation of these gp32s from poly(A), since the concentration of cluster ends decreases exponentially throughout the dissociation (Lohman, 1983, 1984a).[3] The rate equations which describe the change in concentration of protein clusters are

$$-\mathrm{d}[\mathrm{M}_N]/\mathrm{d}t = 2k_e[\mathrm{M}_N] \quad (1)$$

$$-\mathrm{d}[\mathrm{M}_q]/\mathrm{d}t = 2k_e[\mathrm{M}_q] - 2k_e[\mathrm{M}_{q+1}]\ (2 \le q \le N) \quad (2)$$

where M_N is the concentration of clusters containing the maximum number of protein molecules limited by the length of the nucleic acid lattice of N nucleotides, M_q is the concentration of protein clusters of q monomers, where q ranges from 2 to N, and k_e is the rate constant for dissociation of a singly contiguously bound protein molecule (Lohman, 1983). The solutions for these equations are

$$[\mathrm{M}_N] = [\mathrm{M}_N]_0 \exp(-2k_e t) \quad (3)$$

$$[\mathrm{M}_q] = [\mathrm{M}_q]_0 \exp[-2k_e t(1 - p_0)] \quad (4)$$

respectively, where the subscript 0 denotes the initial concentration. The term $1 - p_0$ is the fraction of protein molecules bound at the ends of clusters before dissociation of the complex. As shown in eq 5, $1 - p_0$ is determined by n, the binding site size in nucleotides, ω, the cooperativity parameter, and ν, the binding of density of protein on the lattice. ν is related to f_{sat}, the fractional saturation of the lattice, by $f_{sat} = \nu n$:

$$1 - p_0 = [(n-1)\nu - 1 + R]/[2\nu(\omega - 1)] \quad (5)$$

where $R = [(1 - (n+1)\nu)^2 + 4\omega\nu(1 - n\nu)]^{1/2}$.

In the actual dissociation experiment, the disappearance of bound protein, [PD], is monitored. The rate equation for this reaction reduces to

$$-\mathrm{d}[\mathrm{PD}]/\mathrm{d}t = 2k_e(1 - p_0)[\mathrm{PD}] \quad (6)$$

when the dissociation of isolated and doubly contiguously

[3] At $f_{sat} < 1$, the concentration of cluster ends decreases exponentially throughout the dissociation, giving rise to a first-order recovery of the tryptophan fluorescence of gene 32 protein upon dissociation. This derives from the fact that small clusters ($M_q < 3$) make up a significant portion of the cluster distribution during the dissociation over the entire time course (unpublished simulations). As the dimeric M_2 species dissociates, two cluster ends are lost to solution, giving rise to an exponential decrease of cluster ends (Lohman, 1983).

bound protein is negligible (Lohman, 1983). The apparent rate constant, k_d(app), obtained from a monitoring single exponential decay when the nucleic acid lattice is less than fully saturated ($f_{sat} < 1$) is therefore

$$k_d(\text{app}) = 2k_e(1 - p_0) \quad (7)$$

For an initially fully saturated nucleic acid ($f_{sat} = 1$), the rate equation for the disappearance of [PD] is

$$-\text{d[PD]}/\text{d}t = 2k_e[\text{M}_N]_0 \quad (8)$$

where $[\text{M}_N]_0$ is the concentration of protein clusters at time zero. As can be seen, the rate of dissociation in this case is expected to be constant (zero-order kinetics) as long as the number of cluster ends remains constant. This is expected to be the case at early time points in the dissociation, when the clusters are large. Zero-order kinetics may not however be observed if the length of the nucleic acid molecules is polydisperse, containing even a small substantial population of small oligonucleotides on which only very small protein clusters may form (Lohman, 1983, 1984a).

Equation 7 above shows that the apparent dissociation rate constant depends on the initial binding density ν, the cooperativity of binding ω, and n, the site size, which together determine $(1 - p_0)$, the initial fraction of cluster ends from which a protein molecule can dissociate. Once the binding density dependence of k_d(app) has been determined, eq 7 can then be used to determine the molecular rate constant, k_e. However, this is strongly dependent on how well ω is defined, since the calculated cluster distribution, and thus cluster ends, is so sensitive to the value of ω. This will become important below. In any case, a series of dissociation experiments were performed as a function of the initial fractional saturation of the nucleic acid, f_{sat}, for wild-type, R4K, R4Q, and K3A gp32s at a constant gp32 concentration (0.25−0.3 μM). These B-domain mutants were chosen for this study because f_{sat} could be absolutely determined at the initial [NaCl] of 0.05 M from the ratio of input gp32 to polynucleotide ratio (stoichiometric binding). From earlier fluorescence titration data collected in T/0.05M NaCl for R4T and R4G gp32s and gp32-B, a significant fraction of the total protein is free even at excess poly(A) (Villemain & Giedroc, 1993). Furthermore, due to weak binding, the complete (in particular, high) binding density range cannot be experimentally accessed for these proteins. Since a straightforward interpretation of k_d(app) requires that all gp32 is bound initially and that redistribution does not occur during the time course of dissociation, extremely weak binding mutants were not investigated here since they do not meet these criteria.

