Kinetics and Mechanism of Dissociation of Cooperatively Bound T4 Gene 32 Protein–Single-Stranded Nucleic Acid Complexes. 1. Irreversible Dissociation Induced by Sodium Chloride Concentration Jumps†

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ABSTRACT: The dissociation kinetics of cooperatively bound bacteriophage T4 gene 32 protein from a variety of single-stranded homopolynucleotides has been investigated by stopped-flow techniques. Irreversible dissociation of the complexes was induced by rapidly increasing the salt concentration and monitoring the increase in tryptophan fluorescence upon dissociation of the gene 32 protein. The dependence of the apparent dissociation rate constant on initial fractional saturation of the nucleic acid lattice as well as the observation of zero-order kinetics when the lattice is initially fully saturated with protein indicates that dissociation occurs only from the ends of protein clusters and not from doubly contiguous molecules. The data for the entire time course are quantitatively fit by a kinetics model specifying irreversible dissociation of only singly contiguously bound protein [Lohman, T. M. (1983) *Biopolymers 22*, 1697–1713]. This model is used to extract molecular rate constants for the dissociation of isolated, singly contiguously and doubly contiguously bound protein. It is also shown that the polynucleotide specificity observed for the cooperative binding constant, $K\omega$, and the cooperativity itself are intrinsic properties of the dissociation rate of the various complexes.

Helix destabilizing proteins have been shown to be necessary for replication, recombination, and repair in many organisms and are probably a general component of most replication systems [for a recent review, see Kowalczykowski et al. (1981a)]. This class of proteins binds selectively and, in most cases, cooperatively to single-stranded nucleic acids. One of the most studied helix destabilizing proteins is the product of gene 32 from T4 bacteriophage (Alberts & Frey, 1970). In addition to its role in DNA replication, recombination, and repair, the gene 32 protein (g32p) has also been shown to regulate its own synthesis. This autoregulation, which occurs at the translational level, is dependent upon the high, cooperative binding affinity of gene 32 protein for single-stranded nucleic acids. However, in its role as its own translational repressor, gene 32 protein binds selectively to its own mRNA once its free concentration exceeds ~3 μM (Russel et al., 1976; Lemaire et al., 1978; von Hippel et al., 1982).

Quantitative studies of the equilibrium binding of gene 32 protein to various native and synthetic DNA and RNA molecules (both oligonucleotides and polynucleotides) have been undertaken with particular attention to its cooperative binding behavior (Kelly & von Hippel, 1976; Kelly et al., 1976; Kowalczykowski et al., 1981b; Newport et al., 1981; Bobst et al., 1982). The measured binding affinities of gene 32 protein for single-stranded DNA and RNA as well as double-stranded DNA have been used to successfully model the control of the gene 32 protein concentration during the life cycle of the T4 phage (von Hippel et al., 1982). This indicates that the regulatory role of gene 32 protein seems to be explainable simply on the basis of its relative nucleic acid binding affinities. However, other functions of the T4 gene 32 protein are expected to be sensitive to the kinetic aspects of its binding to nucleic acids. This is certainly the case for its role in DNA replication. The in vivo rate of movement of the replication fork in T4-infected *Escherichia coli* is 500–1000 base pairs per second (Alberts et al., 1977). Although it is not known whether long clusters of gene 32 protein bind to both the leading and the lagging DNA strands, or only to the lagging strand, the protein must either associate and dissociate rapidly from the single-stranded DNA or have an alternate mechanism to keep pace with the rapidly moving fork. Its role in recombination, involving the transient existence of regions of single-stranded DNA, may also involve kinetic considerations.

In an attempt to understand the mechanism and rates of these interactions, we have undertaken a detailed study of the association and dissociation kinetics of gene 32 protein–single-stranded homopolynucleotide binding. The association kinetics (Lohman & Kowalczykowski, 1981) indicate that the binding of gene 32 protein is complex, involving several steps along the pathway to formation of a cooperatively bound nucleic acid complex. The association rate constant for a gene 32 protein monomer to an isolated site on a polynucleotide is $(3–4) \times 10^6$ M^{-1} (nucleotide) s^{-1} and is fairly independent of [NaCl], above 0.10 M. In addition, an analysis of the cooperative binding step suggests that gene 32 protein monomers may have some limited ability to translocate along the single-stranded nucleic acid (Lohman & Kowalczykowski, 1981). In this respect, one can view the gene 32 protein monomer's "search" for a cooperative binding site as similar to the *lac* repressor protein's "search" for its operator site by translocating along the DNA while bound nonspecifically (Richter & Eigen, 1974; Berg & Blomberg, 1976, 1978; Berg et al., 1981; Winter et al., 1981; Barkley, 1981).

In this and the following paper (Lohman, 1984), we present an investigation of the dissociation of cooperatively bound T4 gene 32 protein from various synthetic single-stranded homopolynucleotides, since the association experiments (Lohman & Kowalczykowski, 1981) were not able to yield much information about the dissociation reaction. We make use of the salt sensitivity of the binding constant, $K\omega$, and induce an irreversible dissociation by rapidly increasing the [NaCl] in a stopped-flow experiment, while monitoring the increase in gene 32 protein fluorescence upon dissociation of the complex (Peterman & Wu, 1978; Lohman, 1980; Kowalczykowski et al., 1980). Previous studies of the dissociation kinetics of gene 32 protein have been carried out using single-stranded fd phage

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DNA (Peterman & Wu, 1978). In this study, single-stranded homopolynucleotides have been used in order to avoid the complexities of internal secondary structure in the nucleic acid lattice which makes a quantitative analysis of the kinetics difficult. In this paper the mechanism of dissociation which occurs at high NaCl concentration is characterized as a function of initial fractional saturation of the nucleic acid with gene 32 protein. The data for the entire time course are shown to quantitatively fit a model in which the only pathway for dissociation of cooperatively bound protein is via the ends of protein clusters (Lohman, 1983). From these data we calculate the rate constants for dissociation of singly contiguously bound protein, k_e. In the following paper, we investigate the effects of temperature and a wide range of NaCl concentrations and other salts in order to probe the dissociation mechanism under more physiological conditions. Those results suggest an additional pathway for dissociation.

This work, in addition to its implications for the T4 gene 32 protein, also deals with the analysis of the kinetics of cooperative protein–nucleic acid interactions. The general approaches and the use of salt effects to probe the mechanism of the interaction should be useful in studies of other protein–nucleic acid interactions, particularly those involving cooperativity.

Materials and Methods

Reagents. All chemicals were reagent grade. All solutions were prepared with doubly distilled water.

Buffers. The following buffers were used in this work: buffer T (pH 8.3) is 10 mM Tris [tris(hydroxymethyl)-aminomethane] and 0.1 mM EDTA (ethylenediaminetetraacetic acid); buffer P (pH 6.9) is 5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 0.1 mM EDTA, and 0.75 mM β-mercaptoethanol; buffer B (pH 9.1) is 10 mM Bicine [*N*,*N*′-bis(2-hydroxyethyl)glycine] and 0.1 mM EDTA; buffer H (pH 7.5) is 10 mM Hepes [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid) and 0.1 mM EDTA. The salt concentrations in the buffers were adjusted by addition of NaCl, sodium acetate, or NaF to the indicated value. The pH of the buffer did not vary by more than ±0.1 pH unit over the salt concentration range studied.

