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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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1981; Winter et al., 1981). Investigations of other protein–nucleic acid interactions have revealed evidence suggesting that one-dimensional translocation may be a kinetic feature of *E. coli* RNA polymerase (Belintsev et al., 1980; Park et al., 1982) as well as monomers of the bacteriophage T4 gene 32 protein on single-stranded nucleic acids (Lohman & Kowalczykowski, 1981) and the filamentous phage gene 5 protein on single-stranded nucleic acids (Pörschke & Rauh, 1983). Preliminary evidence also suggests that the *EcoRI* restriction endonuclease may have this ability to translocate (Jack et al., 1982).

Although the effects of many variables must be investigated in any in vitro study of the kinetics of protein–nucleic acid interactions, the variable which usually has the most dramatic effect on the rates of interaction is the ionic composition of the solution. The large effects of the bulk monovalent salt concentration on both the kinetic and equilibrium properties of protein–nucleic acid interactions have long been recognized; however, only recently has a molecular interpretation of these dramatic salt effects been achieved (Record et al., 1976, 1978; Lohman et al., 1978) based on the counterion condensation hypothesis of Manning (1969, 1978). The effects of changes in the monovalent and/or polyvalent cation concentration can be quite useful as a diagnostic tool to decipher possible kinetic mechanisms (Lohman et al., 1978; Berg & Blomberg, 1978; Barkley, 1981; Winter et al., 1981; Lohman & Kowalczykowski, 1981; Roe et al., 1984), although information on the salt dependence of the equilibrium binding constant is necessary to draw firm conclusions. Even in the absence of a molecular interpretation, it is necessary to monitor the effects of the salt concentration if one wishes to use in vitro data to infer the in vivo reaction rates, since the in vivo ionic environment, estimated to be equivalent to ~0.20 M KCl in *E. coli* (Kao-Huang et al., 1977), is often much higher than the salt concentrations used in most in vitro experiments.

In the preceding paper (Lohman, 1984), the dissociation kinetics of bacteriophage T4 gene 32 protein–single-stranded homopolynucleotide complexes were investigated in high, nonphysiological NaCl concentrations. From a quantitative analysis of the data, it was concluded that the major mode of dissociation is via singly contiguous proteins from the ends of protein clusters, and molecular rate constants for this process were extracted by using an analysis based on this model (Lohman, 1983). In the work presented here the dissociation kinetics are investigated under a wide range of conditions ([NaCl], temperature, and pH) in order to probe the process under conditions which more closely approximate the physiological environment. A systematic study of the variation of the dissociation rate constants as a function of [NaCl] is used as a means of probing the mechanism of protein dissociation from cluster ends. Due to direct biding of ions to the protein and nucleic acid and the subsequent release of ions upon formation of a protein–nucleic acid complex, both the equilibrium and kinetic properties of these complexes are extremely sensitive to the bulk salt concentration (Record et al., 1976, 1978; Manning, 1978, Lohman et al., 1978). As a result, the measurement of observed rate constants as a function of [NaCl], in conjunction with appropriate equilibrium data, can be a useful tool in deciphering the mechanism of a protein–nucleic acid interaction. This approach is used in the present study of the T4 gene 32 protein (g32p) kinetics and is a powerful supplement to more standard investigations. Much information on the mechanism of protein–nucleic acid interactions can be obtained by using the salt concentration as a probe since when multiple steps or competing pathways are involved, these can be easily identified if their rate constants vary differently with salt concentration.

Both the equilibrium binding properties as well as the association kinetics of T4 gene 32 protein–single-stranded homopolynucleotide complexes have been well studied, including the effects of [NaCl] on the binding and kinetic constants. Kowalczykowski et al. (1981) and Newport et al. (1981) have shown that the cooperative equilibrium constant $K\omega$ varies with NaCl concentration as $\partial \log K\omega / \partial \log [NaCl] = -6.5 \pm 1$. From a study of the association kinetics, Lohman & Kowalczykowski (1981) have shown that the noncooperative association rate constant, k_1, is essentially independent of salt concentration. With these studies and the preceding paper (Lohman, 1984) as background, the dissociation kinetics, and in particular their dependence on monovalent salt concentration, are used in this work to study the mechanism of interaction of the T4 gene 32 protein with single-stranded homopolynucleotides over a wide range of solution conditions. These studies provide a more direct means of probing the translocation ability of gene 32 protein monomers along single-stranded nucleic acids which was suggested by studies of the association kinetics (Lohman & Kowalczykowski, 1981).

Materials and Methods

All reagents, buffers, and procedures are essentially as described in the preceding paper (Lohman, 1984). Any differences are explained under Results.

Results

As shown in the previous paper (Lohman, 1984) the effects of size fractionation of the homopolynucleotide on both the qualitative and quantitative features of the gene 32 protein dissociation time course are quite dramatic when the nucleic acid is initially fully saturated with protein. The [NaCl] dependence of k_d(app) is also influenced by the length distribution of the nucleic acid population. This is demonstrated in Figure 1, where k_d(app) is plotted vs. the final [NaCl] for two sets of salt-induced dissociation experiments at $f_{sat} = 1$: unfractionated poly(A) and Sepharose 4B fractionated 9.9S poly(A). In addition to the larger values of k_d(app) with unfractionated poly(A) (due to a larger fraction of nucleic acid ends), the [NaCl] dependence is significantly less steep for the unfractionated poly(A). The log–log slope increases from 4.7 ± 0.3 to 6.6 ± 0.3 in Figure 1 when fractionated 9.9S poly(A) is used. The latter slope is equal to the absolute value of the [NaCl] dependence of $K\omega$ (Kowalczykowski et al., 1981; Newport et al., 1981). The basis for this change in slope is not fully understood; however, it may be due to increased binding by gene 32 protein to the ends of the polynucleotide in the unfractionated poly(A) case, since this mode of binding is less [NaCl] dependent than binding to the interior of the polynucleotide (Kowalczykowski et al., 1981). Alternatively, the lower slope may reflect curvature in the log–log plot which is not apparent over the range of [NaCl] used in the unfractionated poly(A) experiments. Curvature in the log–log plots is apparent in the 9.9S fractionated poly(A) experiments at low initial f_{sat} as shown in Figure 2. For both $f_{sat} = 0.09$ and $f_{sat} = 0.045$, the log–log plot is distinctly nonlinear. The extremes of the slopes are ~7 between 0.6 and 0.55 M NaCl and ~4 between 0.45 and 0.4 M NaCl for both $f_{sat} = 0.09$ and $f_{sat} = 0.045$. It is possible that the extreme polydispersity in the unfractionated poly(A) sample in Figure 1 may mask the curvature that is observed for the 9.9S poly(A) at low f_{sat}. In any event, there is a clear quantitative difference in the salt dependences for dissociation of fractionated and unfractionated poly(A)–gene 32 protein complexes. When cooperatively binding proteins are investigated, it is important that this length

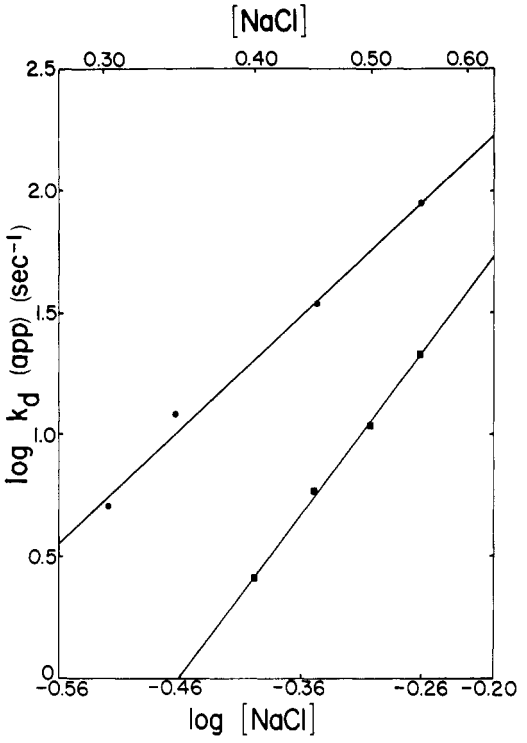

FIGURE 1: Comparison of the [NaCl] dependences of the dissociation rate constant for gene 32 protein–poly(A) complexes, which are initially fully saturated (log–log plot): (●) unfractionated poly(A) (~4.5 S), slope = 4.7; (■) Sepharose 4B fractionated poly(A) (9.9 S), slope = 6.6.