Experimental Results. Initial experiments were carried out with [NaCl] jumps from 0.05 to 0.45 M NaCl over a wide range of binding densities. Representative raw, averaged dissociation kinetic traces obtained for wild-type gp32 from poly (A) at two binding densities, $f_{sat} = 1.0$ or 0.1, are shown in Figure 3. Two points can be made from these data. First, they point out the excellent quality of the data and clearly show that rates of dissociation depend strongly on initial binding density, as expected from Lohman's model (Lohman, 1983). Secondly, clear zero-order kinetics are not observed for wild-type gp32 or B-domain mutants (data not shown) although the sum of the residuals for a single-exponential

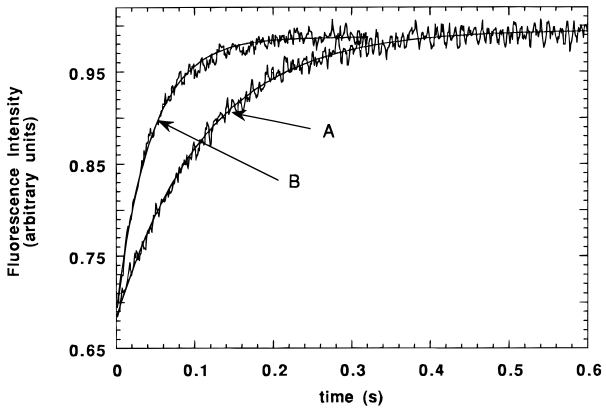

FIGURE 3: Time course of dissociation of wild-type gp32 from poly(A) resulting from [NaCl] jumps from 0.05 to 0.45 M (10 mM Tris, pH 8.1, 0.1 mM EDTA, 20 °C) at f_{sat} of 0.1 (curve B) and 1.0 (curve A). Superimposed on each time course is a best-fit a single exponential decay where k_d(app) = 22.5 ± 0.14 and 8.9 ± 0.08 s^{-1} for $f_{sat} = 0.1$ and 1.0, respectively. Obvious zero-order kinetics are not observed for $f_{sat} = 1.0$ in this case (Lohman, 1984a).

decay are perhaps slightly nonrandom at early time points (data not shown). This suggests that zero-order kinetic behavior may be observed only at very early time points for wild-type gp32 under these conditions and is not easily distinguishable from first-order kinetics when the entire time course is analyzed. Although precautions were taken to use only the longest poly(A) from a column fractionation (Villemain & Giedroc, 1996), the observation of non-zero-order kinetics probably arises from the polydispersity of the poly(A) used in these experiments (Lohman, 1984a).

Measurements of k_d(app) as a function of f_{sat} for wild-type and R4K gp32s (panel A) and R4Q and K3A gp32s (panel B) are shown in Figure 4 at 0.45 M NaCl. A strong binding density dependence of k_d(app) is observed for each of the B-domain substitution mutants. This suggests that the dissociation of cooperatively bound complexes occurs as described previously for wild-type gp32, i.e., from the ends of a ligand cluster (Lohman, 1983). k_d(app) increases as f_{sat} decreases since the number of protein clusters and therefore cluster ends increases. k_d(app) is found to range from 6 to 63 s^{-1} for wild-type gp32, from 13 to 82 s^{-1} for R4K gp32, from 55 to 313 s^{-1} for R4Q gp32, and from 40 to 204 s^{-1} for K3A gp32. As can be seen, k_d(app) for both R4Q and K3A gp32 is significantly faster than for wild-type and R4K gp32s over the entire binding density range, while the differences between wild-type and R4K gp32, while significant, are much smaller.

The significant increase in k_d(app) over the entire binding density range observed for B-domain mutants can be due to either an increase in k_e and/or a decrease in ω, the latter of which would give rise to more cluster ends at equilibrium at any fractional saturation. Two approaches were taken to analyze the data in Figure 4. In one approach, nonlinear least-squares parameter estimation of *both* k_e and ω was carried out using eqs 7 and 5. The smooth curves drawn through the data points are the results of these fits with the optimized parameters collected in the figure legend.[4] Un-

[4] Note that in all cases, the best-fit line misses the experimental data, particularly at high f_{sat}, where the theoretical curve goes to k_d(app) = 0 at $f_{sat} = 1$. This is a direct consequence of the fact the model assumes an infinite-length polynucleotide lattice, which is obviously not attainable experimentally (Lohman, 1984a).

