Mechanism and Role of Cooperative Binding of Bacteriophage fd Gene 5 Protein to Single-Stranded Deoxyribonucleic Acid†

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ABSTRACT: The highly cooperative binding of fd gene 5 to single-stranded DNA was studied kinetically by rapid photo-cross-linking and stopped-flow UV absorption measurements. The observed change in absorbance was shown to be due to the binding by direct evidence of rapid photo-cross-linking of the bound proteins to fd DNA. The bimolecular rate constant obtained for the association was 1.6×10^{10} M^{-1} s^{-1} (in terms of the molecular concentration of DNA), which was concluded to be diffusion controlled. The breakdown of cluster complexes on fd DNA was induced by the addition of large excess amounts of short single-stranded DNA. The breakdown took place in about 1 s. The kinetic process of redistribution of dissociated proteins was monitored by rapid photo-cross-linking and subsequent electrophoresis of the cross-linked complex. The dissociated proteins first formed isolated complexes, but later they were again converted into the cluster. The kinetic results showed that the cooperativity originated from the stabilization of the protein–DNA complex by the cluster formation, not from the accelerated association in the cluster formation. This kind of cooperative binding was shown to perform negative feedback control in the cluster formation. On the basis of the kinetic results obtained, we proposed a model for the regulatory role of the fd gene 5 protein in the synthesis of single-stranded fd DNA.

Multiple binding is one of the most characteristic reactions among various modes of interaction between protein and nucleic acids. Many protein molecules can bind to a single nucleic acid chain, where cooperative binding is often observed. The most well-known example is the helix-destabilizing proteins (Alberts & Sternglanz, 1977) which bind to single-stranded DNA. Some of these proteins were proved to be essential for DNA replication. Biochemical and physicochemical analyses have been most advanced for the bacteriophage fd gene 5 protein and the T4 gene 32 protein [see Kowalczykowski et al. (1982) for example].

The bacteriophage fd gene 5 protein is known to be essential for fd DNA replication from the fd replicative form of DNA (RF DNA)[1] (Pratt & Erdahl, 1968; Salstrom & Pratt, 1971; Mazur & Model, 1973; Mazur & Zinder, 1975). The newly replicated fd DNA is covered with the gene 5 protein and transferred into the cell membrane in a form of the gene 5–DNA complex (Webster & Cashman, 1973; Pratt et al., 1974; Mazur & Zinder, 1975). The protein exists only in infected cells with a copy number of 10^5 per cell (Oey & Knippers, 1972; Alberts et al., 1972).

The bacteriophage fd does not lyse its host cell, and the infected cell still continues to grow. In addition, the two phases of fd replication, one from RF to RF and the other from RF to the single-stranded form, coexist at an early stage of infection (Forsheit et al., 1971; Ray, 1977). Therefore, at most, three replication systems, the host replication and the two phases of fd replication, coexist at a certain stage of infection. This coexistence suggests a regulatory mechanism for fd DNA replication. Recently, the gene 5 protein was shown to be a translational inhibitor for the synthesis of the gene 2 protein (Model et al., 1982; Yen & Webster, 1982). Since the gene 2 protein is the key enzyme in producing the "rolling circle intermediate" from fd RFI DNA and the circular fd DNA from the intermediate (Meyer & Geider, 1982; Geider et al., 1982), the accumulation of the gene 5 protein may contribute in maintaining the host replication. In addition, the gene 5 protein has a stronger affinity for single-stranded DNA than the host helix-destabilizing protein (Geider, 1978) which is essential for host replication (Meyer et al., 1980) and required for in vitro fd replication (Meyer & Geider, 1982). Therefore, the strong and highly cooperative binding of the protein to the single-stranded region of the fd replication intermediate was believed to protect the region from the synthesis of lagging strands (Ray, 1978). From these findings arises a question: why the large amount of gene 5 protein in the cell does not injure the host replication, and the production of fd RF at an early stage of infection. In other words, how is the cooperative binding of the gene 5 protein involved in the regulated synthesis of single-stranded fd DNA?