Nucleic Acids. Homopolynucleotides [except poly(rεA)] were purchased from Miles Biochemicals and Collaborative Research and were used without further purification. Poly(1,N^6-ethenoadenylic acid) [poly(rεA)] was purchased from P-L Biochemicals. For some experiments, the homopolynucleotides were passed through a Sepharose 4B column (20 × 1 cm), and the high molecular weight material, which eluted in the void volume, was collected and concentrated. This procedure eliminated any molecules which were smaller than ~800 nucleotides. Single-stranded DNA from bacteriophage M13 grown in *E. coli* K-37 was isolated essentially as described by Yamamoto et al. (1970) using a 0.5 M NaCl–2.5% PEG 6000 phase separation on the phage-containing supernatant. The M13 phage was banded at equilibrium in a CsCl gradient and dialyzed vs. 10 mM Tris, pH 8.3, 20 mM NaCl, and 3 mM EDTA. The single-stranded M13 DNA was extracted from the phage 5 times with cold phenol, saturated with 10 mM Tris, pH 8.3, 20 mM NaCl, and 3 mM EDTA; 1% sodium dodecyl sulfate (SDS) was added to the M13 phage suspension before mixing with the phenol. The DNA was stored in 10 mM Tris, pH 8.3, 0.1 M NaCl, and 0.1 mM EDTA. Concentrations of the homopolynucleotides were determined by using the extinction coefficients cited by Kowalczykowski et al. (1981b). M13 DNA concentrations were determined by absorbance at 259 nm using $\epsilon = 7370\ cm^{-1}\ M^{-1}$ (phosphate) (Berkowitz & Day, 1974).

T4 Gene 32 Protein. The preparation of the T4 gene 32 protein used in this study is described in Lohman & Kowalczykowski (1981). The source of gene 32 protein was a T4 phage with amber mutations in gene 33 (N134, C18) and gene 55 (BL292). Phage titers were made with *E. coli* CR63 (su^+), and gene 32 protein was prepared from *E. coli* BE infected with the T4 phage (33^-, 55^-), supplied by Drs. D. Rabussay and E. P. Geiduschek.

Sedimentation Velocity. Sedimentation coefficients of the homopolynucleotides were determined by using a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner and multiplexer.

Stopped-Flow Kinetics. The rapid mixing experiments were performed on a modified Durrum-Gibson stopped-flow spectrophotometer with fluorescence detection as previously described (Lohman & Kowalczykowski, 1981). The kinetic experiments monitored changes in the intrinsic tryptophan fluorescence of gene 32 protein, upon dissociation from the nucleic acid, by using an excitation wavelength of 290 nm. All experiments were conducted at 25.0 ± 0.2 °C unless noted otherwise. The range of gene 32 protein concentrations used was 0.1–0.4 μM although we typically worked at 0.2 μM. At these concentrations, >99% of the g32p exists as monomer while free in solution (Carrol et al., 1972, 1975).

Results

Upon binding single-stranded DNA or RNA, T4 gene 32 protein (g32p) undergoes a substantial quenching of its intrinsic tryptophan fluorescence (Kelly & von Hippel, 1976). Lohman & Kowalczykowski (1981) have summarized the evidence which suggests that this fluorescence quenching is due to interactions with the nucleic acid rather than protein–protein interactions which may be involved in the cooperative interaction. On the basis of this evidence, we conclude that the quantum yield of g32p is identical when it is bound to the nucleic acid in any of its three binding modes (i.e., isolated, singly contiguous, or doubly contiguous). The kinetic experiments described in this paper have monitored the increase in intrinsic protein fluorescence ($\lambda_{ex,max}$ = 290 nm; $\lambda_{em,max}$ = 347 nm) resulting from the dissociation of g32p from single-stranded nucleic acids.

NaCl Concentration Jumps Yielding Complete Dissociation of the Gene 32 Protein–Nucleic Acid Complex. The relaxation kinetics of proteins that bind nonspecifically and cooperatively to linear nucleic acid lattices are expected to be quite complex owing to the existence of a minimum of three protein binding modes assuming only nearest-neighbor interactions: isolated, singly contiguous, and doubly contiguous. In order to simplify the interpretation of the dissociation experiments, we have analyzed the dissociation of the various T4 g32p–homopolynucleotide complexes only under conditions which result in complete dissociation of the complexes; i.e., the dissociations were irreversible, and therefore contributions from reassociating protein were negligible. To ensure that these conditions were met, we first determined the minimum [NaCl] that was necessary to completely dissociate the particular complex under study. Two criteria were used to determine this. Under conditions such that the nucleic acid was not fully saturated with g32p, [NaCl] jumps were made, generally starting at ≤0.10 M NaCl (conditions of stoichiometric binding), and the total amplitude of the resulting fluorescence change (ΔF) was observed as a function of the final [NaCl]. In this manner, a minimum [NaCl] ($[NaCl]_{min}$) was determined that resulted in the maximum ΔF for that particular g32p–nucleic acid complex. For jumps to [NaCl] above $[NaCl]_{min}$, the resulting

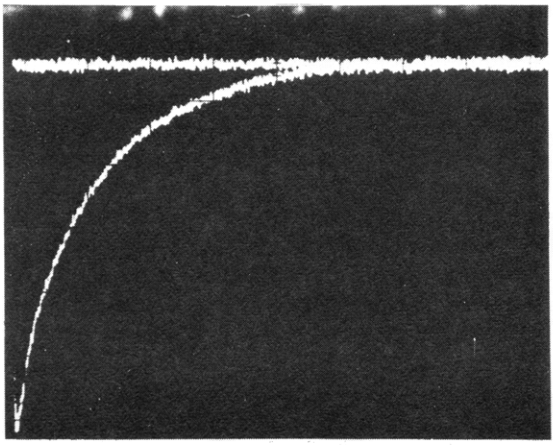

FIGURE 1: Time course for dissociation of a gene 32 protein–poly(dA) complex resulting from a NaCl concentration jump from 0.10 to 0.35 M (10 mM Tris, pH 8.2, and 0.1 mM EDTA). Initial fractional saturation is 0.134.

ΔF remained constant for a given initial f_{sat}, whereas below $[NaCl]_{min}$ the total ΔF decreased as the final [NaCl] decreased. In addition to the amplitude of ΔF, the qualitative behavior of the time course of dissociation was also dependent upon whether the salt jump was sufficient to attain complete dissociation. For initial conditions such that $f_{sat} < 1$, a single exponential decay was observed when the final [NaCl] > $[NaCl]_{min}$ whereas biphasic kinetics were always observed when [NaCl] jumps were insufficient to bring about complete dissociation. Only dissociation experiments resulting from jumps to [NaCl] > $[NaCl]_{min}$ hence satisfying the two criteria of (i) maximum ΔF and (ii) single exponential decay when $f_{sat} < 1$ are reported in this paper, since they represent examples of irreversible dissociation kinetics. A typical time course of the T4 g32p fluorescence is shown in Figure 1 for the irreversible dissociation of a g32p–poly(dA) complex upon jumping the [NaCl] from 0.10 to 0.35 M. No change in fluorescence of the T4 gene 32 protein was observed upon increasing the salt concentration in the absence of nucleic acid.

To confirm that the rate constants determined from these NaCl concentration jumps are true dissociation rate constants, we performed a series of dissociation experiments under identical solution conditions but with increasing g32p and nucleic acid concentrations while maintaining the same initial binding density (constant [g32p]/[DNA]). Identical first-order rate constants were obtained when the concentration of g32p and poly(A) were varied 5-fold, while their ratio was kept constant (data not shown), indicating that we are measuring true first-order dissociation rate constants.

In contrast to the g32p–homopolynucleotide studies described here, we found more complex dissociation kinetics when natural single-stranded DNA such as M13 phage DNA or denatured calf thymus DNA was used. The dissociation of g32p–single-stranded M13 DNA complexes (when $f_{sat} < 1$) always resulted in biphasic kinetics even when the [NaCl] jump was sufficient to ensure complete dissociation of the complex (T. M. Lohman, unpublished results). This behavior has also been observed with bacteriophage fd DNA–g32p complexes (Peterman & Wu, 1978). However, when the M13 DNA is initially fully saturated with g32p, the resulting dissociation kinetics can be described by a single-exponential decay. This more complex kinetic behavior when natural DNA is used as the lattice is presumably due to the formation of secondary structure (when $f_{sat} < 1$) which may play a role in determining the distribution of clusters of g32p on the lattice. To avoid these complications in the analysis of the dissociation kinetics, we have used only homopolynucleotides in the experiments reported here.