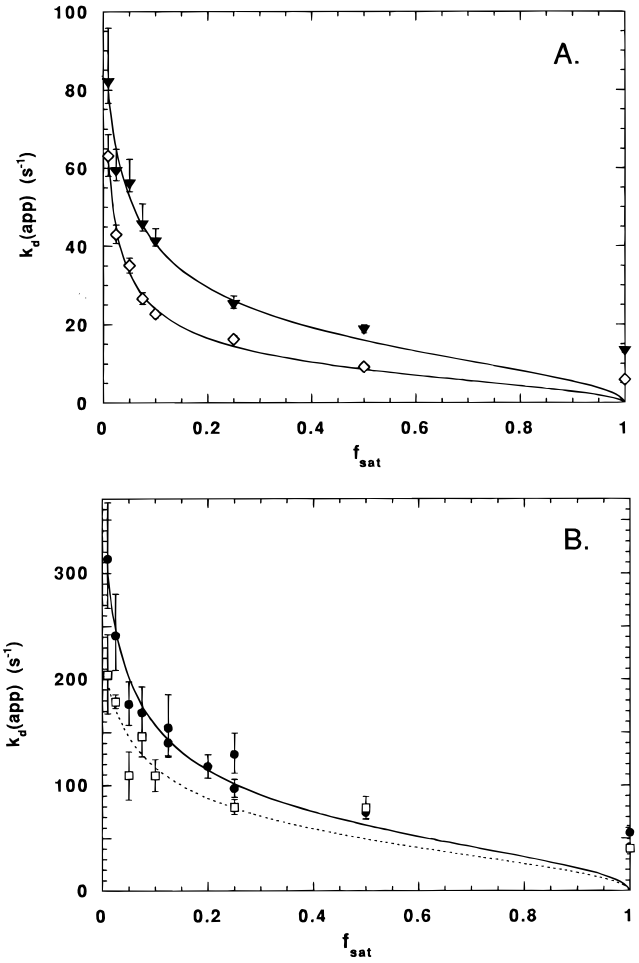

FIGURE 4: Dependence of k_d(app) on the initial fractional saturation (f_{sat}) of the poly(A) for wild-type, R4K, R4Q, and K3A gp32s. [NaCl] jumps were from 0.05 to 0.45 M NaCl. A. Dissociation of wild-type gp32 (◇), $k_e = 62.2 \pm 22.5$ s^{-1}, $\omega = 1530 \pm 1420$; and R4K gp32 (▼), $k_e = 50.2 \pm 7.9$ s^{-1}, $\omega = 246 \pm 137$. B. Dissociation of R4Q gp32 (●), $k_e = 186.5 \pm 25.5$ s^{-1}, $\omega = 220 \pm 102$; and K3A gp32 (□), $k_e = 112.5 \pm 21.7$ s^{-1}, $\omega = 119 \pm 96.4$.

fortunately, the least-squares fit of these data to eqs 7 and 5 is relatively insensitive to the value of the ω over a relatively wide range, and returns, with the exception of wild-type gp32, a severely underestimated value of ω, as judged from previous equilibrium measurements (Villemain & Giedroc, 1993, 1996).

Therefore, a second approach was taken to estimate k_e from the slope of a plot of k_d(app) vs $(1 - p_0)$ (eq 7) where $(1 - p_0)$ is calculated from eq 5 with n (7.5 nucleotides) and ω fixed at their independently determined values (Villemain & Giedroc, 1993, 1996). A representative plot of this kind is shown in Figure 5 where $\omega = 1000$ was chosen for calculation of $(1 - p_0)$, an ω value consistent with the range of values determined for wild-type, R4K, R4Q, and K3A (Villemain & Giedroc, 1993, 1996). Note again, that the linear fit was constrained to pass through the origin since at $f_{sat} = 1$, $(1 - p_0) = 0$, and k_d(app) = 0. Using this approach, k_e determined for R4Q gp32 is 303 ± 33 s^{-1} or ~5-fold larger than wild-type gp32. k_e determined for R4K and K3A gp32s are 78 ± 8 and 207 ± 37 s^{-1} (plots not shown), which are, respectively, indistinguishable and ~3.5-fold larger than wild-type gp32. The optimized value of k_e determined for wild-type gp32 using this approach is 59 ± 6 s^{-1} when $n = 7.5$ and $\omega = 1000$, comparable to that determined above (62 ± 23 s^{-1}; Figure 4). This is expected since the resolved

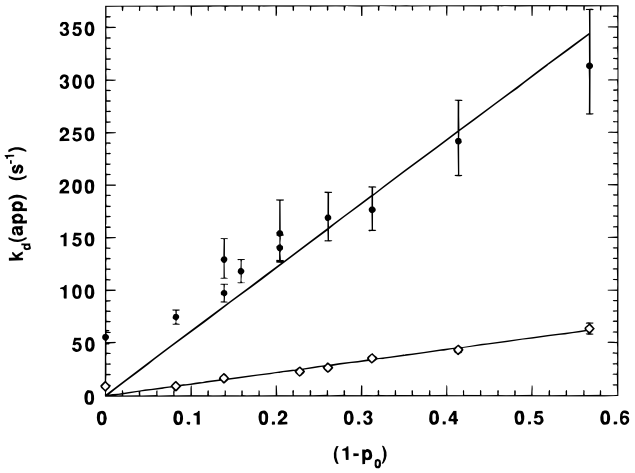

FIGURE 5: k_d(app) versus $(1 - p_0)$ plotted from the data in Figure 3 for wild-type (◇) and R4Q (●) gp32s. Best-fit k_e values (wild-type gp32, 59 ± 3 s^{-1}; R4Q gp32, 303 ± 17 s^{-1}) were obtained by linear least-squares regression analysis of the data using eq 5 which requires that the fit be forced through the origin (see text for details). $(1 - p_0)$ was calculated using $\omega = 1000$ in both cases.

value of ω (~1500) (Figure 4) is similar to the input value used for the linear analysis (Figure 5). Our determination of k_e for wild-type gp32 on poly(A) with ω fixed at 1000 is in excellent agreement with that previously determined (Lohman, 1984a) under nearly identical solution conditions ($k_e = 66$ s^{-1}).