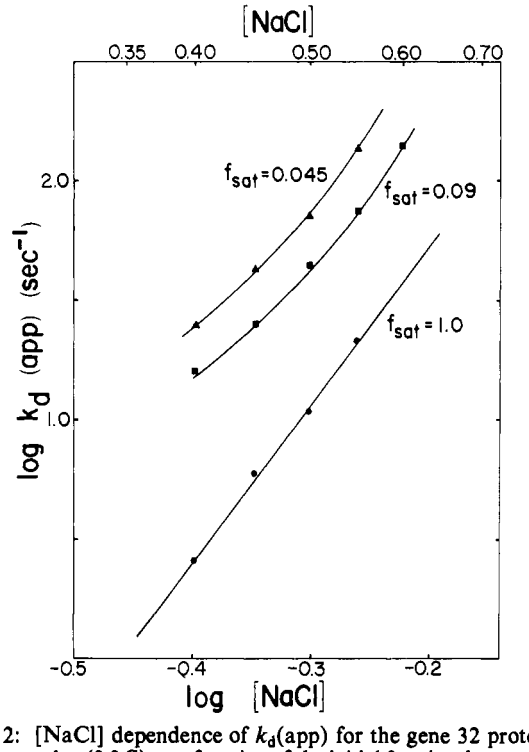

FIGURE 2: [NaCl] dependence of k_d(app) for the gene 32 protein–poly(A) complex (9.9 S) as a function of the initial fractional saturation (f_{sat}) of the poly(A) (log–log plot).

effect be checked, especially when the quantitative value of the salt dependence is used to interpret kinetic data mechanistically. The salt dependences of gene 32 protein dissociation from a number of synthetic homopolynucleotides are listed in Table I. For all of the unfractionated samples, $\partial \log k_d(\text{app})/\partial \log [\text{NaCl}] = 4.2 \pm 0.5$, whereas the fractionated 9.9S poly(A) and 6.5S poly(C) yield slopes of 6.6 and 7.3, respectively. We therefore conclude that the lower slopes of ~4–5 result from

Table I: [NaCl] Dependence of k_d(app)

nucleic acid	fractionated?	$\frac{\partial \log k_d(\text{app})}{\partial \log [\text{NaCl}]}$	f_{sat}
poly(A) (Tris, pH 8)	no	4.7 ± 0.3	1.0
poly(A) (phosphate, pH 7.5)	no	4.5 ± 0.2	1.0
poly(U)	no	4.3 ± 0.2	1.0
poly(dA)	no	3.9 ± 0.2	1.0
poly(dA)	no	3.9 ± 0.2	0.54
poly(dA)	no	3.9 ± 0.2	0.25
poly(dA)	no	3.6 ± 0.2	0.13
poly(dC)	no	3.8 ± 0.2	1.0
poly(rεA)	no	3.6 ± 0.2	1.0
poly(dA-T)	no	3.1 ± 0.1	0.28
ssM13 (wt^+)	yes, intact circles	4.5 ± 0.1	1.0
poly(A)	yes, $s^\circ_{20,w}$ = 9.9 S	6.6 ± 0.2	1.0
poly(A)	yes, $s^\circ_{20,w}$ = 9.9 S	nonlinear; 7 → 4	0.09
poly(A)	yes, $s^\circ_{20,w}$ = 9.9 S	nonlinear; 7 → 4	0.045
poly(C)	yes, $s^\circ_{20,w}$ = 6.5 S	7.5 ± 0.4	1.0

the use of polydisperse samples which contain a significant fraction of low molecular weight nucleic acid. The data presented here were obtained mainly with homopolynucleotides which were fractionated to remove low molecular weight molecules, as described in the previous paper (Lohman, 1984).

Dissociation at Low Salt: Trapping Experiments. To further probe the origin of the curvature in the log–log plots of k_d(app) vs. [NaCl], for fractionated poly(A) at low f_{sat} (see Figure 2), it was necessary to see whether the curvature persisted as the final [NaCl] at which the dissociation occurred was lowered. In order to investigate lower [NaCl] and still work with an irreversible dissociation, competition experiments to measure dissociation rates were needed. The competition experiments take advantage of two properties of the gene 32 protein–homopolynucleotide interaction: (1) Gene 32 protein displays a heirarchy of binding affinities to synthetic homopolynucleotides (Newport et al., 1981; Lohman, 1984). The affinity increases in the order poly(C) < poly(A) ≤ poly(U) < poly(dA) < poly(rεA) < poly(dC) < ssM13 DNA < poly(dT). (2) The percent quenching of the intrinsic tryptophan fluorescence of gene 32 protein also depends on the particular nucleic acid to which it binds. As a result, if gene 32 protein is transferred from a weaker binding nucleic acid to a stronger binding nucleic acid which also has a different extent of fluorescence quenching, then the transfer can be observed. The higher affinity nucleic acid (NA-2), when present in a large exces over the nucleic acid to which gene 32 protein is originally bound (NA-1), will act as a "trap" for protein which dissociates from NA-1. Since the association rate constants for gene 32 protein to the various nucleic acid lattices have been measured (Lohman & Kowalczykowski, 1981), it can be shown that, at low salt conditions, the rate-limiting step in such a mixing experiment is the dissociation from NA-1. In order to check whether the "trapping" nucleic acid was playing any role in the dissociation of gene 32 protein from NA-1, experiments were performed at 5-, 10-, and 25-fold excess of NA-2 over NA-1. In these experiments, preformed gene 32 protein–poly(A) complexes at 0.1 M NaCl (buffer T) were mixed with an excess of poly(rεA) in 0.1 M NaCl (buffer T). The reaction was then monitored via the further quenching of the gene 32 protein fluorescence resulting from the slow dissociation from poly(A) and subsequent fast association to the excess poly(rεA). In all cases, an exponential decrease in fluorescence was observed upon mixing which was quite slow [k_d(app) = 0.07 s^{-1} for f_{sat} = 1] and *independent* of the [poly(rεA)] which served as the trap. Under these conditions (0.1 M NaCl, pH 8.3), if the gene 32 protein had not been bound to poly(A) before mixing with the excess

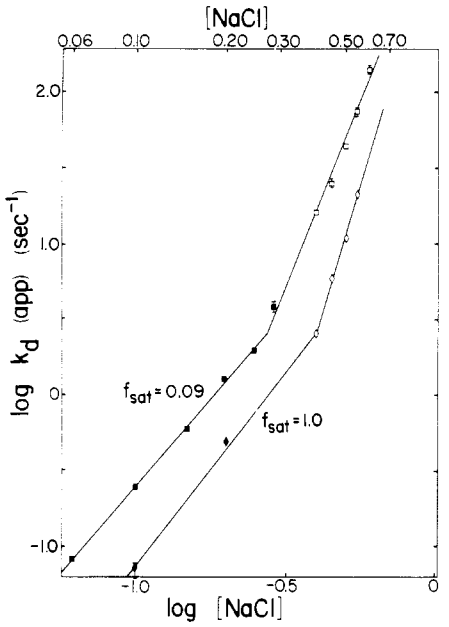

FIGURE 3: [NaCl] dependence of k_d(app) for the gene 32 protein–poly(A) complex (9.9 S) showing the nonlinear behavior of the log–log plots over a wide range of [NaCl]. Open symbols: data obtained by [NaCl] jumps. Filled symbols: data obtained by trapping the dissociated gene 32 protein with excess poly(rεA) or poly(dU).

poly(rεA), the resulting decrease in fluorescence would have been larger with a much higher apparent first-order rate constant of ~10–40 s^{-1} (depending on the exact [poly(rεA)]) (Lohman & Kowalczykowski, 1981). Furthermore, the measured dissociation rate from poly(A) at 0.10 M NaCl was the same irrespective of whether poly(rεA) or poly(dU) was used as the trap. Therefore, the rate constants obtained from the competition experiments do reflect the unperturbed dissociation of cooperatively bound gene 32 protein under low salt conditions.