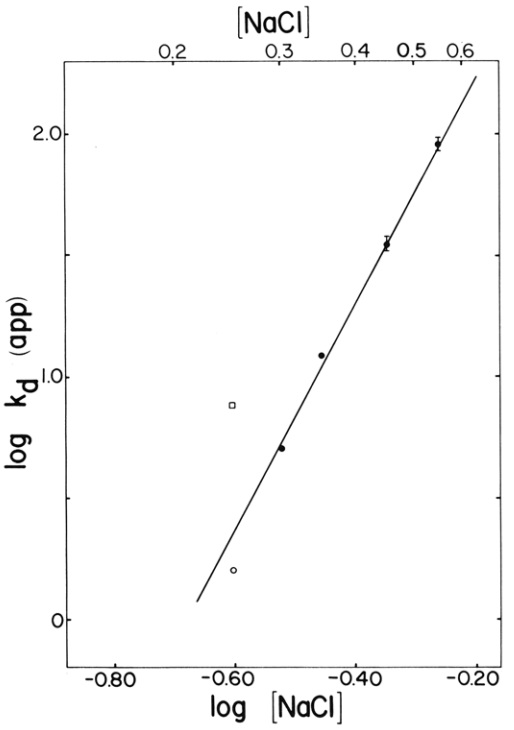

FIGURE 2: Apparent first-order dissociation rate constant [k_d(app)] resulting from a NaCl concentration jump plotted as a function of the final [NaCl] (log–log plot) for the gene 32 protein–poly(A) complex (unfractionated, initial $f_{sat} = 1$). The filled circles represent experiments resulting in complete dissociation and the open circles represent the two relaxation times observed for incomplete dissociation.

Our initial dissociation experiments were performed with homopolynucleotides as they were supplied by the manufacturer; i.e., we did not perform any size fractionation, and hence the population of nucleic acid molecules was extremely polydisperse. Dissociation of gene 32 protein from these polydisperse homopolynucleotide samples, induced by NaCl concentration jumps, results in a single-exponential decay, regardless of the initial f_{sat} of the polynucleotide (including $f_{sat} = 1$), although the magnitude of the rate constant depends on the initial f_{sat}. This is in contrast to polynucleotide samples which were first partially fractionated by passing them over a Sepharose 4B column and collecting the void volume, which eliminates very low molecular weight molecules from the population. Using the fractionated sample, one observes qualitatively different kinetic behavior when the polynucleotide is initially fully saturated ($f_{sat} = 1$) (see below), although when $f_{sat} < 1$, a single-exponential decay is observed as for the unfractionated polynucleotide sample. However, the actual value of k_d(app) is quite sensitive to the size distribution of the lattice, regardless of the initial f_{sat}.

Since we have used both fractionated and unfractionated nucleic acids in these studies, we will designate the state of the nucleic acid sample in the various experiments since the kinetic behavior does depend on the size distribution of the nucleic acid in most cases. This dependence is due to the highly cooperative nature of the binding of the T4 gene 32 protein.

Dissociation Rate Constant Is a Strong Function of the Bulk Salt Concentration. Figure 2 is a plot of the apparent first-order dissociation rate constant, k_d(app), vs. [NaCl] (log–log plot) for the g32p–poly(A) (unfractionated) complex (initial $f_{sat} = 1$). The filled circles represent [NaCl] jumps resulting in complete dissociation, whereas the unfilled circles

are jumps to [NaCl] which did not completely dissociate the complex. [The values of k_d(app) given by the unfilled symbols actually represent the reciprocal relaxation times, τ_f^{-1} and τ_s^{-1}, for the two exponential decays which are observed when complete dissociation of the complex is not achieved.] From Figure 2, one observes that the value of k_d(app) increases with increasing [NaCl] even when the minimum [NaCl] needed to achieve complete dissociation is exceeded (0.3 M NaCl in this case).

The least-squares line in Figure 2, which was calculated by using only the data points corresponding to complete dissociation of the unfractionated poly(A)–T4 g32p complex (f_{sat} = 1), is

$$\log k_d(\text{app}) = 4.7 \log [\text{NaCl}] + 3.17 \quad (1)$$

The dissociation rate constant is extremely salt dependent, increasing by almost a factor of 10^5 for every 10-fold increase in [NaCl]. Experiments with other *unfractionated* homopolynucleotides indicate similar dependences of k_d(app) on [NaCl] with log–log slopes in the range 4.2 ± 0.5 (see Figure 3). However, under identical conditions, the magnitude of k_d(app) varies considerably with the particular homopolynucleotide which is used to form the T4 g32p complex, as discussed below.

In contrast to the unfractionated nucleic acids, larger values of ($\partial \log k_d(\text{app})/\partial \log [\text{NaCl}]$) are found with homopolynucleotides which have been fractionated to eliminate low molecular weight species [see Lohman (1984)]. These fractionated homopolynucleotides yield slopes of ~6–7 at high [NaCl], which are comparable to the absolute values of ($\partial \log K\omega/\partial \log [\text{NaCl}]$) obtained by Kowalczykowski et al. (1981b) and Newport et al. (1981). The molecular interpretation of these salt dependences as well as the different [NaCl] dependences for fractionated vs. unfractionated nucleic acids will be discussed in the following paper (Lohman, 1984).

Dissociation Rate Constant Is Strongly Dependent upon the Nature of the Nucleic Acid. A series of experiments was carried out to test for specificity in the dissociation of T4 g32p from single-stranded nucleic acids. The dissociation rate constant of T4 g32p was measured via [NaCl] jumps as a function of increasing final [NaCl] for a series of *unfractionated* homopolynucleotides and wild-type bacteriophage M13 DNA. In all of these experiments, the lattice was initially fully saturated (f_{sat} = 1), and the initial [NaCl] ≤ 0.10 (buffer T, pH 8.1, 25.0 °C). The results are summarized in Figure 3, where one can see that the log–log slopes of k_d(app) vs. [NaCl] are fairly constant for the various nucleic acid lattices. However, there is a dramatic specificity associated with the nucleic acid lattice. In order of decreasing dissociation rate constant, the ranking is as follows:[1]

poly(C) > poly(A) > poly(U) ≫ poly(dA) ~ poly(ϵA) > poly(dC) ≫ ssM13 DNA > poly(dT)

In general, with the exception of poly(ϵA), T4 gene 32 protein dissociates more rapidly from RNA than from DNA. Certainly, when the corresponding RNA vs. DNA homopolynucleotide of a particular base are compared, the deoxyhomopolynucleotide complex dissociates considerably more slowly. The specificity ranking displayed in Figure 3 is identical with that observed by Newport et al. (1981) for the cooperative equilibrium constant, $K\omega$, with the exception of the position of M13 DNA. The GC contents of M13 DNA and ϕX174 DNA [used by Newport et al. (1981)] are not sufficiently different to account for this difference in the specificity ranking of $K\omega$ vs. k_d(app). As stated above, the qualitative behavior of the dissociation time course is strikingly different for homopolynucleotides vs. natural DNA (fd, M13, and denatured calf thymus) so that the comparison between M13 DNA and the homopolynucleotides may not be justified.

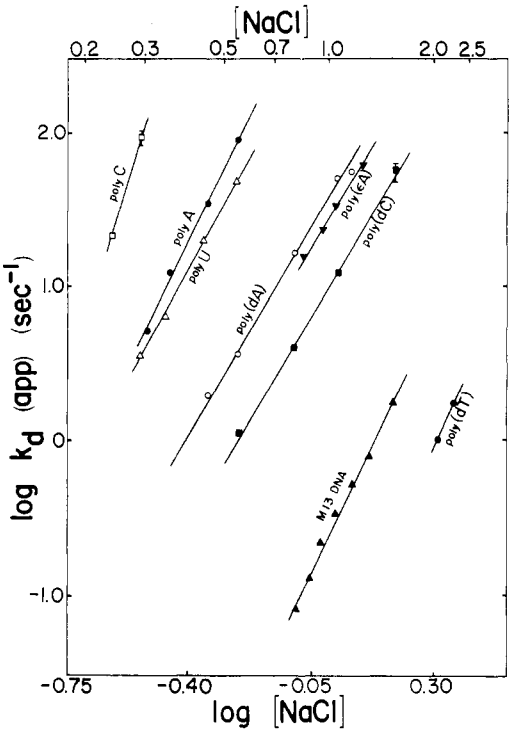

FIGURE 3: Apparent rate constant for gene 32 protein dissociation varies dramatically with the nucleic acid [log–log plot of k_d(app) vs. [NaCl]].

