Ionic Strength Perturbation Kinetics of Gene 32 Protein Dissociation from Its Complex with Single-Stranded DNA†

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ABSTRACT: Equilibrium and kinetic studies of the interaction of gene 32 protein of T4 phage with single-stranded fd DNA were performed monitoring the changes in protein fluorescence. From the fluorescence titrations, it was estimated that a monomer of gene 32 protein covered six nucleotide bases on the DNA and the lower limit for the apparent association constant was 1.9×10^8 M^{-1} with a cooperative parameter of 10^3 in 0.1 M 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride (pH 7) at 25 °C. When an ionic strength jump was applied to the gene 32 protein–fd DNA complex using a stopped-flow apparatus, the complex underwent a dissociation into its individual components accompanied by an increase in protein fluorescence. The kinetics of the dissociation are not consistent with a single first-order process. The data, however, can be analyzed in terms of a model in which gene 32 protein molecules release cooperatively starting from either one or both ends of a cluster of proteins bound to fd DNA. This type of dissociation of gene 32 protein from single-stranded DNA is very efficient and has interesting implications: it could provide a way to facilitate a rapid "zippering" of the two complementary DNA strands during DNA replication and genetic recombination.

The protein product of gene 32 of bacteriophage T4, isolated and characterized by Alberts and co-workers (Alberts et al., 1968; Alberts and Frey, 1970), is essential for DNA replication and genetic recombination (Kozinski and Felgenhauer, 1967; Tomizawa et al., 1966) in T4-infected *Escherichia coli*. Gene 32 protein has a relatively low affinity for native double-stranded DNA but binds very tightly and cooperatively to single-stranded DNA (Alberts et al., 1968; Alberts and Frey, 1970; Jensen et al., 1976; Kelly and von Hippel, 1976; Kelly et al., 1976). By preferential and cooperative binding to single-stranded DNA in the cell, this protein may (a) provide an appropriate conformational arrangement of the DNA for effective T4 DNA polymerase action (e.g., local unwinding of duplex DNA in advance of the replication fork), (b) protect single-stranded DNA from nuclease attack, or (c) prevent the formation of self-complementary hairpin loops. The latter action would facilitate the rate of reassociation of homologous DNA strands, a reaction which might be important to recombination.

One molecule of gene 32 protein contains five tryptophan and nine tyrosine residues (Anderson and Coleman, 1975). When a solution of gene 32 protein is excited at 280 nm, it exhibits a typical tryptophan fluorescence with the emission maximum at 340 nm (Kelly and von Hippel, 1976). Addition of single-stranded DNA to the protein solution produces a partial quenching of the protein fluorescence (Kelly and von Hippel, 1976; Hélène et al., 1976). The fluorescence quenching can be reversed by increasing the ionic strength of the solution, which results in dissociation of the protein–DNA complex. Taking advantage of the fluorescence signal, Kelly et al. (1976) have obtained thermodynamic parameters characterizing the cooperative and noncooperative binding of gene 32 protein to various synthetic poly- and oligonucleotides. In the present studies, we extend this approach to the study of the interaction of gene 32 protein with single-stranded fd DNA. Moreover, the kinetics of dissociation of the gene 32 protein–fd DNA complex was investigated by an ionic strength perturbation using stopped-flow techniques.

Materials and Methods

Samples of *E. coli* strains SW 1485 (Su^-) and CR 63 (Su^+) and phage T4 M41 were generously provided by Dr. D. A. Goldthwait.

Large amounts of T4 M41 phage were prepared by infecting *E. coli* cells CR 63 (Su^+) with T4 M41 phage. After the cells lysed, the debris was removed by centrifugation at 5000 rpm for 10 min, and the phage was collected by centrifugation at 12 000 rpm for 0.5 h. Phage particles so prepared were used to infect (multiplicity of infection ~ 10) *E. coli* cells SW 1485 (Su^-) grown to $\sim 3 \times 10^8$ cells/mL. The cells were harvested and protein was isolated according to the procedure of Alberts and Frey (1970). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber and Osborn, 1969) revealed that the isolated protein was more than 93% homogeneous as determined by the densitometric scanning of the gel. The molecular weight was estimated to be 35 500, which agrees well with the value of 35 000 reported by Alberts and Frey (1970). The concentration of protein was determined spectrophotometrically using an extinction coefficient of 3.7×10^4 M^{-1} cm^{-1} (Jensen et al., 1976).