The simplest regulation may be the negative feedback mechanism by weakly positive cooperativity. Such a regulatory model was proposed for the RF to RF synthesis of the bacteriophage ϕX174 (Ikeda et al., 1979): The gene A protein of the phage binds to ϕXRFI DNA with weakly positive cooperativity; the formation of a protein cluster is essential to the specific nicking activity of the protein; an overproduction of ϕXRFI DNA breaks the cluster because of the weak cooperativity, leading to a suppression of itself. However, for such a strong cooperativity as shown by the fd gene 5 protein, this regulatory mechanism does not work.

There are at least two types of protein–DNA complexes; one is the isolated complex which consists of a single protein molecule bound to an uncomplexed region of DNA, and the other is the cluster complex formed as a contiguous protein sequence along the DNA chain. The ratio of the binding constant of a protein molecule for the cluster complex to that for the isolated complex is known as the cooperativity constant and sometimes denoted as ω. This constant for the gene 5 protein was reported to be as large as 2000 (Dunker, 1975) or 200 (Alma et al., 1983). In this study, we investigated the kinetic mechanism of this strongly cooperative binding by giving special attention to the formation of the two types of

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[1] Abbreviations: RF DNA, replicative form of DNA; RFI DNA, superhelical, double-stranded closed circular DNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; kb, kilobase; Gdn·HCl, guanidine hydrochloride.

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complexes. On the basis of the kinetic results obtained, we propose a dynamic regulatory mechanism that is still effective in the case of strong cooperativity.

Materials and Methods

Preparation of the fd Gene 5 Protein. Escherichia coli K38 was grown to 8×10^8 cells/mL in a 8-L fermentor, infected with bacteriophage fd at a multiplicity of infection of 0.3, and further cultured up to the stationary state. The gene 5 protein of fd was prepared by the method of Alberts et al. (1972) with an additional phosphocellulose column chromatography. The protein was adsorbed to a 2 × 10 cm P-11 (Whatman) column and eluted with a 0–1 M NaCl salt gradient in the presence of 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM 2-mercaptoethanol, and 10% glycerol. The protein thus prepared was estimated to be more than 97% pure from the results of SDS–polyacrylamide gel electrophoresis.

Tritium-labeled gene 5 protein was obtained by the same method from the cells which were cultured in M-9 medium containing 10 μg/mL each of the common amino acids except leucine, isoleucine, and valine. At about 10^8 cells/mL, the cells were infected with the phage, and 0.5 mCi of tritiated leucine was added to 300 mL of culture. They were further grown for 2 h and harvested.

Preparation of Single-Stranded DNAs. Phage fd DNA was prepared according to Knippers & Hoffmann-Berling (1966). Calf thymus DNA was obtained by the method of Zamenhof (1957). It was further deproteinated with Pronase E (Kaken Kagaku, Tokyo) followed by phenol extraction. Short single-stranded calf thymus DNA was obtained from the DNA by denaturation and sonication with a UR-200P sonicator (Tomy Seiko, Tokyo). It was fractionated in size by preparative urea–acrylamide gel electrophoresis with a horizontal gel of 15 × 30 × 1 cm, and DNA was recovered by electrophoresis using dialysis bags. The approximate length of DNA was determined by referring to the migration of 0.75-, 0.46-, 0.41-, 0.25-, and 0.23-kb fragments derived from λ DNA by digestion with *Hpa*I (Takara Shuzo, Tokyo).