In addition to the qualitative similarity in the specificity ranking, the quantitative ratios of $K\omega$ for any two homopolynucleotides (Newport et al., 1981) at a given [NaCl] are quite similar to the ratios of k_d(app) that we measure. As a result, we conclude that the large majority of the binding specificity observed for T4 gene 32 protein resides in the vast differences in its dissociation rate constants for various nucleic acid lattices. Lohman & Kowalczykowski (1981) drew the same conclusion based on a study of the association kinetics of T4 gene 32 protein to homopolynucleotides.

Dissociation Rate Constant Is Dependent upon the Nature of the Anion. Kowalczykowski et al. (1981b) have observed a dependence of $K\omega$ for g32p on the nature of the low molecular weight anion which is present in the binding buffer. At constant [NaX] they observe that $K\omega$ decreases in the order $F^- > CH_3COO^- > Cl^-$. In order to determine whether this anion effect on $K\omega$ results from an effect on the dissociation or association rate constant, we performed the experiments shown in Figure 4. These were salt jump experiments with unfractionated poly(U), under initial conditions of f_{sat} = 1.0. Figure 4 indicates that at a constant [NaX], k_d(app) increases in the order $F^- < CH_3COO^- < Cl^-$. Furthermore, the log–log slopes of k_d(app) vs. log [NaX] also increase in the same order

[1] As discussed in the following paper (Lohman, 1984) the value of k_d(app) is dependent upon the average length of the nucleic acid lattice for a highly cooperative DNA binding protein such as T4 gene 32 protein. This variation could affect the apparent specificity ranking given above as determined by dissociation kinetics. The average lengths of the unfractionated polynucleotides estimated from their sedimentation coefficients do vary by less than a factor of 2 (from ~450 to 800 nucleotides). Therefore, the relative rankings given above should be valid with the possible exception of poly(A) vs. poly(U) and poly(dA) vs. poly(rϵA). The rate constants determined for these pairs are identical within a factor of 2; hence, one can only conclude that poly(A) has comparable specificity to poly(U) and poly(dA) is of comparable specificity to poly(rϵA).

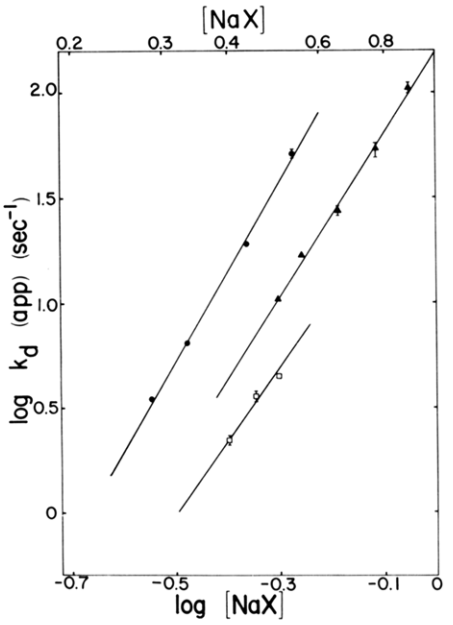

FIGURE 4: Dependence of k_d(app) on the anion for the gene 32 protein–poly(rU) dissociation (log–log plot): (●) log k_d(app) = 4.26 log [NaCl] + 2.85; (▲) log k_d(app) = 3.89 log [NaAc] + 2.19; (□) log k_d(app) = 3.5 log [NaF] + 1.74.

such that $\partial \log k_d(\text{app})/\partial \log [\text{NaX}] = 3.5$ for NaF, 3.9 for CH_3COONa, and 4.3 for NaCl. This behavior is very similar to that observed for $\partial \log K\omega/\partial \log [\text{NaX}]$.

Value of k_d(app) Depends on the Length Distribution of the Nucleic Acid. During the course of these studies, we performed dissociation experiments on several lots of the same polynucleotide. For all of these samples, a single-exponential decay was observed whenever the initial $f_{sat} < 1$. However, occasionally an experiment was undertaken with nucleic acid from a particular lot, under initially fully saturated conditions ($f_{sat} = 1$), where definite deviations from a single-exponential decay were observed. This deviation was not a demonstration of biphasic kinetics, since the apparent rate constant for the reaction seemed to increase at longer times rather than decrease as expected for multiphasic kinetics. This effect was quite reproducible for a given sample lot of homopolynucleotide. The largest deviation observed was for a sample of poly(dT) which had a sedimentation coefficient of $s_{20,w} \sim 7.5$ S. Figure 5 (top panel) shows the time course of dissociation for this sample of poly(dT) which was initially fully saturated with T4 g32p. This particular experiment was carried out in buffer T, pH 8.1, and involved a [NaCl] jump from 0.1 to 1.55 M. The time course shown in Figure 5 (top panel) displays a constant rate of dissociation of the g32p–poly(dT) complex over ~80% of the reaction. This is in dramatic contrast to the single-exponential behavior observed for this same poly(dT) sample when it is not fully saturated with g32p (i.e., $f_{sat} < 1$). This type of deviation from a single-exponential decay seemed to be correlated with the average size of the homopolynucleotide. We never observed this behavior with commercial poly(A) samples which had an average sedimentation coefficient of ~4.5 S, whereas we would occasionally observe it for poly(dA) and poly(dC) samples which had $s_{20,w} \sim 6$–6.5 S.

In order to test whether this deviation from a single-exponential decay was related to the size of the nucleic acid lattice, we fractionated our poly(A) sample using Sepharose 4B as described under Materials and Methods. We collected the poly(A) which came through in the void volume and used it for a series of dissociation experiments. The fractionated