The fd DNA was isolated from fd phage by phenol extraction (Hoffman-Berlin et al., 1963). The concentration of fd DNA was estimated spectrophotometrically using the extinction coefficient of 7.37×10^3 M^{-1} cm^{-1} per phosphate (Berkowitz and Day, 1974).

Absorption spectra were measured with a Cary-118C spectrophotometer. Fluorescence measurements were carried out using a Perkin-Elmer Hitachi MPF-3 spectrofluorometer. The absorbance of samples at the excitation wavelength was always less than 0.05, so that corrections due to inner-filter effects were not necessary.

Kinetic measurements were performed using a Durrum stopped-flow instrument. The original UV–vis source and

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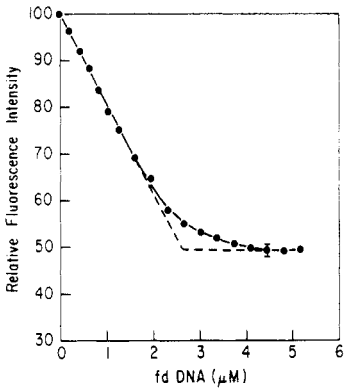

FIGURE 1: Titration of gene 32 protein fluorescence with fd DNA. Protein, 4.7×10^{-7} M, was excited at 280 nm, and fluorescence emission was measured at 337 nm. The titration was carried out in 0.1 M Tris-HCl (pH 7.0) at 25 °C.

monochromator were replaced with a more powerful 200-W xenon–mercury arc lamp (Hanovia) and a Bausch & Lomb high-intensity monochromator. The system, when adjusted properly, has a signal to noise ratio (peak to peak) of at least 1000 in the transmittance mode. To prevent the formation of bubbles during flow, all buffers were degassed.

Results and Data Analysis

Fluorescence Titration. The equilibrium binding of gene 32 protein to fd DNA was studied by fluorescence titration. When gene 32 protein was titrated with fd DNA, approximately 50% of the protein fluorescence was quenched at the saturating concentration of DNA. As shown in Figure 1, the fluorescence intensity of the protein decreased linearly with the initial increase in the concentration of fd DNA but leveled off at higher concentrations. If one assumes an infinite binding affinity for the protein and fd DNA, the fluorescence quenching should be linear with added DNA up to a saturation point after which the fluorescence intensity should remain constant. For a finite, but high-binding affinity such as the case shown here, no sharp break in the titration curve would be observed. However, a stoichiometric point can be obtained from the intercept of two straight lines extrapolated from the linear and saturation portions of the titration curve (Kelly et al., 1976). The stoichiometric point determined this way (Figure 1) indicates that gene 32 protein monomer covers an average of six nucleotide bases on fd DNA. This value is in good agreement with the value of 5 (±1) reported by Kelly et al. (1976) for the binding of gene 32 protein to poly(dA) and with the value of seven reported by Hélène et al. (1976) for the binding of the same protein to denatured *E. coli* DNA.

It is difficult to determine the binding constant from the fluorescence titration data due to the high affinity of gene 32 protein for fd DNA. Nevertheless, a lower limit for the apparent association constant, K_{app}, can still be estimated from the stoichiometric point according to the following equation (Kelly et al., 1976).[1]

$$K_{app} = \frac{\theta}{(1-\theta)^2[P_0]} \quad (1)$$

where θ is the fractional saturation of the protein at the stoichiometric point and $[P_0]$ is the total concentration of the protein. Using this approach, we estimated the lower limit for the apparent association constant to be 1.9×10^8 M^{-1} in 0.1 M Tris-HCl (pH 7.0) at 25 °C. Again, this value is in accord with the value of 2.4×10^8 M^{-1} reported for the binding of gene 32 protein to poly(dA) (Kelly et al., 1976).

A fundamental physical characteristic of gene 32 protein binding to the single-stranded DNA is that this binding is cooperative; i.e., binding of the first protein molecule to DNA facilitates binding of the second. Alberts and co-workers (Alberts et al., 1968; Alberts and Frey, 1970) have demonstrated that the affinity of gene 32 protein for single-stranded DNA increases with increasing protein concentration. That the binding is cooperative was further supported by electron microscopic studies (Delius et al., 1972). At less than saturating levels of gene 32 protein, the distribution of protein along fd DNA was nonrandom; the protein tended to bind in long clusters so that some nearly saturated complexes were formed while other DNA molecules were nearly free of protein. For cooperative binding of the protein to a single-stranded DNA, the cooperativity may be expressed by defining the parameter ω, which represents the increase in binding constant for a second contiguously bound protein molecule above the intrinsic association constant, K_{int}, which would apply in the absence of cooperative interaction. Thus, $K_{app} = K_{int}\omega$ for protein binding to very long DNA under the condition that end effects or overlap binding can be neglected (McGhee and von Hippel, 1974). Taking an average value (2×10^5 M^{-1}) of the association constant for gene 32 protein binding to various dinucleotides (the smallest binding unit for the protein) reported by Kelly et al. (1976) as K_{int}, one can calculate a lower limit of the cooperativity parameter ω for the protein binding to fd DNA to be approximately 10^3. This value agrees with that estimated from the gene 32 protein-perturbed melting transitions of double-helical poly(dA–dT) (Jensen et al., 1976). The value of ω was used in the kinetic analysis described below.