A series of these low [NaCl] competition experiments to measure k_d(app) for gene 32 protein from the fractionated 9.9S poly(A) are shown in Figure 3, along with the high [NaCl] data from [NaCl] jumps. The initial conditions (buffer T, pH 8.3, 0.10 M NaCl) were identical for all of the experiments, and a 10-fold excess of poly(rεA) or poly(dU) was used as a trap in the low salt experiments. Figure 3 shows data for initial f_{sat} = 0.09 and 1.0, and it is clear that at low salt the log–log plot of k_d(app) vs. [NaCl] deviates substantially from the high salt slope. The limiting low salt slope for both f_{sat} = 1.0 and f_{sat} 0.09 is $\partial \log k_d(\text{app})/\partial \log [\text{NaCl}] = 2.4 \pm 0.1$, indicating that the slight curvature observed in the high [NaCl] experiments is real. In Figure 3, the highest [NaCl] for which k_d(app) was measured by the trapping method is the 0.30 M point on the f_{sat} = 0.09 curve. This point falls on the high salt line (determined by [NaCl] jumps) rather than the low salt line, lending support to the conclusion that the values of k_d(app) that have been measured are independent of the method of measurement. If the apparent dissociation rate constants measured by the trapping method did not follow the high salt line after the breakpoint in Figure 3, then there would be cause to suspect that the trapping method is not measuring a true rate of dissociation.

The dependence of k_d(app) on initial f_{sat} at low salt, when measured by the trapping method, is qualitatively similar to the behavior at high salt (Lohman, 1984) as shown in Figure 4; the increase in k_d(app) as the initial f_{sat} decreases at 0.20 M NaCl is diagnostic of a cooperatively binding system. The curve in Figure 4 is the theoretical prediction (using k_e = 5.5 s^{-1} and $\omega = 5 \times 10^3$) based on a model in which only singly contiguously bound protein molecules are observed to dissociate (Lohman, 1983). The model, which is valid for systems with high cooperativity, predicts that

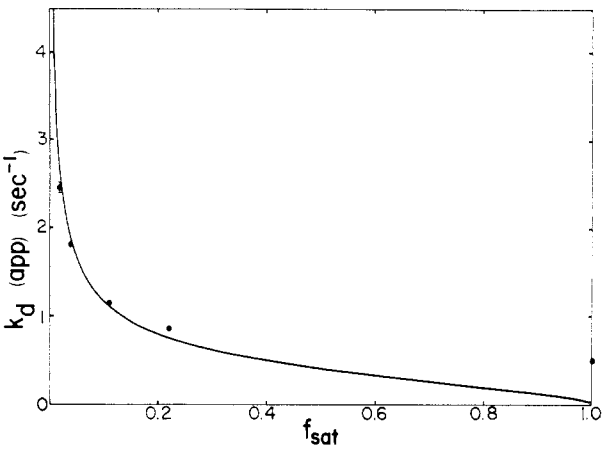

FIGURE 4: Dependence of the apparent dissociation rate constant for the gene 32 protein–poly(A) complex as a function of the initial fractional saturation (f_{sat}) of the poly(A). The smooth curve is generated by using eq 1 with k_e = 5.5 s^{-1}, $\omega = 5 \times 10^3$, and n = 7, based on the model of Lohman (1983).

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

where k_e is the rate constant for dissociation of a singly contiguously bound protein from a cluster end and $1 - p_0$ is the concentration of protein cluster ends at time zero. The expression for $1 - p_0$, which is dependent upon, n, ω, and ν, is given in the preceding paper; the reader is referred to Lohman (1983) for a complete discussion of the model. Plots of k_d(app) vs. $1 - p_0$ using values of $\omega = 10^3$ and 5×10^3 which bracket the range of estimates of ω for T4 gene 32 protein (Kowalczykowski et al., 1981) both represent the data reasonably well (data not shown) with the exception of the f_{sat} = 1.0 point which falls well above the predicted value of zero as it also does at high salt (Lohman, 1984). This results from the finite length of the poly(A) used in the experiments, whereas the theory is based on an infinite lattice model (Lohman, 1983). On the basis of Figure 4, it seems that the low salt dissociation of cooperatively bound gene 32 protein occurs only from ends of protein clusters in a manner similar to the high salt behavior (Lohman, 1984).

Although the dependence of k_d(app) on f_{sat} at low salt is similar to the high salt behavior, one major difference is observed in the low salt dissociation time course. Under fully saturated conditions the low salt dissociation is described very well by a single-exponential decay rather than zero-order kinetics as are observed at high salt (Lohman, 1984). Therefore, in addition to a change in the [NaCl] dependence of k_d(app) upon lowering the [NaCl], a change in the qualitative behavior of the dissociation time course when f_{sat} = 1 is also observed.

In Figure 5, it is shown that the decrease in $\partial \log k_d(\text{app})/\partial \log [\text{NaCl}]$ at low [NaCl] occurs for poly(C) and poly(dA) as well as poly(A). The high salt log–log slopes for poly(C), poly(A), and poly(dA) are 7.3, 6.6, and 3.8, respectively, whereas the low salt slopes are 2.1, 2.6, and 2.4, respectively. The poly(dA) sample used for the experiment in Figure 5 was not fractionated, although it had an $s_{20,w}$ = 6.5 S; hence, the high salt slope of 3.8 is likely to be an underestimate of the fractionated value. Newport et al. (1981) report values of $\partial \log K\omega/\partial \log [\text{NaCl}]$ for poly(C), poly(A), and poly(dA) of -7.0 ± 0.3, -6.8 ± 0.2, and -5.8 ± 0.1, respectively. Although the dissociation from all three homopolynucleotides undergoes

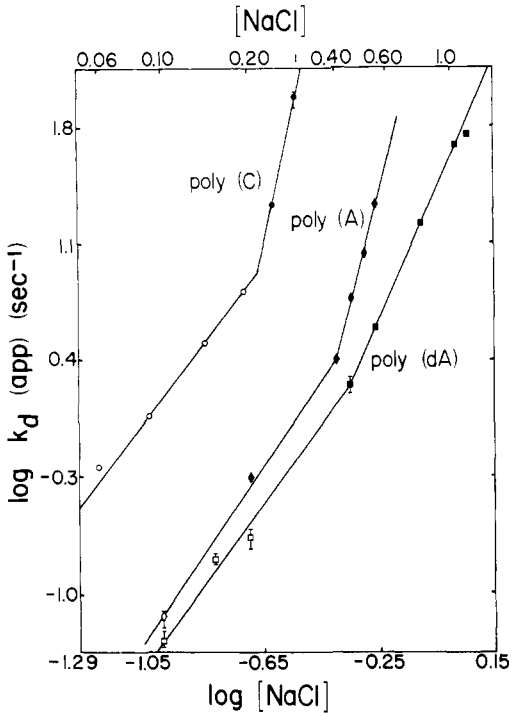

FIGURE 5: [NaCl] dependence of k_d(app) (log–log plot) over a wide range in [NaCl] for poly(C), poly(A), and poly(dA), indicating that the apparent breakpoint in the curves is dependent upon the nucleic acid and occurs at different [NaCl]. Filled symbols: data from [NaCl] jumps. Open symbols: data from trapping method using excess poly(rϵA).

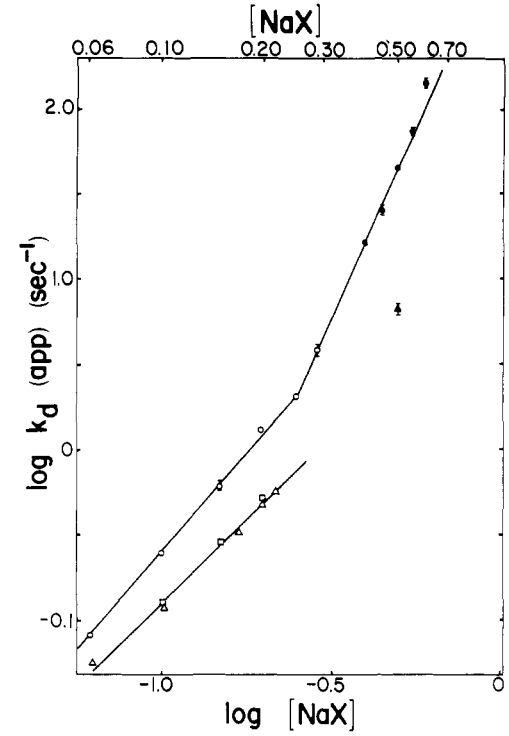

FIGURE 6: Effect of anion concentration on the dissociation of the gene 32 protein–poly(A) (9.9 S) complex (initial f_{sat} = 0.09). (△, ▲) Sodium acetate; (□) NaF; (○, ●) NaCl. Filled symbols: data obtained by salt jumps. Open symbols: data obtained by trapping with excess poly(rϵA).

a transition to a smaller salt dependence at low salt, the approximate breakpoints in the log–log plots for the three occur at distinctly different NaCl concentrations.