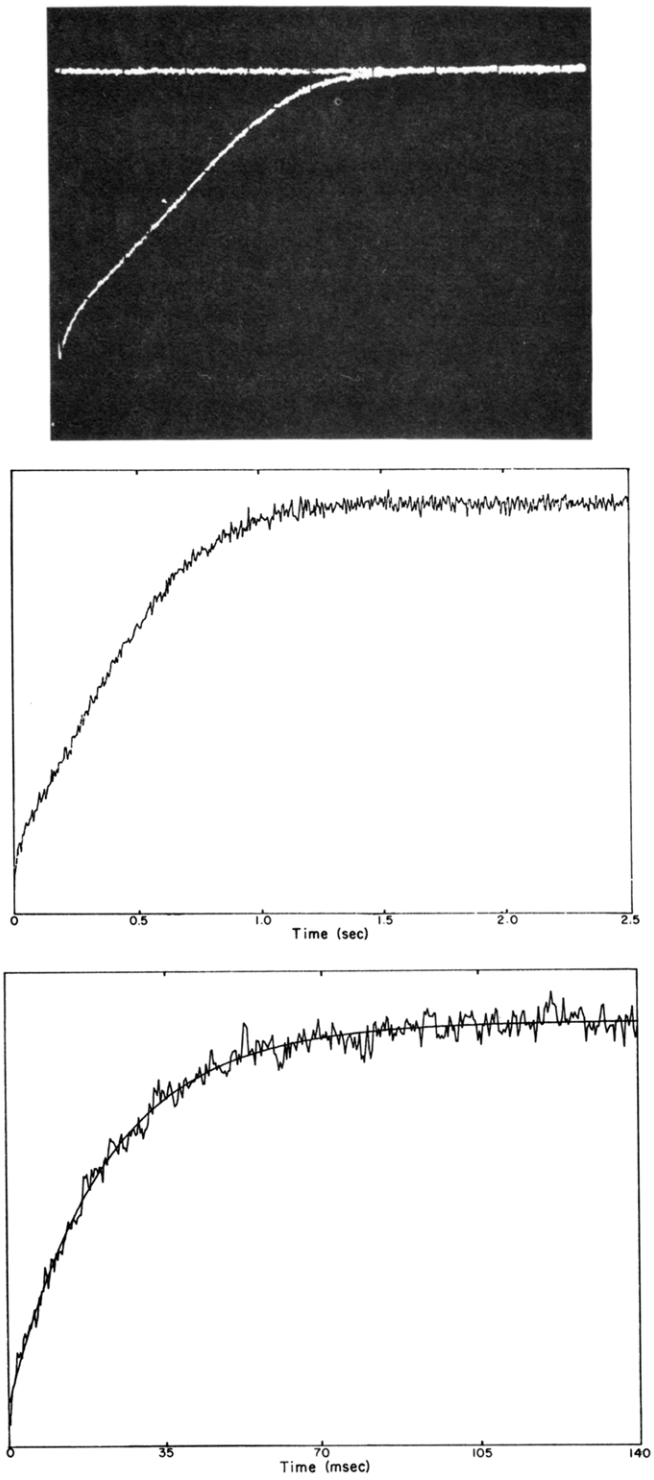

FIGURE 5: Time course of dissociation for gene 32 protein from high molecular weight nucleic acids ($f_{sat} = 1$) showing zero-order kinetic behavior. (Top) 7.5S poly(dT), $f_{sat} = 1$; middle) 9.9S poly(rA), Sepharose 4B fractionated, $f_{sat} = 1$; (bottom) 9.9S poly(rA) as in the middle panel, but $f_{sat} = 0.06$, 11.9 °C. The solid line represents a single-exponential fit to the data with k_d(app) = 45.7 s^{-1}.

poly(A) had an $s°_{20,w} = 9.9$ S as determined by boundary sedimentation velocity in 1.0 M NaCl, which corresponds to a length of 1000 ± 200 nucleotides, by using the studies of Eisenberg & Felsenfeld (1967) as a calibration.

When we used the 9.9S poly(A) in the dissociation experiments, the time courses were qualitatively similar to the 7.5S poly(dT) case. With $f_{sat} = 1$, a constant rate of dissociation (zero-order kinetics) was observed over 80–85% of the time course as shown in the middle panel of Figure 5. When this

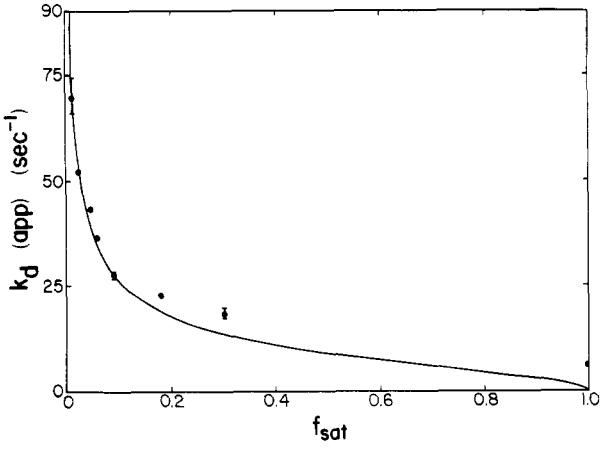

FIGURE 6: Dependence of k_d(app) on the initial fractional saturation (f_{sat}) of the nucleic acid [9.9S fractionated poly(rA)]. Dissociation was induced by [NaCl] jumps from 0.10 to 0.45 M (10 mM Tris, pH 8.2, and 0.1 mM EDTA, 25.0 °C).

same poly(A) sample was used in an experiment where the initial $f_{sat} < 1$, a single-exponential decay was observed as shown in the bottom panel of Figure 5. In addition, when two identical experiments were performed at the same initial f_{sat}, but with 9.9S poly(A) vs. *unfractionated* poly(A), the k_d(app) for the T4 g32p–9.9S poly(A) complex was significantly lower (by a factor of ~8) than for the *unfractionated* poly(A) complex. On the basis of the above we conclude that the observation of a constant rate of dissociation of T4 g32p from a nucleic acid lattice when $f_{sat} = 1$ (as in Figure 5, top and middle panels) is not dependent on the type of lattice (i.e., nucleotide) but rather is a function of the length of the lattice. Furthermore, zero order kinetics seem to be observed only when (i) the average length of the nucleic acid lattice is larger than a critical value and (ii) the population of small oligonucleotides is extremely low. When these two criteria are not met, apparent first-order kinetics are observed, even when $f_{sat} = 1$. Interestingly, the dissociation of T4 g32p from intact fully saturated M13 DNA (approximately 6400 nucleotides) does not display zero-order kinetics even though it clearly meets the two criteria listed above (T. M. Lohman, unpublished results). This may be related to an effect of some very stable secondary structure in M13 single-stranded DNA which remains even in the presence of the gene 32 protein.

As mentioned above, an additional effect of fractionating the nucleic acid is an increase in the salt dependence of k_d(app) from 4.2 to approximately 6–7. The reasons for this are not fully understood, but possibilities are discussed in the following paper (Lohman, 1984).

Dependence of the Dissociation Rate Constant on Initial Fractional Saturation of the Nucleic Acid. By use of the fractionated 9.9S poly(A), a series of dissociation experiments were performed as a function of the initial fractional saturation (f_{sat}). These were [NaCl]-induced dissociations starting at 0.1 M NaCl and performed at constant T4 g32p concentration (0.2 μM). The range of initial f_{sat} was achieved by varying the input concentration of poly(A). The values of k_d(app) as a function of f_{sat} are shown in Figure 6 for [NaCl] jumps from 0.10 to 0.45 M. Since the time course for the $f_{sat} = 1$ experiment is not a single exponential, rather it is zero order, the value of 6 s^{-1} given in Figure 6 for k_d(app) at $f_{sat} = 1$ was calculated by using $k_d(\text{app}) = (1/[\text{PD}]_{total})\, d[\text{PD}]/dt$; that is, the constant rate of dissociation (M s^{-1}) was divided by the initial bound protein concentration. Hence, this value of k_d(app) at $f_{sat} = 1$ is not simply related to the values of k_d(app) for the $f_{sat} < 1$ data which were obtained by fitting the time course to a single-exponential decay with time constant, $\tau = 1/k_d(\text{app})$.

The data in Figure 6 are indicative of the dissociation of a cooperatively bound protein–nucleic acid complex, by virtue of the increase in k_d(app) as the initial f_{sat} decreases. The values of k_d(app) range from 6 s^{-1} at $f_{sat} = 1$ to 70 ± 5 s^{-1} at 1.12% saturation of the poly(A) (25.0 °C, 0.45 M NaCl, pH 8.1). Over the range from 100% to 18% saturation, k_d(app) varies by ~3.8-fold, whereas below 18% saturation, the variation in k_d(app) is much more dramatic. Qualitatively, this dependence of k_d(app) on f_{sat} is due to the increase in the number of protein clusters and hence cluster ends as the initial binding density decreases. As we show below, the data fit a model in which the major pathway for dissociation is via singly contiguous proteins from the cluster ends (Lohman, 1983). Hence, as the fraction of cluster ends increases upon decreasing the initial f_{sat} (at the start of the dissociation), then k_d(app) also increases. This same qualitative behavior was observed with all of the homopolynucleotides we tested which included poly(C), poly(U), poly(A), poly(ϵA), poly(dC), poly(dA), poly(dT), and M13 DNA.