Measurements of the Stoichiometry of Gene 5 Protein Binding to DNA. All the binding reactions between the protein and DNAs were made at 30 °C in a buffer containing 10 mM Tris-HCl (pH 8.0), 0.15 M KCl, and 0.2 mM dithiothreitol, unless otherwise noted. The stoichiometry of binding between gene 5 protein and fd DNA was determined by fluorescence titration of the protein. A fixed amount of fd DNA was successively added to a 1.2 μM sample of the protein, and the fluorescence intensity at 305 nm with excitation at 276 nm was monitored with a MPF-4 Hitachi fluorescence spectrophotometer. Two straight lines crossing at a point were obtained when the intensity was plotted against the DNA concentration. The concentration of DNA at the point was considered to be stoichiometric for the protein present. The size of the protein binding site on the DNA was calculated with the extinction coefficients of 7300 M^{-1} cm^{-1} for gene 5 protein at 276 nm (Oey & Knippers, 1972) and 8000 M^{-1} cm^{-1} for fd DNA per nucleotide at 260 nm (Day, 1973). The values obtained varied from 3.8 to 4.3 nucleotides per bound protein molecule depending on the protein preparations, but they are in good agreement with the results determined with UV absorption and circular dichroism (Day, 1973).

Stopped-Flow UV Absorption Measurements. The time course of binding between the gene 5 protein and fd DNA was measured by the UV absorption stopped-flow technique. The absorbance change at 276 nm was measured with a stopped-flow spectrophotometer, Model RA-401 (Union Giken, Osaka). Solutions of the protein and the DNA were driven by nitrogen gas of 3 atm. After rapid mixing, the solution was led to an observation cell of 1-cm path length. The original system of RA-401 produced an artifact relaxation due to incomplete mixing when fd DNA was mixed with the buffer. The artifact was eliminated by inserting a small Teflon disk with three pinholes in it between the mixer and the cell. Signals were processed with a System 77 microcomputer (Union Giken). They were recorded with double time bases with 250 points per time base. Signals were averaged over 20–40 measurements. The averaged signals were fitted to a theoretical curve as mentioned under Results. The dead time of this system was determined to be 4 ms from the measurements of the reduction kinetics of 2,6-dichlorophenol–indophenol by ascorbic acid. For a survey of slower relaxations, a Hitachi Model 320 spectrophotometer was used.

The amplitude of the kinetic relaxation was also compared with the static difference absorbance. Solutions for difference spectroscopy were prepared by mixing common original solutions by weight in order to attain an improved accuracy.

Rapid Photo-Cross-Linking between Gene 5 Protein and fd DNA and Analyses of the Cross-Linked Material by Ultracentrifugation and SDS–Polyacrylamide Gel Electrophoresis. A hand-built multimixing apparatus with three channels was used for the rapid photo-cross-linking of the gene 5–fd DNA complex. Three solutions, respectively containing the labeled protein, fd DNA, and quencher, were simultaneously driven by nitrogen gas of 1.5–3 atm. The first two solutions were mixed at mixer 1 and led to mixer 2. The third solution was then mixed with the mixture, and the final mixture was collected in a 10-mL syringe.

A cylindrical cell of 2-mm path length was located in the fluid path between the two mixers. Light from a 450-W xenon arc lamp was focused on the cell to photo-cross-link the protein–DNA complex formed by the first mixing. Two layers of thin poly(vinylidene chloride) film were used as a filter since UV light with a wavelength shorter than 240 nm would decompose the photo-cross-linked complex (Paradiso et al., 1979). A mirror was placed behind the cylindrical cell to best utilize the transmitted light. Photo-cross-linking was made with a light dose of 1.9×10^4 erg/mm^2 or more in most cases. In order to photo-cross-link the complex at 0.2 s or later after the first mixing, the purging of the three solutions was interrupted, and the mixture of the protein and DNA was aged in the channel between mixer 1 and the cell. After the aging, the solutions were purged again, led to the cell for photo-cross-linking, and then quenched at mixer 2.

Half of the photo-cross-linked material was centrifuged with a Hitachi 70P-72 ultracentrifuge using an RPV65T vertical rotor at 62 000 rpm at 15 °C for 2 h. Particles with sedimentation coefficients larger than 5 S are pelletted in this condition. The solutions before and after the centrifugation were each mixed with Univergel (Nakarai Chemical, Kyoto), and radioactivity was counted with a liquid scintillation counter, Model LSC-701 (Aloka, Tokyo). The counting efficiency was corrected by the external standard method to convert cpm into dpm.