Does the decrease in $\partial \log k_d(\text{app})/\partial \log [\text{NaCl}]$ from 6–7 at high [NaCl] to 2.5 at low [NaCl] reflect a change in the mechanism of dissociation or simply a change in the ion binding properties of gene 32 protein and/or the nucleic acid? The [NaCl] dependence of $K\omega$ (Kowalczykowski et al., 1981) and k_d(app) indicates that when the gene 32 protein–nucleic acid complex dissociates, Na^+ must rebind to the nucleic acid and since there is a large anion effect on $K\omega$ (Kowalczykowski et al., 1981) and k_d(app) (Lohman, 1984), chloride ions probably also rebind to the gene 32 protein. If the extent of ion binding to either the gene 32 protein or the nucleic acid decreases upon decreasing the [NaCl] as mass action would predict, then the value of $\partial \log k_d(\text{app})/\partial \log [\text{NaCl}]$ is expected to decrease.

On the basis of measurements of $K\omega$ as a function of $MgCl_2$, NaCl, NaF, and sodium acetate, Kowalczykowski et al. (1981) have interpreted the salt dependence of $K\omega$ in the following manner. The value of $\partial \log K\omega/\partial \log [\text{NaCl}] = -7 \pm 0.5$ seems to result from a release of three to four Na^+ from the nucleic acid and three to four Cl^- from the gene 32 protein upon formation of the cooperative complex. Assuming this to be the case, then it is conceivable that as the [NaCl] is lowered, the extent of anion binding to the gene 32 protein might become insignificant. If the Na^+ binding to the single-stranded nucleic acid can be described by a condensation phenomenon (Manning, 1969, 1978) as seems to be the case for duplex DNA (Anderson et al., 1978), then one might expect $\partial \log k_d(\text{app})/\partial \log [\text{NaCl}] = 2$–3 at low enough [NaCl], as we observe in Figures 3 and 5, this salt dependence being due to the reassociation of three to four Na^+ ions onto the nucleic acid. One prediction of this hypothesis is that there should be no effect of anion concentration on k_d(app) in the low salt region where $\partial \log k_d(\text{app})/\partial \log [\text{NaCl}] = 2.5$. This was tested by measuring k_d(app) for the gene 32 protein–poly(A) dissociation as a function of sodium acetate and NaF in this low salt region. In Figure 6, these data are compared with the NaCl data. The values of k_d(app) for poly(A)–gene 32 protein dissociation decrease when NaF or sodium acetate are substituted for NaCl; however, in the low salt region ($\leq$0.20 M) k_d(app) values are identical in the NaF and sodium acetate solutions. The linear low salt lines describing the data in Figure 6 in the range 60– to 200 mM are

$$\log k_d(\text{app}) = 2.3 \log [\text{NaCl}] + 1.68$$

and

$$\log k_d(\text{app}) = 1.93 \log [\text{NaF/NaOAc}] + 1.03$$

The salt dependences in the presence of NaF or sodium acetate are identical and are slightly smaller (1.9) than with NaCl (2.3). Clearly there is an effect of anions on k_d(app) at low salt, indicating that the salt effect in this region is not just a result of Na^+ interaction with the nucleic acid. In addition, if the curvature in the log k_d(app) – log [NaCl] plots were due solely to a mass action effect of anion binding to the gene 32 protein, then the breakpoint in the curves should depend only on the gene 32 protein and hence be independent of the nucleic acid from which it dissociates. This is not the case as indicated in Figure 5. The breakpoint in the poly(C) dissociation occurs at a much lower [NaCl] (0.21 M) than for the poly(A) dissociation curve (0.40 M). Since the high salt slopes for poly(A) and poly(C) are comparable (7.3 vs. 6.6), the ion binding properties of poly(C) and poly(A) are similar, indicating that the different breakpoints are not likely due to differences in Na^+ binding to poly(A) vs. poly(C). Therefore, although part of the curvature observed in the log k_d(app) – log [NaCl] plots may be due to less ion binding by gene 32 protein at low salt, this cannot be the entire explanation. A further possibility is that the change in salt de-

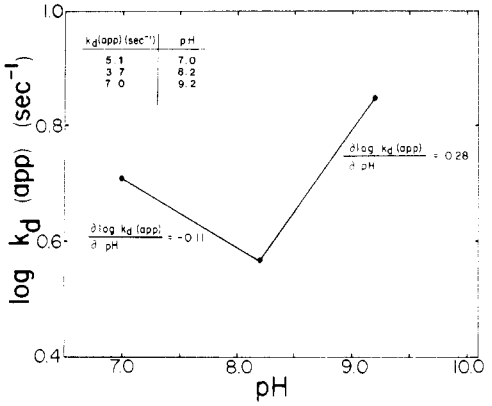

FIGURE 7: pH dependence of k_d(app) for the gene 32 protein–poly(dA) dissociation (f_{sat} = 1) over the range pH 7–9.2. The dissociations were made by [NaCl] jumps to a final [NaCl] of 0.55 M.

pendence could reflect a change in the rate-limiting step or an alternate pathway of dissociation at low salt.

Dissociation Activation Energy Is Dependent upon [NaCl]. To probe this question further, the temperature dependence of k_d(app) was measured as a function of [NaCl] to see if the apparent activation energy changes at low salt, which might indicate a change in mechanism. If no change in mechanism occurs with changing [NaCl], a fairly constant temperature dependence should be found as a function of [NaCl]. Rate constants were measured over the temperature range 12–30 °C for the fractionated poly(A)–g32p dissociation at 0.60, 0.45, and 0.2 M NaCl at low f_{sat} (0.06–0.09) and also at f_{sat} = 1. As the [NaCl] is decreased from 0.60 to 0.45 to 0.20 M, the activation energy increases from 14.4 ± 1 to 18.5 ± 2 to 27.0 ± 1 kcal/mol. This dramatic effect of [NaCl] on the activation energy is not expected if only a change in the extent of ion binding to gene 32 protein or poly(A) were occurring at low salt, since the ΔH for ion binding is close to zero. In addition to the dramatic change in activation energy with [NaCl], a change also occurs in the qualitative nature of the dissociation time course for fully saturated poly(A) at $T > 25$ °C. At these elevated temperatures, single-exponential dissociation curves are observed, whereas at $T < 25$ °C, zero-order kinetics are observed when the initial f_{sat} = 1. This may indicate either a decrease in the cooperativity parameter at high temperatures or some change in the dissociation mechanism.

It is possible that the observed increase in E_a as the [NaCl] decreases may result from an effect on the protonation of an amino acid on gene 32 protein that is required for binding. Protonation events have a large negative ΔH associated with them (Shiao & Sturtevant, 1976), and this may contribute to a large E_a at low [NaCl]. The effect of pH on k_d(app) was determined in order to probe this question. The salt dependence of k_d(app) is independent of pH between pH 7 and pH 8; however, there is a small but complex effect of pH on k_d(app) which is shown in Figure 7; k_d(app) has a minimum near pH 8.2 and increases slightly at higher and lower values. The small effect of pH on k_d(app) seems to rule out any substantial linkage to protonation in the binding reaction.