Analysis To Obtain the Dissociation Rate Constant from a Cluster End, k_e. The dissociation data presented above and that obtained for all of the homopolynucleotides studied support the following model for the irreversible dissociation of cooperatively bound T4 gene 32 protein–single-stranded homopolynucleotide complexes at high [NaCl]: (1) The dominant mode of dissociation is that of singly contiguously bound protein from the ends of protein clusters. (2) Each protein cluster (M_q), regardless of its size, provides two sites for dissociation, by virtue of its two ends (q denotes the number of g32p monomers in a cluster, M_q). (3) Doubly contiguously bound g32p does not dissociate within the time course of the experiment. (4) Isolated, bound gene 32 protein is not observed as a dissociable species for two reasons: (i) isolated molecules are not present under most of the initial binding densities of the experiments, since ω is large (10^3–10^4); (ii) even when the fraction of bound protein which is present as isolated molecules is not negligible (e.g., at ~1% saturation, approximately 10% of the bound protein is present as isolated molecules), these molecules dissociate within the dead time of the stopped-flow experiment (~2.5 ms) and hence are not observed. (5) No redistribution of bound T4 gene 32 protein occurs during the dissociation process. Thus, large clusters do not break up into smaller bound clusters, and hence, an increase in cluster ends does not occur during dissociation. This conclusion is justified by our observation of zero-order kinetics in the dissociation experiments from fractionated homopolynucleotides when $f_{sat} = 1$.

The quantitative aspects of this model have been described in detail in an analysis which also accounts for the overlap of potential protein binding sites on the nucleic acid (Lohman, 1983). The resulting equations which are used in the analysis of the T4 g32p dissociation experiments are given below. These equations describe the entire dissociation time course for the model outlined above, which is valid for high cooperativity, $\omega > \sim 500$ (Lohman, 1983).

Initial Conditions: $f_{sat} < 1$. Under conditions where the dissociation of isolated protein is negligible (high ω and/or moderate binding density), the time course for dissociation of bound protein PD is described by

$$-d[\text{PD}]/dt = 2k_e(1 - p_0)[\text{PD}] \qquad (2)$$

where k_e is the rate constant for dissociation of a singly contiguously bound protein (Lohman, 1983). The term $1 - p_0$ represents the fraction of cluster ends which are present before

dissociation of the complex and is given by

$$1 - p_0 = \frac{(n-1)\nu - 1 + R}{2\nu(\omega - 1)} \quad (3)$$

where the notation is that of McGhee & von Hippel (1974) such that n = the number of nucleotides occluded by the protein ($n = 7$ for gene 32 protein), ν = the binding density, and $R = [(1 - (n+1)\nu)^2 + 4\omega\nu(1 - n\nu)]^{1/2}$.

Equation 2 indicates that one should observe a single-exponential decay with initial binding densities such that $f_{sat} < 1$. This behavior results from the fact that cluster ends only disappear when a cluster of size $q = 2$ (M_2) dissociates, and the time dependence of the concentration of clusters of any size ($q > 2$) is given by a single-exponential decay (Lohman, 1983). Since there is always some finite concentration of M_2 clusters in the distribution, when $f_{sat} < 1$ (even when $\omega = 10^3$–10^4), cluster ends are eliminated in an exponential fashion as soon as the irreversible dissociation begins. From eq 2 we see that the apparent dissociation rate constant for this model is given by

$$k_d(\text{app}) = 2k_e(1 - p_0) \quad (4)$$

so that k_e can be obtained from the slope of a plot of k_d(app) vs. $(1 - p_0)$.

Initial Conditions: $f_{sat} = 1$. When the nucleic acid lattice is fully saturated with a cooperatively bound protein and the cooperativity parameter, ω, is sufficiently large as in the case of the T4 gene 32 protein, a constant rate of dissociation (i.e., zero-order kinetics) is predicted if dissociation occurs only via singly contiguously bound molecules. The expression for the dissociation time course is (Lohman, 1983)

$$-d[PD]/dt = 2k_e[M_N]_0 \quad (5)$$

where $[M_N]_0$ is the concentration of protein clusters at time zero. Each cluster contains a maximum number of protein molecules, N, which is determined by the size of the nucleic acid lattice. Since the entire nucleic acid is saturated, at time $t = 0$, $[M_N]_0$ is simply the concentration of nucleic acid molecules, each molecule containing one protein cluster with two cluster ends. The rate $-(d[PD]/dt)$ is constant as eq 5 indicates, since all of the protein clusters are large when $f_{sat} = 1$, and when one protein dissociates from a cluster end, no change in the concentration of ends occurs. Cluster ends only begin to be depleted when clusters of size M_2 (i.e., dimers) are formed and subsequently dissociate; this does not occur until the majority of the protein has dissociated. The data for the $f_{sat} = 1$ case, shown in Figure 5, indicate a constant rate of dissociation for 80–85% of the time course. Deviation from zero-order kinetics only occurs in the final 15–20% of the dissociation for the poly(dT) and poly(A) samples used in this study. Of course, the persistence of a constant rate (zero-order kinetics) is dependent on the length of the nucleic acid used, since this will determine the initial fraction of cluster ends. Higher molecular weight samples of nucleic acid will have a lower fraction of cluster ends when fully saturated with protein; hence, a constant dissociation rate will be observed for a greater percentage of the time course.

The molecular rate constants for dissociation of singly contiguously bound T4 gene 32 protein can be estimated from the data by using eq 2 (when $f_{sat} < 1$) and 5 ($f_{sat} = 1$). In Figure 7 we have replotted the binding density dependence data of Figure 6 as k_d(app) vs. $1 - p_0$, using $n = 7$ and $\omega = 5 \times 10^3$ to calculate values of $1 - p_0$. A reasonable fit to a straight line with zero intercept is observed as predicted by eq 2 and 4. The slope of the line in Figure 7 equals $2k_e$, and a value of $k_e = 126 \pm 8$ s^{-1} is calculated for the dissociation

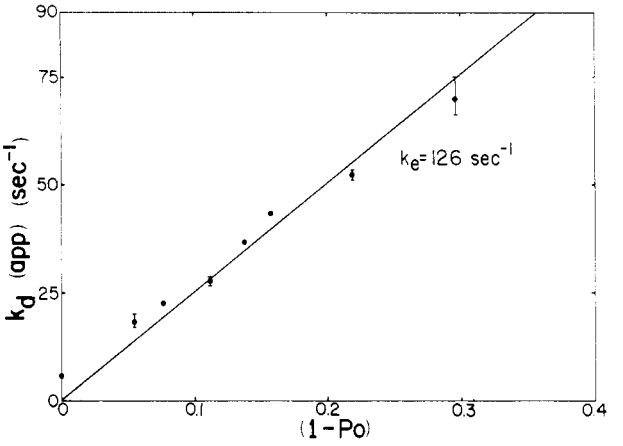

FIGURE 7: Data from Figure 6 have been replotted as k_d(app) vs.1 $- p_0$ according to eq 4 in the text.

of a singly contiguously bound T4 g32p from poly(A) in 0.45 M NaCl (pH 8.1, 25 °C).

It is apparent in Figure 7 that the dissociation rate constant under initially fully saturated conditions is well above the zero intercept. This results from the fact that the theoretical treatment used in the derivation of eq 2 assumes an infinitely long nucleic acid lattice. A fully saturated infinitely long lattice has no ends, and hence $1 - p_0 = 0$. However, the poly(A) used in our experiments, although fractionated and possessing an average length of ~1000 nucleotides, is not infinitely long. The nonzero value of k_d(app) (6 s^{-1}) when $f_{sat} = 1$ results from the small fraction of singly contiguously bound T4 gene 32 protein which is present due to the finite length of the nucleic acids in the population. As the initial binding density is decreased, the contribution to k_d(app) resulting from the finite length of the poly(A) becomes insignificant, and the theory is in better agreement with experiment.