The cross-linked samples were also analyzed by SDS–polyacrylamide gel electrophoresis. Samples were precipitated with 9.1% (final concentration) trichloroacetic acid in the presence of 50 μg/mL hen egg white lysozyme and 40 μg/mL yeast RNA as carriers. After centrifugation at 3000 rpm for 30 min, the pellet was washed twice with acetone and resuspended in 0.1 M NaOH. The solution was mixed with a solution to finally make 10% glycerol, 10 mM dithiothreitol, and 1% SDS. The mixture was incubated at 37 °C for 10 min

before being loaded onto a 12.5 × 14 × 0.15 cm gel of 15% polyacrylamide. After electrophoresis according to the method of Laemmli (1970), the gel was sliced, and each slice was separately solubilized by autoclaving in the presence of a minimum amount of 30% hydrogen peroxide at 105 °C for 15 min. In this procedure, the tritium in a vial was retained by more than 80% if it was tightly sealed with silicone rubber lining.

Rapid Photo-Cross-Linking of Gene 5 Protein to DNA for Analyses by Urea–Polyacrylamide Gel Electrophoresis. The photo-cross-linking of the gene 5–DNA complex for urea–polyacrylamide gel electrophoresis was carried out with the same multimixing apparatus as described above with the following modifications unless otherwise noted. The third channel was not used for quenching to avoid an increase in volume. Instead, the mixture was quickly transferred to a tube containing urea and all the reagents necessary for gel electrophoresis and mixed thoroughly with a Vortex mixer (Scientific Industries). Such a manual quenching of photo-cross-linking was satisfactory for the stable cluster complex and for the isolated complex formed in the presence of a large amount of single-stranded DNA as well.

Urea–Polyacrylamide Gel Electrophoresis for Monitoring Cluster Formation. The cross-linked complex described above was applied onto a 5% polyacrylamide gel. The gel composition was similar to the SDS gel except that SDS was replaced by 7 M urea. Sample solutions contained 7 M urea, 125 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, and 0.02% bromphenol blue, including about 0.1 M KCl arising from the cross-linking reaction mixtures. The running buffer contained 0.3% Tris base and 1.44% glycine. A sample solution of 2 mL was poured into a slot with a wide opening shaped like a prism. Since samples were condensed into a sharp band in the Laemmli gel system (Laemmli, 1970), such a slot was used for in situ concentrating the solution.

Theoretical Simulation of the Time Course of Binding Reactions. The time courses of complex formation for the theoretical models described under Discussion were simulated with the microcomputer. The frequency of each binding or dissociating event for a unit time interval was determined stochastically by using a random library function of the computer. The function generated a random number between zero and twice the assumed rate of the reaction. The integer part of the number was used as the frequency of the reaction. The binding locus was also chosen by a similar stochastic procedure. Thus, the number of isolated complexes and the protein molecules in clusters were calculated at each time interval.

In the association-controlled model, the association rate constant for the flanking sites of clusters was assumed to be 2000 times larger than that for the isolated binding site. In the dissociation-controlled model, the dissociation rate constant for the cluster complex was assumed to be 2000 times smaller than that for the isolated complex. The equilibrium constant between free protein molecules and the isolated complex was tentatively assumed to be unity (molecules^{-1}). The actual time courses thus simulated exhibited small stochastic fluctuations, but they were neglected.