Detailed examination of the kinetic traces obtained for rapidly dissociating R4Q and K3A gp32s at 0.45 M NaCl (Figure 4B) reveals that a reasonable portion (up to 35−40%) of the dissociation reaction for these mutants, particularly at low fractional saturations ($f_{sat} \leq 0.10$), appears to occur within the dead time of the instrument. This is signified by low amplitudes obtained from the single exponential fits of the data to obtain k_d(app) at $f_{sat} \leq 0.10$ (data not shown), suggesting that k_d(app) for R4Q and K3A gp32s may have been underestimated at low f_{sat}; this in turn may underestimate k_e and ω since it is these data points which largely dictate the resolved magnitude of these parameters. With this in mind, a second set of dissociation experiments was carried out for R4Q and K3A gp32s with [NaCl] jumps from 0.05 to 0.31 M NaCl. From the [NaCl]-dependence of k_d(app) for wild-type and the mutant proteins, plotted as $\partial \log k_d(\text{app})/\partial \log[\text{NaCl}]$ (Figure 7, see below), 0.31 M NaCl was chosen as a [NaCl] for which k_d(app) for R4Q and K3A gp32s would be comparable in magnitude to the range in k_d(app) values determined for wild-type and R4K gp32s at 0.45 M NaCl (Figure 4B). This should allow for a more direct comparison. Knowing the dependence of k_d-(app) on [NaCl] for each protein then allows results obtained for R4Q and K3A gp32s at 0.31 M NaCl to be compared to values obtained for wild-type and R4K gp32s at 0.45 M NaCl.

The binding density dependences of k_d(app) for R4Q and K3A gp32s at 0.31 M NaCl are shown in Figure 6 with k_d-(app) ranging from 16 to 146 s^{-1} for R4Q gp32 and from 14 to 111 s^{-1} for K3A gp32. These data were fit by nonlinear least-squares analysis using eqs 7 and 5 to obtain values of k_e and ω (Figure 6). For R4Q gp32, $k_e = 121 \pm 24$ s^{-1} and $\omega = 822 \pm 463$, while for K3A gp32, $k_e = 102 \pm 31$ s^{-1} and $\omega = 1160 \pm 950$. Although there remains a significant uncertainty associated with the determinations of

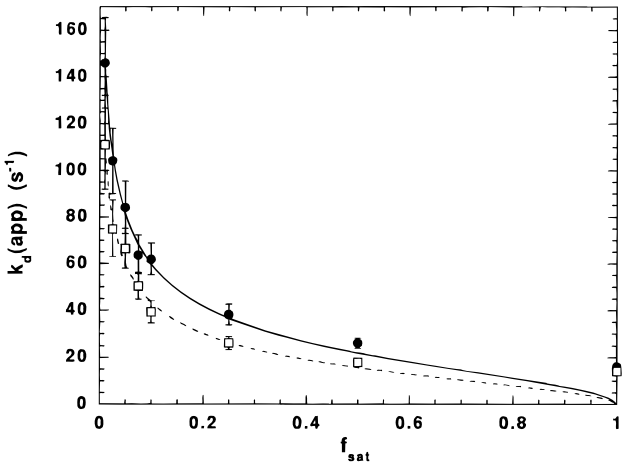

FIGURE 6: Dependence of k_d(app) on f_{sat} of poly(A) for R4Q (●) and K3A (□) gp32s with [NaCl] jumps from 0.05 to 0.31 M (10 mM Tris, pH 8.1, 0.1 mM EDTA, 20 °C). R4Q gp32, $k_e = 120.5 \pm 23.8$ s^{-1}, $\omega = 822 \pm 463$; K3A gp32, $k_e = 101.5 \pm 30.7$ s^{-1}, $\omega = 1158 \pm 946$.