Another independent estimate of k_e for the T4 g32p–poly(A) dissociation in 0.45 M NaCl can be obtained from the dissociation of the fully saturated poly(A) lattice in which zero-order kinetics are observed. In order to calculate k_e from the data in Figure 5 (top and middle panels), using eq 5, we must estimate $[M_N]_0$, which is the concentration of nucleic acid lattices and also the concentration of protein clusters, since $f_{sat} = 1$. An estimate of $[M_N]_0$ is

$$[M_N]_0 = \frac{[\text{nucleotide}]}{\langle\text{length}\rangle} = \frac{n[PD]_0}{\langle\text{length}\rangle} \quad (6)$$

where ⟨length⟩ represents the average length of the nucleic acid lattice, n is the site size of the T4 gene 32 protein, and $[PD]_0$ is the initial concentration of bound protein. Upon insertion of eq 6 into eq 5 and rearranging, we obtain

$$\frac{1}{[PD]_0}\left(\frac{-d[PD]}{dt}\right) = k_d(\text{app}) = \frac{2nk_e}{\langle\text{length}\rangle} \quad (7)$$

For the poly(A) experiment shown in the middle panel of Figure 5, k_d(app) = 6 s^{-1}, $n = 7$, and ⟨length⟩ = 1000, from which we calculate $k_e = 429$ s^{-1}, at 0.45 M NaCl (pH 8.1, 25.0 °C). This value for k_e is a factor of 3.4 higher than the value of $k_e = 126$ s^{-1} which was calculated from k_d(app) as a function of binding density. More than likely, this reflects the polydispersity of the poly(A) sample. Even though the poly(A) had been fractionated to eliminate fairly small molecules, it is still not monodisperse and contains more molecules than we estimate on the basis of its average length as determined from its $s^\circ_{20,w}$. Certainly the value of $k_e = 126$ s^{-1} is more reliable since it is based on data over a range of binding densities where

Table I: Rate Constant for Dissociation of Singly Contiguous Protein

nucleic acid	final [NaCl] (M)	k_e (s^{-1})	ω
poly(A) fractionated	0.40	36	10^3
poly(A) fractionated	0.40	73	5×10^3
poly(A) fractionated	0.45	66	10^3
poly(A) fractionated	0.45	126	5×10^3
poly(A) fractionated	0.50	103	10^3
poly(A) fractionated	0.50	214	5×10^3
poly(A) fractionated	0.55	183	10^3
poly(A) fractionated	0.55	383	5×10^3
poly(U) unfractionated	0.34	63	10^3
poly(U) unfractionated	0.34	104	5×10^3
poly(dA) unfractionated	0.45	6.4	10^3
poly(dA) unfractionated	0.45	12.8	5×10^3
poly(dA) unfractionated	0.55	22.4	10^3
poly(dA) unfractionated	0.55	44.6	5×10^3
poly(dT) unfractionated	2.03	18.9	10^3
poly(dT) unfractionated	2.03	33.6	5×10^3

errors due to the finite size of the lattice are less important.

Table I lists values of k_e for a few homopolynucleotides obtained from an analysis of the binding density dependence of k_d(app) by using eq 4. The value of k_e which is estimated from the data is sensitive to the value of ω used to calculate the cluster distribution and hence $1 - p_0$. Values of $\omega = 10^3$ and 5×10^3 were used for the calculations in Table I since these cover the range of estimates of ω for gene 32 protein. This uncertainty in ω (although not great) contributes substantially to the uncertainty in our estimates of the value of k_e when eq 4 is used, since ω is needed to calculate $1 - p_0$. For example, the same poly(A) data shown in Figure 7 also yields reasonable straight lines when $1 - p_0$ is calculated by using values of $\omega = 10^4$ or 10^3. The calculated values for k_e from these plots are 66 ($\omega = 10^3$) and 168 s^{-1} ($\omega = 10^4$). Hence, the uncertainty in ω limits the confidence in our estimations of k_e, since the calculated cluster distribution is so sensitive to the value of ω.

Discussion

Major Pathway of Dissociation of Cooperatively Bound T4 Gene 32 Protein Is via the Ends of Protein Clusters. The irreversible dissociation kinetics of gene 32 protein–single-stranded homopolynucleotide complexes, resulting from a NaCl concentration jump, display a single-exponential decay when the gene 32 protein fluorescence is monitored and the nucleic acid is initially not fully saturated (see Figure 1). This behavior is quite different from the biphasic dissociation kinetics observed for complexes of gene 32 protein–single-stranded fd DNA (Peterman & Wu, 1978) and M13 DNA (T. M. Lohman, unpublished results). Since it has been shown that T4 gene 32 protein binds cooperatively to single-stranded homopolynucleotides (Kowalczykowski et al., 1981a,b) as well as natural single-stranded DNA, it is likely that the more complex dissociation kinetics observed with natural DNA result from influences of secondary structure. In order to simplify the interpretation of these kinetics experiments, only the synthetic homopolynucleotides have been used in this study. When the homopolynucleotides are initially fully saturated with T4 g32p, a constant rate of dissociation (zero-order kinetics) is observed upon complete dissociation via a salt jump (see Figure 5, top and middle panels). These kinetics are dramatically different than those observed when $f_{sat} < 1$. All of the stopped-flow data presented here indicate that the only cooperatively bound species which dissociate are singly contiguous proteins from the ends of protein clusters. Furthermore, protein clusters which are preformed at low (≤0.10 M NaCl) do not redistribute along the nucleic acid upon jumping to a higher salt concentration nor do they redistribute substantially during the dissociation process. The time courses of dissociation both when the nucleic acid is initially fully saturated ($f_{sat} = 1$) and when $f_{sat} < 1$ are well described by a model which assumes only singly contiguous and isolated protein molecules can dissociate (Lohman, 1983). When cooperativity is high, as in the case of T4 gene 32 protein, a single exponential is observed when $f_{sat} < 1$, since very few isolated molecules are formed. From an experimental point of view, the dissociation of isolated protein molecules occurs within the dead time of a stopped-flow experiment, and hence, they are not observed even when they represent a reasonable fraction of the population. The conclusion that no substantial redistribution of cooperatively bound T4 gene 32 protein occurs and that doubly contiguous molecules do not dissociate from the interior of clusters comes from the observation of a constant rate of dissociation (zero-order kinetics) of protein when $f_{sat} = 1$ (and the nucleic acid is sufficiently long). This observation of zero-order kinetics is the most striking evidence in support of the proposed mechanism as discussed by Lohman (1983). If redistribution of bound protein occurred or if the dissociation of doubly contiguously bound protein occurred to a measureable degree, then zero-order kinetics would not be observed under any conditions. In the limit of an infinitely fast redistribution such that lattice equilibrium is maintained throughout the course of dissociation, one predicts that the rate of dissociation will constantly increase throughout the time course. This would be due to the breakup of large protein clusters into smaller ones with the concomitant increase in the fraction of molecules contained in cluster ends (Epstein, 1979; Lohman, 1983). This is clearly not observed in our studies of the T4 gene 32 protein.

Balazs & Epstein (1984) have recently shown that deviations from single-exponential behavior are observed when the dissociation of doubly contiguous molecules occurs in addition to singly contiguous protein. They have shown that the contribution due to the dissociation of doubly contiguous protein is only significant at high binding densities in systems with low values of the cooperativity parameter ($\omega < 100$), which supports the conclusions reached in this study for gene 32 protein which has $\omega > 10^3$.

The observation of zero-order kinetics when $f_{sat} = 1$ is a good indicator for the mechanism proposed here. However, this behavior can be masked if a polydisperse sample of nucleic acid is used. Therefore, it is very important to fractionate samples of homopolynucleotides and use only the high molecular weight fraction in order to detect zero-order kinetics under saturating protein conditions. This is because a polydisperse sample of nucleic acid contains some fraction of fairly short molecules, so that, even under saturating protein conditions, small protein clusters are present due to the short length of these nucleic acid molecules. As a result, upon dissociation, these small clusters are lost sooner than the longer clusters and a change in cluster end concentration occurs. This will produce near-exponential behavior rather than a constant rate of dissociation and therefore may lead to incorrect conclusions about the mechanism of dissociation.