Results

Binding Kinetics Obtained by Stopped-Flow UV Absorption Measurements. The binding between the gene 5 protein and fd DNA was investigated by the stopped-flow method with UV absorption as a probe. Day (1973) has shown that light scattering contributed to the absorbance change on complex formation, and the change was as large as 30% of the total absorbance of the protein. We confirmed his results and found

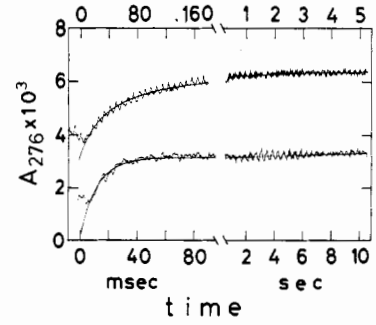

FIGURE 1: Time courses of the absorbance change at 276 nm due to the binding of fd gene 5 protein to fd DNA. The gene 5 protein (1.2 μM final concentration) and an excess amount of fd DNA were mixed in a stopped-flow apparatus at 30 °C in a buffer containing 10 mM Tris-HCl (pH 7.9), 0.1 M KCl, and 0.2 mM dithiothreitol. (Upper curve) 1.6 times excess DNA (7.7 μM nucleotide) was mixed. The solid line represents the best-fit curve of eq 2 with 2.62×10^6 M^{-1} s^{-1} for k_{sec} and 2.8×10^{-3} for C. (Lower curve) 6.4 times excess DNA (30.7 μM nucleotide) was mixed. The solid line represents the best-fit relaxation curve of eq 1 with 84.4 s^{-1} for k and 2.8×10^{-3} for C. The instance when the reaction was started is shown as time zero.

that the difference spectrum was affected by the optical alignment of the spectrophotometer used. We chose the wavelength of 276 nm for the measurement with the stopped-flow spectrophotometer. The signal to noise ratio was good, and the apparent change in absorbance was proportional to the amount of the complex as mentioned later.

We examined the time region covered from 4 ms to 2 min. When 1.2 μM (final concentration) gene 5 protein was mixed with fd DNA in excess amounts in terms of the binding sites, an increase in absorbance in the wavelength region between 260 and 285 nm was observed (Figure 1). Control experiments performed by mixing the buffer either with fd DNA or with the gene 5 protein gave no relaxations. The increase also disappeared by the addition of 0.75 M KCl.

In the presence of a DNA concentration greater than 30.7 μM, a pseudo-first-order reaction was observed, and the relaxation rate constants were calculated by fitting the data to an exponential relaxation curve:

$$\text{absorbance change} = X = C[1 - \exp(-kt)] \qquad (1)$$

where C is the total absorbance change and k is the relaxation rate constant. It is expected, in contrast, that if the number of binding sites on DNA is not much in excess of that of the protein second-order kinetics will result. The data obtained with DNA in 1.6 or 3.2 times excess in fact gave a better fitting to the following second-order relaxation curve than eq 1 (Figure 1):

$$(C - X)/(D - X) = (C/D) \exp[-k_{sec}(D - C)t] \qquad (2)$$

Here, X and C have the same meanings as in eq 1, k_{sec} is the second-order rate constant, and D is the product of C and the ratio of the amount of binding sites on DNA to that of the protein. In other words, D represents an imaginal absorbance change when all the DNA binding sites are occupied by the protein. The left-hand side of eq 2 was first calculated from the observed values of X and C and then fitted to the right-hand side of eq 2.

In the pseudo-first-order kinetics at higher DNA concentrations, the relaxation rate constant becomes proportional to the DNA concentration if the dissociation is negligible. The proportional constant should be equal to k_{sec} obtained in the second-order kinetics at lower DNA concentrations. The values of k/[DNA] obtained by using eq 1 at high DNA concentrations were compared in Table I with the values of

Table I: Kinetic Parameters Obtained from Stopped-Flow UV Absorption of 1.2 μM Gene 5 Protein at 30 °C

[fd DNA] (nucleotide μM)	order of reaction	k_{sec} or k/[fd DNA] ($\times 10^6$ M^{-1} s^{-1})	total relaxation amplitude, A_{276} ($\times 10^{-3}$)
7.7	second	2.62 ± 0.05[a,c]	2.8[d]
15.4	second	2.64 ± 0.06[a,c]	2.9[d]
30.7	pseudo first	2.75 ± 0.02[a,c]	2.8[d]
46.1	pseudo first	2.43 ± 0.20[b,c]	2.6[d]
61.4	pseudo first	2.46 ± 0.18[b,c]	2.7[d]
7.7	second	2.91 ± 0.11[a,f]	1.4[d]
7.7	static[e]		2.8