Value of the Cooperativity Parameter Is Independent of [NaCl] in the Range 0.05 M < [NaCl] < 0.3 M. Another possible explanation for the decrease in the [NaCl] dependence of k_d(app) at low salt is that the cooperativity parameter, ω, which has a value of 10^3 to 5×10^3 at high [NaCl], decreases at lower [NaCl]. This decrease in ω would result in an increase in the fraction of protein cluster ends at low f_{sat} and hence an increase in k_d(app). It is difficult to measure ω at low [NaCl] since the noncooperative equilibrium constant is so large. However, it is possible to see whether ω changes as a function of [NaCl] by using kinetic techniques. To test this, gene 32 protein–poly(dT) complexes were formed at low f_{sat} (~0.2) at various [NaCl] between 50 mM and 0.30 M, conditions for which all of the gene 32 protein is bound to the poly(dT). [NaCl] jumps were then made to the *same* final [NaCl] (2.5 M), and the values of k_d(app) were measured. Since the final solution conditions were identical, the only cause for differences in k_d(app) would be different initial cluster distributions due to the different initial NaCl concentrations. The values of k_d(app) were independent of the initial [NaCl] in the range 50 mM to 0.30 M, indicating identical cluster distributions and hence identical values of ω. Therefore, the change in salt dependence, which occurs in some cases within the 50 mM–0.30 M NaCl range, is not due to a change in ω, unless the binding of poly(C), poly(A), and poly(dA) to gene 32 protein is affected differently by [NaCl] changes than the binding to poly(dT). All previous binding studies indicate that this is not the case (Kowalczykowski et al., 1981; Newport et al., 1981).

Table II: Different Dissociation Behavior at Low vs. High Salt for Gene 32 Protein–Poly(A)

low [NaCl]	high [NaCl]
(1) $\partial \log k_d(app)/\partial \log$ [NaCl] = 2.5 ± 0.3	$\partial \log k_d(app)/\partial \log$ [NaCl] = 6.5 ± 1
(2) E_a = 27 ± 2 kcal/mol	E_a = 14 ± 2 kcal/mol
(3) initial f_{sat} = 1; observe: single-exponential decay	initial f_{sat} = 1; observe: constant rate of dissocation (zero-order kinetics)

Discussion

A summary and comparison of the relevant differences at low [NaCl] vs. high [NaCl] in the cooperative dissociation of T4 gene 32 protein from single-stranded homopolynucleotides are given in Table II. There are three major differences in the dissociation based on the fractionated poly(A)–gene 32 protein data. (1) $\partial \log k_d(app)/\partial \log$ [NaCl] is reduced from its value of 6–7 at high [NaCl] to 2.5 at low [NaCl]. (2) The apparent activation energy increases from 14 ± 1 kcal/mol at 0.6 M NaCl to 27 ± 1 kcal/mol at 0.2 M NaCl. (3) When the poly(A) is initially fully saturated with gene 32 protein, a constant rate of protein dissociation (zero-order kinetics) is observed at high [NaCl] (Lohman, 1984), whereas a single-exponential decay is observed at low [NaCl]. Since the initial conditions are identical in each of the low salt/high salt comparisons, the initial protein cluster distributions must be identical in each case, and the observed differences can only be due to the final [NaCl] at which dissociation occurs. Since the gene 32 protein cooperativity parameter, ω, does not change over the [NaCl] range 50 mM to 0.30 M (see above), these observations strongly suggest a change in the dissociation mechanism at low [NaCl]. A loss of zero-order kinetics is also observed at temperatures above 25 °C, even at high [NaCl]; hence, the change in mechanism may also be temperature dependent.

Possible Explanations of the Low Salt Dissociation Behavior. The high [NaCl] slope of 6–7 which we find for the fractionated poly(A) and poly(C) is a strong indication that singly contiguous proteins dissociate directly into solution, at high [NaCl], without an intermediate step. This follows from the fact that $\partial \log K\omega/\partial \log$ [NaCl] = −6.5 ± 1 (Kowalczykowski et al., 1981; Newport et al., 1981) and that $\partial \log k_1/\partial \log$ [NaCl]) ≃ 0 (Lohman & Kowalczykowski, 1981), where k_1 is the bimolecular association rate constant for formation

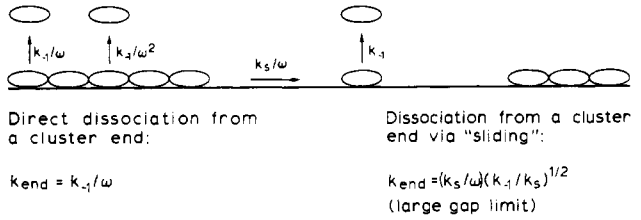

FIGURE 8: Schematic depicting four possible modes of dissociation of a cooperative protein nucleic acid complex, considering only nearest-neighbor cooperativity.

of noncooperative gene 32 protein–nucleic acid complexes. Furthermore, the salt dependence of the dissociation rate constant, k_{-1}, for a protein which dissociates directly into solution is predicted to be approximately equal to $-(\partial \log K/\partial \log [NaCl])$ (Lohman et al., 1978). Therefore, in the gene 32 protein–poly(A) dissociation $\partial \log k_{-1}/\partial \log [NaCl]$ is predicted to be 6–7 if the protein dissociates without an intermediate step.

One explanation for the curvature in the log k_d(app)–log [NaCl] plots is that the pathway for dissociation of gene 32 protein from the ends of protein clusters changes as a function of salt. It is possible that at high [NaCl] the protein dissociates directly from a cluster end whereas at low [NaCl] the singly contiguously bound protein slides off the end of a cluster to first form an isolated intermediate which subsequently dissociates as depicted in Figure 8. That is, dissociation may occur via two alternate pathways. If these two pathways possess different salt dependences, this may result in the observed curvature; the [NaCl] would determine the fraction of molecules which dissociate via direct dissociation vs. sliding.

From the theoretical treatment of Berg (Berg & Blomberg, 1976; Berg et al., 1981) the expression for the rate constant for dissociation of g32p from a cluster end via sliding to first form an isolated intermediate is given by

$$k_{e,s} = (k_s/\omega)(k_{-1}/k_s)^{1/2} = \omega^{-1}(k_s k_{-1})^{1/2} \qquad (2)$$

where k_{-1} is the rate constant for dissociation of an isolated protein and k_s is a "sliding" rate constant, which is defined in the Appendix along with a derivation of eq 2. Equation 2 is valid under conditions where the protein which slides away from a cluster end does not collide with another cluster before undergoing dissociation. These conditions should hold at very low binding densities, where protein clusters are well separated or possibly at very high binding densities where only one large cluster exists on each nucleic acid lattice.

The rate constant for dissociation from a cluster end via direct dissociation into solution, $k_{e,d}$, is

$$k_{e,d} = k_{-1}/\omega \qquad (3)$$

In eq 2 and 3 we have assumed that the cooperativity involved in dissociation, ω_r, is equal to ω^{-1}, the inverse of the equilibrium cooperativity parameter. This assumes that the bimolecular association rate constant is noncooperative, which seems to be the case for the T4 gene 32 protein (Lohman & Kowalczykowski, 1981; Lohman, 1984). The overall rate constant for dissociation from a cluster end, k_e, is the sum of eq 2 and 3:

$$k_e = \omega^{-1}[k_{-1} + (k_{-1}k_s)^{1/2}] \qquad (4)$$

The dissociation from a cluster end can therefore occur in either of two ways, and both pathways will contribute to k_e, although, under a given set of conditions, one pathway may dominate.

In order to evaluate the salt dependence of k_e, by use of eq 4, the salt dependence of k_s must be known. The only quantitative data available concerning the salt dependence of sliding is from the *lac* repressor–operator kinetic studies. Comparisons of theory with experiment indicate that the one-dimensional diffusion of *lac* repressor while bound nonspecifically is essentially independent of the bulk salt concentration (Barkley, 1981; Winter et al., 1981). The one-dimensional diffusion coefficient which best fits the data in NaCl buffers is $D_1 \sim 10^{-9}$ cm^2/s which corresponds to a sliding rate constant $k_s \sim 10^6$ s^{-1}. Therefore, as a first approximation, it is assumed that if gene 32 protein slides along single-stranded nucleic acids, k_s is also independent of the bulk [NaCl].