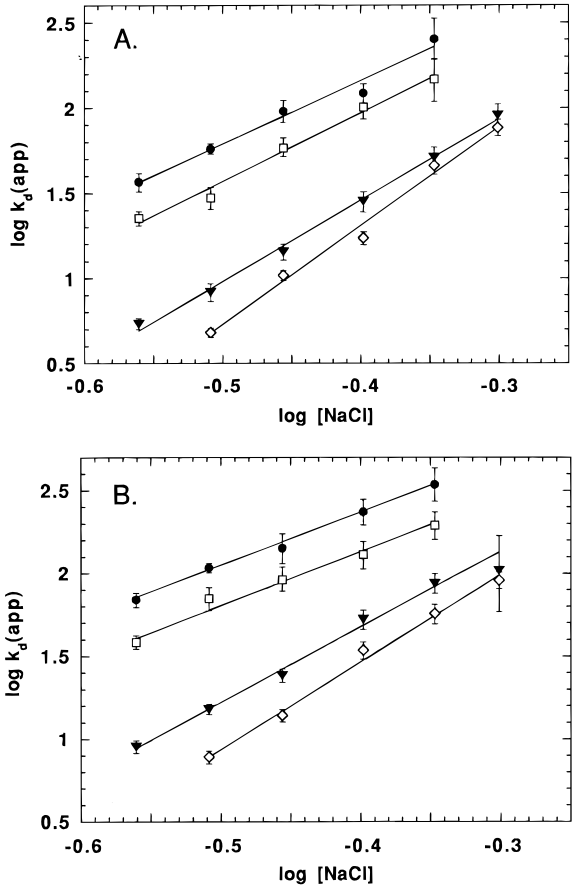

FIGURE 7: [NaCl] dependence of k_d(app) for wild-type (◇), R4K (▼), R4Q (●), and K3A (□) gp32s at 20 °C plotted as log k_d(app) versus log[NaCl]. A. $f_{sat} = 0.25$; wild-type gp32, slope $= 5.8 \pm 0.4$; R4K gp32, slope $= 4.8 \pm 0.2$; R4Q gp32, slope $= 3.7 \pm 0.3$; K3A gp32, slope $= 4.0 \pm 0.3$. B. $f_{sat} = 0.075$; wild-type gp32, slope $= 5.3 \pm 0.3$; R4K gp32, slope $= 4.6 \pm 0.3$; R4Q gp32, slope $= 3.2 \pm 0.4$; K3A gp32, slope $= 3.3 \pm 0.4$.

ω, they are much more consistent with that determined by equilibrium binding studies (Villemain & Giedroc, 1993, 1996). As expected, a linear analysis [(k_d(app) vs $1 - p_0$ with $\omega = 1000$; see Figure 4) of the data shown in Figure 6 yields identical values of k_e, 129 ± 8 and 96 ± 8 s^{-1}, for R4Q and K3A gp32s, respectively. By using the relationship in eq 7, $k_d(\text{app}) = 2k_e(1 - p_0)$, and assuming that ω is independent of [NaCl] (Villemain & Giedroc, 1996), k_e at 0.45 M NaCl can be estimated from k_e at 0.31 M NaCl for R4Q and K3A gp32 from

$$k_d(\text{app})^{0.31\text{ M NaCl}}/k_d(\text{app})^{0.45\text{ M NaCl}} = k_e{}^{0.31\text{ M NaCl}}/k_e{}^{0.45\text{ M NaCl}}$$

Using this relationship, the mean values for k_e at 0.45 M NaCl taken over the f_{sat} range 0.075−1.0 are 384 ± 34 and 328 ± 36 s^{-1} for R4Q and K3A gp32s, respectively. Note that k_e for R4Q gp32 estimated in this way is much closer to value estimated from the direct dissociation data at 0.45 M NaCl (Figure 3B) and assuming an $\omega = 1000$ (Figure 4); this speaks to the internal consistency of the determination of k_e at 0.45 M NaCl. These values of k_e reflect ∼6- and ∼5-fold increase, respectively, in the rate of dissociation of an R4Q and K3A gp32 monomer from the end of a cooperatively bound cluster relative to wild-type gp32 at 0.45 M NaCl. Due to the fact that k_d(app) was determined with higher precision over the entire fractional saturation range at 0.31 M NaCl and resolved ω values are comparable to those determined at equilibrium, we would argue that k_e calculated in this way for R4Q and K3A gp32s at 0.45 M NaCl reflects a more accurate estimation.

[NaCl]-Dependence of k_d(app). The [NaCl]-dependence of the apparent dissociation rate constant was measured for wild-type, R4K, R4Q, and K3A gp32s at two different fractional saturations of poly(A), $f_{sat} = 0.25$ and 0.075. This was done in order to facilitate comparisons such as that outlined above as well as determine if the dependence of k_d(app) on [NaCl] correlates with the dependence of the equilibrium constant, K_{app}, on [NaCl] measured previously (Villemain & Giedroc, 1993, 1996). Previous studies with wild-type gp32 showed that at ≥0.25 M NaCl and at $f_{sat} = 1$ on poly(A), $\partial \log k_d(\text{app})/\partial \log[\text{NaCl}] \approx -\partial \log K_{app}/\partial \log[\text{NaCl}]$. Since ω is independent of salt concentration, this requires that $\partial \log k_e/\partial \log[\text{NaCl}] \approx -\partial \log K_{int}/\partial \log[\text{NaCl}]$, revealing that the entire salt-dependence in K_{int} resides in the rate of dissociation of gp32 monomers from the ends of protein clusters (Lohman, 1984a). In addition, two fractional saturations were investigated since earlier measurements of the [NaCl]-dependence of k_d(app) for wild-type gp32 at low fractional saturations ($f_{sat} = 0.045$ and 0.09) detected nonlinearity in the $\partial \log k_d(\text{app})/\partial \log[\text{NaCl}]$ plot not detected at $f_{sat} = 1$ (Lohman, 1984b).