Ionic Strength Perturbation Kinetics. Both gene 32 protein and fd DNA are charged macromolecules, so one would expect their interaction to be sensitive to the ionic strength of the solution. When 0.6 M NaCl was added to a solution containing the gene 32 protein–fd DNA complex at low ionic strength (0.1 M Tris-HCl, pH 7.0), the fluorescence intensity of the solution increased to the value corresponding to that of the free protein, indicating that the system had undergone a transition to a state where separate components were formed. Since no significant change in the fluorescence intensity of free protein was noted by the addition of the same concentration of salt, the observed fluorescence enhancement can be used as an index of the extent of dissociation of the complex. Monitoring this fluorescence change, we have carried out kinetic studies of the dissociation of the complex after an ionic strength jump produced by rapid mixing of the complex with NaCl using a stopped-flow apparatus. A typical oscilloscope trace of the ionic strength jump kinetics is shown in Figure 2. As can be seen, the kinetic curve does not appear to be a single exponential decay, suggesting that the dissociation process is not a simple first-order reaction, rather it is quite complex. This is not surprising, since the kinetic mechanisms involved in cooperative binding of ligands to linear biopolymers is known to be very complicated and may involve many intermediate states (Schwarz, 1968). Thus, one would expect that the gene 32 protein–fd DNA dissociation kinetics may also be complicated, representing the summation of many individual steps. Quantitative analysis of such complex kinetics is very difficult; however, as will be shown below, some useful information can still be obtained by analyzing the early

[1] As indicated in the paper of Kelly et al. (1976), this formulation ignores the effect of "overlap" binding described by McGhee and von Hippel (1974). For highly cooperative binding situations, e.g., the binding of gene 32 protein to fd DNA studied here, the effect of "overlap" becomes insignificant.

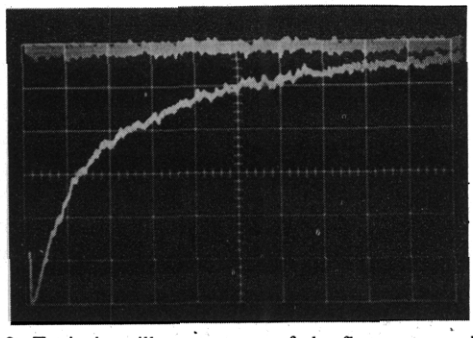

FIGURE 2: Typical oscilloscope trace of the fluorescence change accompanying the dissociation of gene 32 protein from fd DNA after ionic strength perturbation. One syringe contained 3.3×10^{-7} M protein and 3×10^{-5} M (phosphate) fd DNA in 0.1 M Tris-HCl (pH 7.0). The other syringe contained 0.1 M Tris-HCl (pH 7.0) and 1.2 M NaCl. After rapid mixing of these two solutions with the stopped-flow apparatus, the change in fluorescence at 337 nm was recorded on the oscilloscope. The horizontal line shown on the top is the trace obtained after the dissociation is complete. The time scale is 0.1 s/large division and the vertical scale is 0.2 V/large division.

portion of the kinetic curve based on a cooperative dissociation model.

A kinetic model for the cooperative dissociation of gene 32 protein from its complex with fd DNA can be formulated as follows. It has been shown that due to the high cooperativity in binding gene 32 protein molecules are bound contiguously on fd DNA to form long clusters (Delius et al., 1972). The release of protein from the complex can be described using a notation similar to that introduced by Schwarz (1970) for cooperative binding of ligands to a linear biopolymer:

$$\text{- - - uaa - - -} \underset{k_R}{\overset{k_D}{\rightleftharpoons}} \text{- - - uua - - - + A} \qquad (2)$$

where uaa represents a segment of fd DNA having one empty (u) and two occupied (a) protein binding sites, and A represents the free protein. The rate equation for the dissociation of bound protein from the protein–DNA complex is:

$$-\frac{dC_a}{dt} = 2k_D C_{uaa} - 2k_R C_A C_{uua} \qquad (3)$$

where C_a and C_A are the concentrations of bound and free protein, and C_{uaa} and C_{uua} are the concentrations of uaa and uua segments, respectively. The factor 2 takes into account that each "a" sequence provides two ends as potential sites for protein release or rebinding (note that $C_{uua} = C_{auu}$ and $C_{uaa} = C_{aau}$). If one considers only the initial velocity of the dissociation of the complex induced by the ionic strength perturbation, then the second term in eq 3 is negligible so that the equation becomes:

$$-\frac{dC_a}{dt} = 2k_D \bar{C}_{uaa} \qquad (4)$$

where the overbar denotes the equilibrium value at low ionic strength, which is the initial condition of the reaction. Dividing both sides of the equation by $\bar{C}_a$, one can write the rate of dissociation immediately after the perturbation in terms of the reciprocal of an initial relaxation time, τ:

$$-\frac{1}{\bar{C}_a}\frac{dC_a}{dt} = 2k_D\left(\frac{\bar{C}_{uaa}}{\bar{C}_a}\right) = \frac{1}{\tau} \qquad (5)$$

The equilibrium concentration, $\bar{C}_{uaa}$, can be derived utilizing an analytical procedure of statistical mechanics. For a limiting case where ligand molecules bind cooperatively to a very long polymer, $\bar{C}_{uua}$ can be expressed as (Schwarz, 1970):

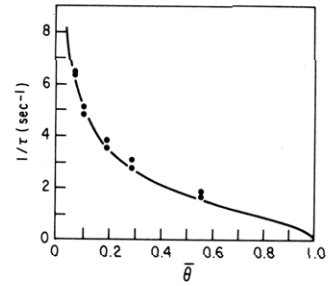

FIGURE 3: Plot of the reciprocal of initial relaxation time, $1/\tau$, as a function of the fractional saturation, $\bar{\theta}$. The concentration of protein (C_A^0) was held constant at 1.65×10^{-7} M in all kinetic runs and the concentration of fd DNA (C_B^0) was varied from 2.9×10^{-7} M to 2.5×10^{-6} M. The solid line represents the values of $1/\tau$ calculated according to eq 11 assuming $k_D = 27\ s^{-1}$ and $\omega = 1000$.

$$\bar{C}_{uua} = \frac{1}{\lambda_0^2}\left(\frac{S}{\omega}\right)^{1/2}[\bar{\theta}(1-\bar{\theta})]^{1/2} C_B^0 \qquad (6)$$

where C_B^0 is the total concentration of protein binding sites on fd DNA and $\bar{\theta}$ is the fractional saturation of binding sites. λ_0 is defined as

$$\lambda_0 = 1 + \left(\frac{S}{\omega}\right)^{1/2}\left(\frac{\bar{\theta}}{1-\bar{\theta}}\right)^{1/2} \qquad (7)$$

and S is related to $\bar{\theta}$ and ω by

$$\frac{S}{(1-S)^2} = \omega\left[\frac{\bar{\theta}(1-\bar{\theta})}{(1-2\bar{\theta})^2}\right] \qquad (8)$$

For cases with strong cooperativity, both λ_0 and S become nearly equal to unity. Under these conditions, one can use the relationship of $\bar{C}_{uua} = S\bar{C}_{uaa}$ (Schwarz, 1970) to obtain the following expression for $\bar{C}_{uaa}$

$$\bar{C}_{uaa} = \left[\frac{\bar{\theta}(1-\bar{\theta})}{\omega}\right]^{1/2} C_B^0 \qquad (9)$$

At or near the initial equilibrium ($C_A \simeq 0$)

$$\bar{\theta} = \frac{\bar{C}_a}{C_B^0} \simeq \frac{C_A^0}{C_B^0} \qquad (10)$$

where C_A^0 is the total concentration of protein added to fd DNA. Inserting eq 9 and 10 into eq 5, the expression for the reciprocal of initial relaxation time immediately after the ionic strength perturbation becomes

$$\frac{1}{\tau} = 2\left(\frac{k_D}{\omega^{1/2}}\right)\left(\frac{C_B^0}{C_A^0} - 1\right)^{1/2} \qquad (11)$$