From eq 2, the known [NaCl] dependence of k_1 and the assumption that k_s and ω are independent of [NaCl], it is predicted that singly contiguously bound gene 32 protein which dissociates only by sliding to first form an isolated intermediate would have the following dependence on [NaCl]:

$$\frac{\partial \log k_{e,s}}{\partial \log [NaCl]} = \frac{1}{2}\frac{\partial \log k_{-1}}{\partial \log [NaCl]} = \sim 3\text{–}3.5 \qquad (5)$$

Furthermore, since k_s is assumed to be independent of [NaCl], eq 4 predicts that the "sliding limit" salt dependence (eq 5) should be reached as the [NaCl] is lowered. Hence, at high salt, k_d(app) is predicted to have a [NaCl] dependence (log–log) of 6–7 (similar to $1/K\omega$) whereas as the [NaCl] is lowered, the [NaCl] dependence (log–log) will reach the sliding limit of 3–3.5. The [NaCl] range over which these two limits hold will depend on both the absolute and the relative values of k_s and k_{-1}. The experimental [NaCl] dependence, of k_d(app) for the g32p–poly(A) dissociation fits the model described above reasonably well. Figures 3 and 5 show high salt slopes of 6–7 which display curvature and reach limiting low salt slopes of 2–2.5.

A comparison of the theoretical prediction of eq 4 with the g32p–poly(A) data ($f_{sat} = 0.09$) is shown in Figure 9, where k_d(app) = 2 $k_e(1 - p_0)$. Equation 6 was used for the [NaCl]

$$\log k_{-1} = 7.0 \log [NaCl] + 7.5 \qquad (6)$$

dependence of k_{-1}. It is calculated from the salt dependence of $K\omega$ (Kowalczykowski et al., 1981) (using $\omega = 5 \times 10^3$) and the measured bimolecular association rate constant, k_1, which is essentially salt independent and equal to 4×10^6 M^{-1} (nucleotide) s^{-1} (Lohman & Kowalczykowski, 1981). The best fit to the data is achieved with a value of $k_s = 2 \times 10^6$ s^{-1} (assumed to be independent of [NaCl]), which is the same value of k_s required to fit the association kinetic data (Lohman & Kowalczykowski, 1981). Therefore, two independent sets of experiments yield the same quantitative result.

The low salt part of the curve fits the data least well as the limiting slope of the theoretical prediction (3.5) is considerably higher than for the experiment (2.5). This reflects the fact that the theoretical curve does not change slope as abruptly as does the experimental curve. The basis for this has several possible explanations, the most likely of which is a lowered extent of anion binding to gene 32 protein at lower salt concentrations, which is not accounted for in eq 4.

Also shown in Figure 9 is the expected dependence of k_d(app) on [NaCl] (log–log) if dissociation occurred only from the ends of clusters, but without any contribution from the sliding pathway. Although the contribution to k_d(app) from sliding is certainly much greater at low salt for poly(A)–g32p, sliding still contributes to the dissociation rate even at the highest [NaCl] used in the poly(A) experiments. Therefore, both pathways contribute to k_d(app) over the entire experimental range of [NaCl], although direct dissociation dominates at high [NaCl], and the sliding pathway dominates at low [NaCl].

Table III: Molecular Dissociation Rate Constants Estimated from the Two-Pathway Model (25 °C, pH 8.2)

nucleic acid	[NaCl] (M)	k_e (s^{-1})	$K\omega$[a] (M^{-1})	$k_{d.c.}$ (min^{-1})	k_{-1}/ω (s^{-1})[b]	ω ($\times 10^{-3}$)	k_s (s^{-1})[d]
poly(A) (fractionated)	0.40	36	6.9×10^5	2.2	5.8	1	1.6×10^5
	0.40	73	6.9×10^5	0.88	5.8	5	3.9×10^6
	0.45	66	2.9×10^5	4.0	14	1	1.9×10^5
	0.45	126	2.9×10^5	1.5	14	5	4.6×10^6
	0.50	103	1.37×10^5	6.2	29	1	1.9×10^5
	0.50	214	1.37×10^5	2.6	29	5	5.6×10^6
	0.55	183	6.84×10^4	11.0	58	1	2.7×10^5
	0.55	383	6.84×10^4	4.6	58	5	9.1×10^6
poly(U) (unfractionated)	0.34	62.8	7.44×10^5	3.8	5.4	1	6.1×10^5
	0.34	104.3	7.44×10^5	6.3	5.4	5	9×10^6
poly(dA) (unfractionated)	0.45	6.4	2.02×10^6	0.38	2.0	1	1.0×10^4
	0.45	12.8	2.02×10^6	0.15	2.0	5	3.0×10^5
poly(dA) (unfractionated)	0.55	22.4	6.8×10^5	1.3	5.9	1	4.6×10^4
	0.55	44.6	6.8×10^5	0.54	5.9	5	1.3×10^6
poly(dT) (unfractionated)	2.03	18.9	6.1×10^5	1.1	6.6	1	2.3×10^4
	2.03	33.6	6.1×10^5	0.40	6.6	5	5.5×10^5
poly(A) (unfractionated)	0.20	2.8	1×10^8[c]	2.4×10^{-3}	0.04	1	1.9×10^5
	0.20	5.3	1×10^8[c]	4.8×10^{-4}	0.04	5	3.5×10^6

[a] Data from Kowalczykowski et al. (1981) and Newport et al. (1981). [b] Calculated from $k_{-1}/\omega = k_1/K\omega$ with $k_1 = 4 \times 10^6$ M^{-1} (nucleotide) s^{-1}. [c] This value of $K\omega$ was obtained by linear extrapolation of the higher salt data from Kowalczykowski et al. (1981) and hence, it may be an overestimate [see Lohman & Kowalczykowski (1981)]. [d] Obtained by solving for k_s, using $k_e = (1/\omega)[k_{-1} + (k_{-1}k_s)^{1/2}]$.

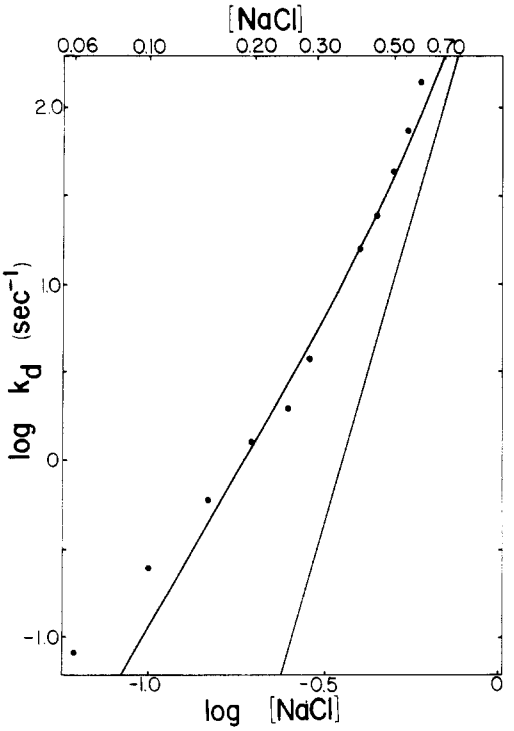

FIGURE 9: Theoretical prediction of the [NaCl] dependence of k_d(app) = $2k_e(1 - p_0)$ (log–log plot) assuming the dissociation from cluster ends occurs by both direct dissociation and sliding to form a noncooperative intermediate (see Figure 8). Data points are for the gene 32 protein–poly(A) (9.9 S) complex (f_{sat} = 0.09). The straight line without points is the expected dependence of k_{-1} on [NaCl] (see eq 6 in the text).

In Table III, the molecular rate constants for a number of homopolynucleotides have been listed along with the value of k_s that is calculated by using eq 2. The values of k_{-1}/ω are calculated from the known association rate constant, $k_1 = 4 \times 10^6$ M^{-1} (nucleotide) s^{-1} (Lohman & Kowalczykowski, 1981), and equilibrium constant, $K\omega$ (Kowalczykowski et al., 1981; Newport et al., 1981). In every case, the experimental determination of k_e reported in this work is significantly greater than k_{-1}/ω, indicating that dissociation of gene 32 protein from the end of the cluster does not occur solely by direct dissociation of protein into solution. In fact, other pathways must also be available for the dissociation of singly contiguous molecules, e.g., sliding off the end to first form an isolated

Table IV: Comparison of $K\omega$ and k_d(app) for Gene 32 Protein–Poly(rU) at 0.4 M

$[K\omega(NaF)/K\omega(NaCl)]^{1/2}$[a]	7.5	$k_d(NaCl)/k_d(NaF)$[b]	6.4
$[K\omega(NaOAc)/K\omega(NaCl)]^{1/2}$[a]	3.2	$k_d(NaCl)/k_d(NaOAc)$[b]	3.3
$[K\omega(NaF)/K\omega(NaOAc)]^{1/2}$[a]	2.4	$k_d(NaOAc)/k_d(NaF)$[b]	2.0

[a] Data from Kowalczykowski et al. (1981). [b] Data from Lohman (1984).

complex. The values of k_s which are needed to fit the data, point by point, generally range from $\sim 10^5$ to 10^7 s^{-1}, depending on the value of ω used in the calculation (10^3 or 5×10^3). The values of k_s for poly(dA) and poly(dT) are lower than for poly(A) and poly(U), although due to the lack of sufficient data no significance is placed on this at this time, especially since some of the lattices are unfractionated.