Figure 7A and 7B show the $\partial \log k_d(\text{app})/\partial \log[\text{NaCl}]$ plots for both $f_{sat} = 0.075$ and 0.25 over the [NaCl] range from 0.275 to 0.50 M NaCl for wild-type and the B-domain mutant gp32s. Table 1 compiles the slopes of the log−log plots measured at each fractional saturation compared with the value of $\partial \log K_{app}/\partial \log[\text{NaCl}]$ measured previously (Villemain & Giedroc, 1993, 1996). In contrast to the earlier work (Lohman, 1984b), our data suggest that $\partial \log k_d(\text{app})/\partial \log[\text{NaCl}]$ is linear for all proteins tested at both $f_{sat} = 0.25$ and 0.075. However, it has been noted that the average length of the nucleic acid will affect the slope of the log−log plot and significant polydispersity of the nucleic acid may mask any nonlinearity (Lohman, 1984b). As found previously, there is a slight diminution in the values of $\partial \log k_d(\text{app})/\partial \log[\text{NaCl}]$ in going from $f_{sat} = 0.25$ to $f_{sat} = 0.075$, which may be more pronounced for the weaker binding R4Q and K3A gp32s. In addition, with the possible exception of R4K

Table 1: $\partial \log k_d(app)/\partial \log[NaCl]$ Values for Wild-Type, R4K, R4Q, and K3A Gp32s on Poly(A) at $f_{sat} = 0.25$ and 0.075 (10 mM Tris, pH 8.1, 0.1 mM EDTA, 20 °C) Compared with $\partial \log K_{app}/\partial \log[NaCl]$ Values Measured Previously at [NaCl] ≥ 0.2 M[a]

gp32	$\partial \log k_d(app)/\partial \log[NaCl]$ $f_{sat} = 0.075$	$\partial \log k_d(app)/\partial \log[NaCl]$ $f_{sat} = 0.25$	$\partial \log K_{app}/\partial \log[NaCl]$[b]
wild-type	5.3 (±0.3)	5.8 (±0.4)	−6.6 (±1.0)
R4K	4.6 (±0.3)	4.8 (±0.2)	−4.7 (±0.9)
K3A	3.3 (±0.4)	4.0 (±0.3)	−5.4 (±1.1)
R4Q	3.2 (±0.4)	3.7 (±0.3)	−4.2 (±0.4)
R4T	nd[c]	nd[c]	−4.3 (±0.6)

[a] Determined from the data shown in Figure 6. [b] Data from Villemain and Giedroc (1993, 1996). [c] nd, not determined.

gp32, all $\partial \log k_d(app)/\partial \log[NaCl]$ values determined at $f_{sat} = 0.25$ are slightly smaller than the absolute value of $\partial \log K_{app}/\partial \log[NaCl]$ determined from equilibrium binding studies at [NaCl] ≥ 0.20 M (Villemain & Giedroc, 1993, 1996). Regardless of whether or not this is the case, a comparison of the dissociation kinetic data with the equilibrium studies suggests a trend toward smaller absolute values of both $\partial \log k_d(app)/\partial \log[NaCl]$ and $\partial \log K_{app}/\partial \log[NaCl]$ for weaker binding mutants. As discussed below, this reduction in the absolute value of $\partial \log k_d(app)/\partial \log[NaCl]$ observed here for B-domain mutants relative to wild-type gp32 may reflect a change in the dominant pathway of lattice dissociation available to B-domain mutants which does not occur for wild-type gp32 until much lower [NaCl] (Lohman, 1984b).

DISCUSSION

Lohman's model for the kinetics of irreversible dissociation of cooperatively bound protein−nucleic acid complexes predicts that the dominant pathway of protein dissociation is from the end of clusters of bound protein (Lohman, 1983). The dissociation of wild-type gp32 from ss polynucleotides induced by high salt concentration jumps was previously found to be well-described by this model (Lohman, 1984a,b). In this paper, we show that the mechanism of high-salt-induced dissociation of three B-domain mutant gp32s, R4K, R4Q, and K3A gp32s, from poly(A), is unchanged relative to wild-type gp32. This is consistent with the highly cooperative binding previously observed for these gp32s to poly(A) at equilibrium (Villemain & Giedroc, 1993, 1996) which gives rise to a significant increase in k_d(app) as the fractional saturation of the polynucleotide is decreased (Figures 4 and 6). This binding density dependence of k_d(app) derives from an increase in the number of protein clusters and therefore cluster ends from which protein molecules may dissociate as the fractional saturation decreases. Since gp32 cluster size and distribution is determined entirely by the magnitudes of ω and ν, at any one ν and indistinguishable ω values for any two proteins, an increase in k_d(app) must arise from an increase in the molecular rate constant k_e, the rate at which gp32s dissociate from cluster ends. This is precisely what is observed for R4Q and K3A substitution mutants.