The initial relaxation time, τ, was determined experimentally by drawing a tangent to the observed kinetic curve at $t = 0$, which intersects the final equilibrium line at $t = \tau$. Figure 3 shows that the reciprocal of initial relaxation time, $1/\tau$, obtained experimentally is a complex function of the fractional saturation, $\bar{\theta}$. If the dissociation of gene 32 protein from fd DNA were a simple first-order process, one would expect $1/\tau$ to be independent of $\bar{\theta}$. When $1/\tau$ is plotted against $(C_B^0/C_A^0 - 1)^{1/2}$, the plot can be fitted to a straight line (Figure 4) showing good agreement between theory and experiment. The slope of the linear plot is equal to $2k_D/\omega^{1/2}$. Using the value of $\omega = 10^3$ determined at low ionic strength (0.1 M Tris-HCl, pH 7.0), one can calculate the apparent dissociation rate constant $k_D = 27\ s^{-1}$.

Discussion

Our data from ionic strength perturbation studies of the gene 32 protein–fd DNA complex are not consistent with simple, first-order dissociation kinetics, suggesting that the release of

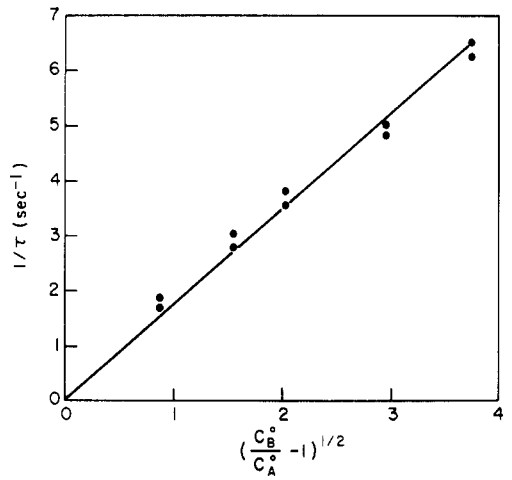

FIGURE 4: Relationship between $1/\tau$ and $(C_B^0/C_A^0 - 1)^{1/2}$. The final concentration (after mixing) of C_A^0 was kept constant at 1.65×10^{-7} M, and C_B^0 was varied from 2.9×10^{-7} to 2.5×10^{-6} M.

individual protein molecules from the complex is not a random, independent process. The data can, however, be analyzed in terms of a kinetic model in which the protein molecules dissociate cooperatively. The large number of possible intermediate states involved in this model is expected to lead to complex kinetics for the overall process. We have avoided difficulties due to the nonlinearity of the kinetic rate equations by restricting ourselves to small deviations from the initially existing chemical equilibrium. Fortunately, for the cases of very strong cooperativity, the initial part of the overall dissociation process can, in principle, be approximated by first-order kinetics and thus be described by a single relaxation time. Such an initial relaxation time can be conveniently determined from the initial slope of the pertinent experimental curve. It is, therefore, possible to obtain valuable kinetic information by a relatively simple analysis of what appear to be rather complicated experimental data. Although other more complex kinetic mechanisms have not been ruled out rigorously, the model presented here provides some interesting implications for the biological functions of gene 32 protein.

According to our model, the cooperative dissociation of gene 32 protein from its complex with fd DNA can be envisioned as a sequential, nonrandom release of protein starting from either one or both ends of a bound-protein cluster. When the ionic strength jump is applied, the long clusters of bound protein peel off from fd DNA in an all or none manner, similar to the kinetic behavior of helix-coil transitions of polypeptides (Schwarz, 1965) and polynucleotides (Saunders and Ross, 1960; Ross and Sturtevant, 1960). This kind of mechanism represents a very rapid and efficient way for dissociation of a large number of proteins from a linear array of binding sites.

Alberts and Frey (1970) observed that the presence of gene 32 protein accelerates the renaturation of T4 DNA under physiological conditions. It was proposed that gene 32 protein induces a conformation of the single strands of T4 DNA which is responsible for the facilitation of renaturation. Since gene 32 protein shows a very low affinity for native double-stranded DNA [three orders of magnitude lower than that for single-stranded DNA (Jensen et al., 1976)], it seems likely that the bound protein is rapidly displaced from the rewinding single strands as the double helix forms. Rewinding of the double helix in locally unwound regions of native DNA and in replication forks is a necessary step in genetic recombination and DNA replication. In order to facilitate a rapid "zippering" of the two complementary DNA strands, the cooperative release of gene 32 protein described above may be essential. Although there is no evidence that local ionic strength fluctuations occur near replication forks or recombination sites, it is not inconceivable that propagation of the helix formation may be caused by local perturbations similar to the ionic strength jump.

Further work on the kinetics of cooperative binding of gene 32 protein to fd DNA is in progress in our laboratory.

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