The model of two competing pathways for the dissociation of singly contiguously bound g32p also provides an explanation for the different [NaCl] breakpoints observed for poly(C), poly(A), and poly(dA) in Figure 5. Equation 4 indicates that the apparent breakpoint in the log–log plot of k_d(app) vs. [NaCl] should occur when $k_s = k_{-1}$. Therefore, if k_s is similar for sliding of gene 32 protein along these three homopolynucleotides, then the relative breakpoints will be dependent only upon the relative values of k_{-1}. The values of k_{-1} display nucleic acid specificity (Lohman, 1984) and the [NaCl] at which $k_{-1} = 2 \times 10^6$ s^{-1} is lowest for poly(C), higher for poly(A), and the highest for poly(dA), which is the same ranking observed in Figure 5.

Additional support for this model comes from a comparison of the relative values of $K\omega$ and k_d(app) in NaCl, sodium acetate, and NaF. A prediction of the sliding model is

$$k_d(\text{app}) \propto (K\omega)^{-1/2} \quad (7)$$

under conditions such that only dissociation by sliding occurs. A complete set of equilibrium (Kowalczykowski et al., 1981) and kinetic (Lohman, 1984) data are available to test eq 7 for poly(rU). In Table IV the predicted ratios of k_d(app) in NaCl, sodium acetate, or NaF based on the $K\omega$ values are compared with the measured ratios of k_d(app). The agreement is quite good. If sliding did not contribute to k_d(app), an inverse first power dependence would be expected.

There are still a number of unknown aspects of the interaction of gene 32 protein with single-stranded nucleic acids. In particular, there are likely to be conformational changes

which occur in the protein upon binding to and dissociation from the nucleic acid. Such conformational changes may have complex temperature dependences which might explain the dramatic effect of NaCl concentration on the activation energy for dissociation which is observed. This remains a possibility which cannot be excluded at this time.

Molecular Aspects of Protein Translocation along Nucleic Acids. What are the molecular features that enable a particular protein to translocate along its nucleic acid lattice? As pointed out by Hill & Tsuchiya (1981) it is not necessary that the protein binds weakly to the nucleic acid but rather that the activation energy for lateral movement be low. As long as this requirement is met, even proteins which possess a large binding constant are capable of sliding. The most likely way of maintaining a high binding constant along with a low activation barrier for lateral movement is if the binding free energy is exclusively electrostatic. In this case, one expects that the nucleic acid would be viewed by the protein as an isopotential surface (Winter et al., 1981) with very little energy barrier to lateral movement. This situation seems to exist for the nonspecific binding of *lac* repressor to duplex DNA. Through a comparison of DNA-binding properties of oligolysines and *lac* repressor, de Haseth et al. (1977) have shown that *lac* repressor binds to duplex DNA, in its nonspecific binding mode, in an almost exclusively electrostatic manner. The favorable binding free energy results from the increased entropy due to the release of condensed counterions on the DNA into solution upon forming the protein–DNA complex (Record et al., 1976, 1978; Manning, 1969, 1978). Therefore, even though the binding constant for the *lac* repressor nonspecific DNA is $\sim 10^8$ M^{-1} at 50 mM NaCl, 25.0 °C, pH 8 (de Haseth et al., 1977), it should have very little problem sliding along the DNA. This is consistent with the kinetic data (Riggs et al., 1970; Barkley 1981; Winter et al., 1981) which strongly indicates that *lac* repressor does indeed slide in its nonspecific binding mode. Unfortunately, no temperature dependence studies of the kinetics are available so that the prediction of a low activation energy to sliding remains unverified.

Is it necessary that the binding free energy be totally electrostatic in order for a protein to slide, or is there simply a minimum electrostatic component to the binding free energy which is necessary for translocation? In the case of enzymes which clearly have the ability to translocate such as polymerases and helicases, they also would seem to have a large nonelectrostatic component, although the necessary binding experiments have not been done to test this. Is it possible that gene 32 protein is able to translocate? For gene 32 protein, the dramatic dependence of $K\omega$ on the nucleic acid lattice (Newport et al., 1981) suggests that there is clearly a large nonelectrostatic component to its binding free energy. However, since other DNA binding proteins which must translocate also have nonelectrostatic components (although these have not been quantified), one cannot rule out the possibility for gene 32 protein simply on these grounds. Certainly gene 32 protein also has a reasonable electrostatic binding component which is reflected in the large [NaCl] dependence of its binding constant (Newport et al., 1981). Additionally, as outlined by Winter et al. (1981) for *lac* repressor, it is entirely possible that rapid fluctuations between two binding modes exist for proteins which are able to slide, such that in one mode the protein can translocate (bound mainly electrostatically) while in the other mode the protein is relatively immobile with a larger nonelectrostatic component. This may also apply to gene 32 protein.

Normally, one can use the observed dependence of k_d(app) on [NaCl] to obtain quantitative information about the number of ionic interactions formed in a protein–nucleic acid interaction (Lohman et al., 1978). In this case, however, the interpretation is complicated due to the contributions of anion binding to gene 32 protein (Kowalczykowski et al., 1981: see also Figure 6). Until the anion binding properties of gene 32 protein are better understood, a quantitative interpretation of the [NaCl] dependence is not possible. The qualitative features of the salt dependence of the dissociation rate constants deserve comment, however. First of all, essentially all of the [NaCl] dependence of $K\omega$ originates in the dissociation reaction, at least at high salt concentrations where the data is available to make comparisons. This is exactly the expected result for a dissociation reaction which occurs via a single step, i.e., $\partial \log k_{-1}/\partial \log [NaCl] = -\partial \log K_{obsd}/\partial \log [NaCl]$ (Lohman et al., 1978). Furthermore, the dissociation rate increases with increasing [NaCl] even above the [NaCl] which is necessary for complete dissociation of the complex. This results from the requirement for cations to rebind to the nucleic acid as well as anions (and possibly cations) to rebind to the gene 32 protein upon dissociation of the protein–nucleic acid complex. The fact that raising the [NaCl] increases the rate constant for dissociation of this single-step process indicates that there is a direct competition between the protein and cations for the nucleic acid.

Relation of the Measured Rate Constants to the Replication Process. The dissociation kinetics experiments reported in this and the preceding paper (Lohman, 1984) as well as the association kinetics experiments (Lohman & Kowalczykowski, 1981) can be used to estimate the rate of fork movement assuming that the recycling or treadmilling (Hill & Tsuchiya, 1981) of gene 32 protein, is rate limiting. Using 0.15 M KCl and 3 mM Mg^{2+} as equivalent to the in vivo ionic environment in *E. coli* (Kao-Huang et al., 1977), then we estimate $k_1 = 5.5 \times 10^6$ M^{-1} (nucleotide) s^{-1} and $k_e \sim 10$ s^{-1} for gene 32 protein at 37 °C [using the poly(dC) data as the closest approximation to natural single-stranded DNA]. Given that the gene 32 protein concentration in T4-infected *E. coli* is autoregulated at ~2–3 μM (Russel et al., 1976; Lemaire et al., 1978, von Hippel et al., 1983), an approximate association rate of gene 32 protein is 15–20 s^{-1} which is close to the estimate for k_e given above.[1] These rates are well below the estimated in vivo fork rate of 500–1000 base pairs s^{-1}, as well as the highest observed rates for in vitro replication by the seven protein T4 replication complex, which are near 400 base pairs s^{-1} (Liu et al., 1978). One possible explanation for this difference is that recycling is not limited by the unperturbed lifetime of the gene 32 protein–single-stranded DNA complex, rather gene 32 protein may be displaced from the single-stranded DNA during replication so that the dissociation rates that have been reported may not be directly applicable to the in vivo situation. It is entirely reasonable that such a displacement may occur on the lagging strand by the replication complex engaged in lagging strand synthesis. On the leading strand, however, gene 32 protein may not even bind in large clusters. It may function as a "subunit" of the replication complex and move processively with the complex as DNA is replicated (Alberts et al., 1980). Therefore, recycling may not even be necessary on the leading strand. Not enough is known about the detailed structure and positioning of the