As previously discussed, Lohman's dissociation kinetic model has features which distinguish it from an alternative model proposed by Epstein (1979). Epstein's model, denoted the Instantaneous Establishment of Lattice Equilibrium (IELE) model, also describes the kinetics of binding (or dissociation) of cooperative ligands to a linear lattice. This model incorporates the provision that proteins can translocate infinitely quickly along a nucleic acid lattice such that the distribution of clusters of bound protein is always at equilibrium. As a result, the IELE model predicts that for highly cooperative binding systems, the cluster size distribution would change throughout the time course of a dissociation event since the binding density would be continuously changing. Instead of an exponential decay, the IELE model predicts an initial lag period in the time course of dissociation of a cooperatively bound protein from the ss nucleic acid (Epstein, 1979; Lohman, 1983). Since Lohman's model (Lohman, 1983) predicts a single exponential decay at low initial f_{sat}, these two models are readily distinguished from one another. Since a single exponential decay quantitatively fits the dissociation kinetic data obtained for B-domain mutants well, in addition to the strong-binding density dependence of the k_d(app), we conclude that the major pathway for the dissociation of the mutant gp32s from poly(A), like that for wild-type gp32, is via the dissociation of singly contiguous protein monomers from protein cluster ends without significant redistribution of the protein clusters during the time course of dissociation. A more general treatment of Lohman's non-redistributing model in which significant dissociation is permitted to occur from the interior of clusters is only operative at high initial fractional saturation and low cooperativity of binding (Balazs & Epstein, 1984). Since wild-type and all mutant proteins studied here bind with high and largely indistinguishable cooperativities (Villemain & Giedroc, 1993, 1996), dissociation of gp32s from the interior of clusters is unlikely to represent a major pathway of dissociation.

Since the mechanism of the high-salt-induced dissociation of the B-domain mutants is unchanged relative to wild-type gp32, a comparison of the *apparent* rates of dissociation can yield direct insight into the relative magnitudes of *molecular* dissociation rates. Figure 4 shows that the rates of dissociation of R4Q and K3A gp32s from poly(A) are dramatically faster than those determined for wild-type and R4K gp32s at 0.45 M NaCl. For example, the apparent dissociation rates at $f_{sat} = 1$ for wild-type and R4K proteins from poly(A) show at most a modest 2-fold difference (6 s^{-1} for wild-type versus 13 s^{-1} for R4K gp32). Differences in k_d(app) for wild-type (6 s^{-1}), R4Q gp32 (55 s^{-1}), and K3A gp32 (40 s^{-1}) at $f_{sat} =$ 1, 0.45 M NaCl, are much greater, corresponding to 9- and 7-fold faster apparent rates of dissociation of the saturated complex for R4Q and K3A gp32s, respectively.

In contrast to the clear differences observed among the apparent rates of dissociation for wild-type and R4K gp32s at 0.45 M NaCl, the molecular dissociation rate constants, k_e, are nearly indistinguishable at 63 ± 16 and 78 ± 8 s^{-1}, respectively, for these two proteins. Since k_d(app) values are consistently larger for R4K gp32 relative to wild-type gp32 over the entire fractional saturation range, it seems possible the R4K mutation incurs a small reduction in the value of ω and therefore cluster size distribution since k_d(app) depends on n, ω, and ν. Resolution of K_{int} and ω for R4K gp32 from previous equilibrium binding data lends support to this possibility (Villemain & Giedroc, 1993), consistent with the resolved values of k_e and ω (Figure 4A). In contrast to R4K gp32, the k_e values for R4Q and K3A gp32s, 384 ± 34 and 328 ± 36 s^{-1}, respectively, correspond to a ∼6- and ∼5-fold reduction, respectively, in k_e relative to wild-type gp32, which appears to reflect the full ∼5-fold

diminution in K_{app} determined under the same solution conditions (pH 8.1, 0.45 M NaCl) (Villemain & Giedroc, 1996). We conclude that a faster rate of dissociation of R4Q and K3A gp32s from the ends of cooperatively bound clusters accounts for entire diminution in K_{app} reported for these mutants.

Comparison of the [NaCl]-dependence of k_d(app) at [NaCl] > 0.20 M (Table 1) with the [NaCl]-dependence of K_{app} reported previously (Villemain & Giedroc, 1993, 1996) reveals that the trend in the absolute magnitudes of $\partial \log k_d(app)/\partial \log[NaCl]$ vs $\partial \log K_{app}/\partial \log[NaCl]$ for wild-type and B-domain mutants parallel one another nicely. From an earlier comparison of the [NaCl] dependence of K_{app} and k_d(app) for wild-type gp32 (Lohman, 1984b), Lohman concluded that, under conditions where $\partial \log k_d(app)/\partial \log[NaCl]$ and $\partial \log K_{app}/\partial \log[NaCl]$ for wild-type gp32 could be directly compared ([NaCl] ≥ 0.35 M), essentially all of the [NaCl]-dependence of K_{app} resides in k_e. Our data suggest that the same conclusion can be drawn for the B-domain mutants, consistent with the finding that the mechanism of dissociation relative to wild-type gp32 at high salt appears unchanged.