Nucleic Acid Specificity of T4 Gene 32 Protein Resides in the Dissociation Rate Constant. As is obvious from Figure 3, there is a tremendous variation in the dissociation rate constant of g32p from the several nucleic acids which have been studied. Of the ones we have studied, dissociation is fastest from poly(C) and slowest from poly(dT); the rate constants differ by approximately 5×10^5 at a given salt

Table II: Nucleic Acid Specificity Is Found in the Dissociation Rate Constant

nucleic acid	[NaCl] (M)[a]	nucleic acid	[NaCl] (M)[a]
poly(C)	0.22[b]	poly(rεA)	0.76[b]
poly(A)	0.34	poly(dC)	1.01[b]
poly(U)	0.37	M13 DNA	2.22[b]
poly(dA)	0.71	poly(dT)	3.29[b]

[a] Values for which k_d(app) = 10 s^{-1}. [b] These values are extrapolations to k_d(app) = 10 s^{-1}.

concentration. Another way of comparing these is to calculate the salt concentration at which the apparent dissociation rate constant, k_d(app), equals 10 s^{-1}. These are listed in Table II. The [NaCl] necessary to attain k_d(app) = 10 s^{-1} ranges from 0.22 M for poly(C) to 3.3 M for poly(dT).

The specificity ranking that appears in Figure 3 is identical with that found for $K\omega$ by Newport et al. (1981). Furthermore, within a factor of 2, the ratio of $K\omega$ for two polynucleotides at a given [NaCl] is equal to the reciprocal of the ratio of k_d(app) for the same two polynucleotides. On the basis of this, as well as previous evidence that the bimolecular rate constant, k_1 = (3–4) × 10^6 M^{-1} (nucleotide) s^{-1} is independent of the type of polynucleotide (Lohman & Kowalczykowski, 1981), we conclude that the observed specificity is entirely in the dissociation rate constant, k_{-1}. Additional evidence in support of this is the anion dependence of k_d(app) shown in Figure 4, which indicates the same dependence of $1/k_d$(app) on the anion as seen for $K\omega$ by Kowalczykowski et al. (1981a,b). This is thought to reflect differential anion binding by the T4 gene 32 protein when it is free and bound, but the details of this effect have not been investigated.

We conclude the following from these studies: (1) Cooperatively bound T4 gene 32 protein dissociates only from the ends of protein clusters under the conditions of our experiments (high [NaCl]). (2) No major redistribution of bound protein occurs during the course of dissociation. (3) The model given by Lohman (1983) for the irreversible dissociation of cooperatively bound protein (valid for high ω) describes the T4 gene 32 protein data quantitatively, including the observation of zero-order kinetics when f_{sat} = 1. One can obtain quantitative estimates of k_e, the rate constant for removal of a singly contiguous protein from a cluster end, through analysis of the dependence of k_d(app) on the initial binding density [see eq 4 and Lohman (1983)].

In a previous analysis of the dissociation of T4 gene 32 protein–single-stranded fd DNA, Peterman & Wu (1978) conclude that the biphasic kinetics which they observe is a result of the cooperative nature of the interaction. This is clearly not the case since we observe single-exponential behavior when single-stranded homopolynucleotides are used as the lattice. At this point we are not certain of the origin of the biphasic dissociation time courses when natural DNA is used. Peterman & Wu (1978) did analyze their g32p–fd DNA dissociation data by considering only the initial part of the dissociation, rather than the entire time course as we do here, and also concluded that cooperatively bound gene 32 protein dissociates mainly from cluster ends. However, their analysis to obtain estimates of k_e does not account for the overlap of potential protein binding sites on the nucleic acid. Schwarz & Watanabe (1983) have recently given an analysis of the irreversible dissociation kinetics of cooperatively bound ligand–DNA interactions. Although different in form, their equation yields numerically equivalent results to the treatment of Lohman (1983) for f_{sat} < 1 and high ω.

In our discussions we have referred to the rate constant for removal of a protein from a cluster end as k_e rather than k_{-1}/ω. If the singly contiguous protein dissociates directly into solution, then $k_e = k_{-1}/\omega$; however, if an intermediate or alternate pathway is involved, then k_e may not be equal to k_{-1}/ω. This possibility is discussed further in the following paper (Lohman, 1984) which investigates the effects of solution variables and temperature on the dissociation rate, under conditions which more closely approximate those found in vivo.

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Kinetics and Mechanism of Dissociation of Cooperatively Bound T4 Gene 32 Protein–Single-Stranded Nucleic Acid Complexes. 2. Changes in Mechanism as a Function of Sodium Chloride Concentration and Other Solution Variables†

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ABSTRACT: The dissociation kinetics of bacteriophage T4 coded gene 32 protein–single-stranded nucleic acid complexes have been examined as a function of monovalent salt concentration, temperature, and pH in order to investigate the details of the dissociation of cooperatively bound protein. Fluorescence stopped-flow techniques were used, and irreversible dissociation was induced by a combination of [NaCl] jumps and mixing with excess nucleic acid competitor. This made it possible to directly investigate the irreversible dissociation process over a wide range of NaCl concentrations [e.g., from 50 mM to 0.60 M for the gene 32 protein–poly(A) complex], in the absence of reassociation. Over the entire salt range, the only dissociable species observed is the singly contiguously bound gene 32 protein which dissociates from the ends of protein clusters. However, the [NaCl] dependence of the dissociation rate constant suggests that two competing pathways exist for dissociation of cooperatively bound gene 32 protein from the ends of protein clusters. At high monovalent salt concentrations, dissociation is dominated by a single-step process, with $\partial \log k_e / \partial \log [NaCl] = 6.5 \pm 0.5$; i.e., the dissociation rate constant increases with increasing NaCl concentration due to the uptake of approximately six monovalent ions upon dissociation. This indicates that singly contiguous protein dissociates directly into solution. However, at much lower [NaCl] the data suggest that gene 32 protein, when bound at the end of a protein cluster, dissociates by first sliding off the end to form a noncooperatively bound intermediate which subsequently dissociates. A quantitative model which incorporates the sliding pathway [Berg, O. G., Winter, R. B., & von Hippel, P. H. (1981) *Biochemistry 20*, 6929–6948] in the dissociation mechanism fits the data reasonably well and suggests that noncooperatively bound monomers of gene 32 protein may be capable of one-dimensional translocation along single-stranded nucleic acids as suggested by independent kinetic data on the association reaction [Lohman, T. M., & Kowalczykowski, S. C. (1981) *J. Mol. Biol. 152*, 67–109]. It is also observed that both the absolute dissociation rate constant for T4 gene 32 protein and its salt dependence are sensitive to the average molecular weight and polydispersity of the nucleic acid sample used. This is a general phenomenon exhibited by proteins that bind to nucleic acids in a highly cooperative manner.

The kinetics of protein–nucleic acid interactions are likely to be important in the regulation of gene expression at many levels including transcriptional and translational control. Recombination, repair, replication, and other processes involving transient intermediates are also certain to be influenced by kinetic aspects of the interactions of particular proteins with nucleic acids although the details of these kinetic questions have not been addressed in most cases. In addition to obtaining information concerning how fast a particular protein or other ligand may bind to or dissociate from its nucleic acid target, it is important to know the mechanism by which these events occur since this information can be used to infer what motions and movements particular proteins are capable of undergoing while in the vicinity of the DNA or when actually associated with the DNA.

One kinetic aspect of particular interest is the mobility of proteins while they are bound to nucleic acids. Clearly enzymes whose functions require processive action such as DNA and RNA polymerases and helicases (Geider & Hoffman-Berling, 1981) must possess the ability to translocate along the nucleic acid while remaining "bound" in some sense although the molecular aspects of this motion are unknown. There is recent evidence that proteins which are not involved in processive processes and are not ATPases also appear to be able to undergo a quite rapid, random one-dimensional translocation along DNA. The most convincing evidence for such motion has been obtained in the case of the *Escherichia coli lac* repressor which seems able to translocate or "slide" while in its nonspecific binding mode with a pseudo-one-dimensional diffusion coefficient of $\sim 10^{-9}$ cm^2 s^{-1}, which corresponds to an average rate of $\sim 10^3$ base pairs s^{-1} in one direction (Riggs et al., 1970; Berg & Blomberg, 1978; Barkley,

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