[1] Under conditions of excess gene 32 protein, $k_1 \sim 10^7$ M^{-1} s^{-1} (S. C. Kowalczykowski, unpublished results); hence, the association rate may be a factor of 2 faster than the rate which is calculated on the basis of the data obtained under conditions of excess nucleic acid.

various replication proteins at the fork to comment further on these possibilities. From these studies, however, it is clear that the lifetimes of singly contiguously bound T4 gene 32 protein which have been measured in this work are too long to enable it to be recycled through an intermediate which is free in solution. Either gene 32 protein is displaced by the processively moving replication complex or it may move with the complex itself, as may be the case in leading strand synthesis. The kinetic data in this paper, as well as previous work (Lohman & Kowalczykowski, 1981), suggests that monomers of gene 32 protein may be able to translocate along single-stranded nucleic acids, indicating that movement of gene 32 protein monomers with the replication fork is a possibility.

The work presented here as well as other studies on the kinetics of protein–nucleic acid interactions (Barkley, 1981; Winter et al., 1981; Lohman & Kowalczykowski, 1981; Roe et al., 1984) demonstrates that effects of salt concentration on the observed rate constants can be a valuable tool in deciphering a mechanism. Even in the absence of a detailed molecular model for how salt interacts with the two macromolecules, one can still learn much from such an investigation. What is essential, however, is quantitative information about the salt dependence of the binding constant under the identical conditions used to study the rate constants. With this information, the presence of intermediate steps in a reaction pathway can be more easily inferred. As Roe et al. (1984) point out, the large dependences of the equilibria and kinetic properties of protein–nucleic acid complexes suggest a possible role of low molecular weight ions in the regulation of processes involving protein–nucleic acid interactions.

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Appendix

This derivation follows the treatment of Berg et al. (1981) for the dissociation of *lac* repressor from tandem operators. Consider the dissociation of a singly contiguously bound protein from the end of a cluster into a gap of length L_0 nucleotides as depicted in Figure 8. The protein remains bound to the nucleic acid in its isolated binding mode. In order to calculate the rate constant for dissociation from the nucleic acid, it is necessary to calculate P_d, the probability that the protein will dissociate from the nucleic acid at some point upon entering the gap, before it returns to its original cluster or completely crosses the gap and becomes a part of the neighboring cluster. The differential equation describing this process is

$$\frac{\partial u(x,t)}{\partial t} = D_1\left(\frac{\partial^2 u(x,t)}{\partial x^2}\right) - k_{-1}u \qquad 0 < x < L_0 \qquad \text{(A1)}$$

where $u(x,t)$ = probability distribution for protein within the gap, D_1 = one-dimensional diffusion coefficient for bound gene 32 protein monomers, and k_{-1} = dissociation rate constant for an isolated protein. The boundary conditions are

$$u(0,t) = u(L_0,t) = 0$$

and the initial conditions are

$$u(x,0) = \delta(x - l)$$

which assumes that, at $t = 0$, the protein has moved from the cluster end at $x = 0$ to the nearest isolated binding site at $x = l$, where l is the length of a nucleotide.

The expression for the probability P_d is

$$P_d = k_{-1}\int_0^{L_0} \tilde{u}(x)\,dx$$

where $\tilde{u}(x) = \int_0^\infty u(x,t)\,dt$. In the case where $(k_{-1}l^2/D_1)^{1/2} \ll 1$, which is when the protein is able to slide a distance much greater than a single nucleotide without dissociating, and in the limit of infinite gap length, $L_0 \to \infty$, Berg et al. (1981) obtain the result

$$P_d = (k_{-1}l^2/D_1)^{1/2} \qquad \text{(A2)}$$

Therefore, the overall dissociation rate constant from a cluster end via the sliding pathway, $k_{e,s}$, is given by

$$k_{e,s} = (1/\omega)(D_1/l^2)P_d = \left(\frac{k_s}{\omega}\right)\left(\frac{k_{-1}}{k_s}\right)^{1/2} \qquad \text{(A3)}$$

where a sliding rate constant, k_s, is defined as

$$k_s = D_1/l^2 \qquad \text{(A4)}$$

Equation A3 should be valid at low binding densities where the gap size is large.

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Controlled Proteolysis of Tubulin by Subtilisin: Localization of the Site for MAP_2 Interaction†

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ABSTRACT: The treatment of tubulin with subtilisin resulted in a significant decrease in the ability of tubulin to assemble. The addition of taxol reduced the effect of subtilisin on the assembly of digested protein. Limited proteolysis of tubulin by subtilisin affected simultaneously both α- and β-subunits, and it resulted in the appearance of two major cleavage fragments (32 and 20 kilodaltons) or an alternative pattern yielding two fragments (48 and 4 kilodaltons). The smallest peptide (4 kilodaltons) and also the 20-kilodalton fragment are localized in the C-terminal region of the tubulin α-subunit. Digested tubulin can assemble into sheet-shaped polymers, which cannot incorporate MAP_2. On the other hand, the isolated C-terminal fragments can bind to MAP_2. These results suggest that the carboxyl-terminal domain of the tubulin molecule is the site for the MAP_2 interaction.

Tubulin, the major component of microtubules, can self-associate, bind nucleotides and specific drugs, or interact with other proteins associated with microtubules (MAPs).[1] It is very important to know the characteristics of the tubulin domains involved in these interaction processes. One approach has been done to study the functional role of specific tubulin residues in those associations (Maccioni et al., 1981a,b; Maccioni & Seeds, 1981, 1982; Mann et al., 1978; Palanivelu & Ludueña, 1982; Wadsworth & Sloboda, 1982; Mellado et al., 1980, 1982). Recently, limited proteolysis with trypsin and chymotrypsin (Brown & Erickson, 1983; Maccioni & Seeds, 1983) has proven very useful to fractionate the tubulin molecule into fragments and to analyze the tubulin structure as related to microtubule assembly. A similar approach has been previously used (Vallee & Borisy, 1977, 1978) to study the binding domain of the microtubule-associated protein MAP_2 to tubulin. Vallee (1980) has found that MAP_2 binds to tubulin through its cationic terminal region. However, nothing is known about the tubulin domain bound to MAP_2. For the present report, we have done a limited cleavage at specific sites of the tubulin molecule with subtilisin to study the structural domains of tubulin involved in the interaction with MAP_2. Our results indicate that the carboxyl-terminal region of tubulin is involved in the interaction with MAP_2.

Materials and Methods

Purification of Tubulin. Tubulin from pig brain was prepared by temperature-dependent cycles of assembly–disassembly following the procedure of Shelanski et al. (1973) and stored as pellets at −70 °C. Microtubule pellets were resuspended in 0.1 M MES (pH 6.4), 2 mM EGTA, and 0.5 mM $MgCl_2$ (buffer A) prior to their use, and a third cycle of assembly–disassembly was performed. Tubulin depleted of MAPS was obtained by phosphocellulose chromatography (PC-tubulin) as described by Weingarten et al. (1975). The microtubule-associated protein MAP_2 was isolated according to the procedure of Sandoval & Weber (1980).

The protein concentration of microtubule protein was determined by the procedure of Lowry et al. (1951) and the concentration of PC-tubulin by $A_{280} = 1.15$ mg·mL^{-1} (Appu Rao et al., 1978).

Radioactive Labeling of Tubulin. The ^{125}I-iodinated tubulin used in some experiments was prepared according to the procedure of Carlier et al. (1980). The radiolabeling resulted

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTP, guanosine 5′-triphosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; C-terminal, carboxyl terminal; N-terminal, amino terminal; $NaDodSO_4$, sodium dodecyl sulfate; MAPs, microtubule-associated proteins; PMSF, phenylmethanesulfonyl fluoride; PC-tubulin, phosphocellulose-purified tubulin.

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