Interestingly, the absolute magnitudes of $\partial \log k_d(app)/\partial \log[NaCl]$ and $\partial \log K_{app}/\partial \log[NaCl]$ for B-domain mutants appear significantly and consistently smaller than that observed for wild-type gp32 (Table 1) over the same range in [NaCl]. Lohman has previously observed through the use of polynucleotide-trapping experiments that the [NaCl]-dependence of k_d(app) for wild-type gp32 from poly(A) at [NaCl] ≤ 0.25 M becomes significantly smaller than that observed at [NaCl] ≥ 0.25 M NaCl (Lohman, 1984b). The same trend in the [NaCl]-dependence of K_{app} for wild-type gp32 as well as for all B-domain mutants has also been observed, although the breakpoint for B-domain mutants appears to occur at lower salt, at ~0.20 M NaCl (Villemain & Giedroc, 1996). From these findings and additional considerations, Lohman has argued that the dissociation of gp32 from poly(A) at [NaCl] ≤ 0.25 M reflects the presence of an additional dissociation pathway available to gp32, in which gp32 dissociates from cluster ends via translocation or sliding to form an isolated site bound intermediate, which subsequently dissociates from the lattice (see Figure 2). Since the salt-dependence of sliding has been shown to essentially zero in some systems (Winter et al., 1981), this would decrease the [NaCl]-dependence of k_d(app) as this pathway becomes the dominant one, relative to direct dissociation from cluster ends. This also explains satisfactorily why $\partial \log k_d(app)/\partial \log[NaCl]$ is modestly dependent on fractional saturation of the lattice (Table 1): as f_{sat} is decreased, the gaps between clusters becomes much larger, enabling extensive sliding without collision with another cluster.

One plausible interpretation of the data compiled in Table 1 is that the smaller $\partial \log k_d(app)/\partial \log[NaCl]$ values obtained for N-terminal domain mutants may be reporting on a larger contribution of the sliding pathway relative to direct dissociation from cluster ends on poly(A) over the same concentration of [NaCl] relative to wild-type gp32. This makes qualitative sense since the [NaCl] at which sliding vs direct dissociation from cluster ends becomes comparable depends only on the relative magnitudes of k_{-1}, the rate of dissociation of an isolated gp32 monomer, and k_s, the sliding rate constant, provided one assumes that k_s for B-domain mutants is comparable to wild-type gp32 (Figure 2) (Lohman, 1984b). Considering that k_e is equal to k_{-1}/ω in the absence of sliding, ω for these gp32s are indistinguishable from the wild-type protein (Figure 6) (Villemain & Giedroc, 1993, 1996), and k_e defines the molecular defect in R4Q and K3A gp32s, k_{-1} must be larger for B-domain mutants. Additional dissociation kinetic studies at much lower salt concentrations using polynucleotide trapping techniques are required to determine the extent of correspondence between $\partial \log k_d(app)/\partial \log[NaCl]$ and $\partial \log K_{app}/\partial \log[NaCl]$ (Villemain et al., 1996) over a much wider range of [NaCl], from which further support for the presence of two alternate pathways of lattice dissociation could be obtained.

Moderately defective gp32s have provided us with tools with which to assess how the N-terminal B-domain modulates the ssDNA binding affinity of gp32. We conclude that single amino acid substitutions in the N-terminal domain of gp32 significantly affect the rate at which gp32 monomers dissociate from the ends of protein clusters on the polynucleotide lattice. This reveals that the B-domain modulates the lifetime of mutant gp32−ss nucleic acid complexes in a manner apparently *independent* of the magnitude of the cooperativity parameter. These findings have important implications for the structural organization of cooperatively bound gp32, and in particular the disposition of the N-terminal domain relative to the rest of the complex. Consideration of the recent crystal structure of the gp32−(A+B)−ss nucleic acid complex (Shamoo et al., 1995) and the relative position of the ssDNA binding groove and Gly^{22}, which defines the N-terminus of the core fragment, suggests that the N-terminal domain (residues 1−21) may function as a gate that closes over the bound ssDNA thereby extending the lifetime of the complex. Since this domain is also required for cooperative binding by gp32 to ss nucleic acids, we further speculate that the B-domain of one gp32 monomer may form part of the gate extending over its nearest-neighbor(s). High-resolution crystallographic studies of a functional cooperatively bound gp32−ss nucleic acid complex are required to substantiate these ideas. Finally, if these findings can be extended to physiological salt concentration as expected (Lohman, 1984b), they suggest the possibility that these kinetic deficiencies may profoundly affect the functional activity of the molecule. As such, we are currently investigating to what extent the mechanistic deficiencies outlined here for, in particular R4Q and K3A gp32s, affect the ability of gp32 to perform its accessory role in well-defined in vitro reconstituted replication and recombination systems.[3]

ACKNOWLEDGMENT

We thank Drs. Tom Baldwin for generous use of his SLM 8000C stopped-flow fluorimeter and Paul Fitzpatrick for his constructive comments on the manuscript.

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BI961482N