[a] The values were calculated by least-squares fitting to the second-order relaxation curve, eq 2. [b] The values were obtained by fitting to eq 1. [c] The weighted average of these values gives a value for the second-order rate constant of (1.6 ± 0.1) × 10^{10} M^{-1} s^{-1} in terms of the molecular concentration of fd DNA. [d] The values were obtained from the best-fit curve by taking into account the dead time for the rapid mixing. [e] Difference absorbance measurements by alternating flushings. See Results for details. [f] The concentration of the protein was 0.6 μM.

k_{sec} obtained by using eq 2 at nearly stoichiometric concentrations. The good agreement suggests that the relaxation actually arises from the binding step which is second order at low DNA concentrations and becomes pseudo first order at high DNA concentrations. The molecular second-order binding rate constant was calculated to be (1.6 ± 0.1) × 10^{10} M^{-1} s^{-1} when the molecular concentration of DNA was used. This value is one of the largest association rate constants of the protein–DNA interaction ever observed and reaches the limit of diffusion control as will be discussed later.

When the dead time of the mixing was taken into account, the total change in absorbance at 276 nm, C, was about 2.8 × 10^{-3}, which was independent of DNA concentration (Table I). To make sure that there were no more detectable relaxations, a static measurement of the difference absorbance was made by using the same stopped-flow apparatus because the same optical alignment was required for the exact measurements. When a valve at the end of a reservoir was closed, the solution in the other reservoir could be flushed into the observation cell. The absorbance of the protein was measured by alternate flushings either of the protein solution or of the buffer. A similar measurement was made with the DNA solution in place of the buffer. The result obtained was 2.8 × 10^{-3} at 276 nm at 7.7 μM DNA in exact agreement with the kinetic result (Table I). In addition, the total change became half when the amount of the protein was diminished to half. This result confirmed that there were no more detectable relaxations and suggested that the absorbance change was proportional to the formation of the complex. In fact, no relaxations were found up to 1 min with the stopped-flow apparatus and up to 1 h with a conventional spectrophotometer.

Rapid Photo-Cross-Linking between Gene 5 and fd DNA. The results obtained from the rapid kinetics with the spectroscopic probe suggest that the observed relaxation is due to the binding. We tried to confirm this interpretation by using more direct probes than the spectroscopic one. Since strong UV light cross-links the gene 5–DNA complex in vitro (Paradiso et al., 1979) and in vivo (Lica & Ray, 1977; Paradiso & Konigsberg, 1982), a rapid photo-cross-linking was used to monitor the amount of the gene 5–DNA complex formed. The experimental system, system a, is schematically illustrated in Figure 2a. The tritium-labeled gene 5 protein (1.2 μM) was first mixed with fd DNA (30 μM) at mixer 1, and the mixture was made to flow across the UV light beam

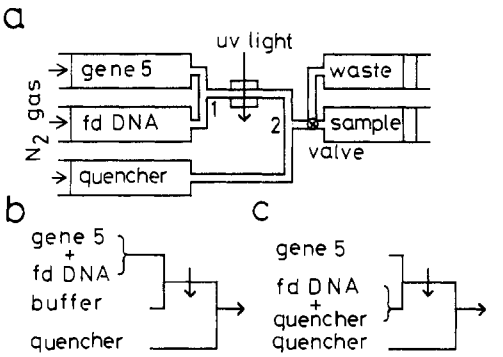

FIGURE 2: Schematic illustration of the rapid photo-cross-linking experiment. (a) fd gene 5 protein and fd DNA were driven pneumatically into the first mixer (1) by nitrogen gas and then irradiated with UV light for photo-cross-linking. The mixture was then mixed with the quencher at the second mixer (2). The quenched solution was driven into the sample syringe. The valve was switched from the waste syringe to the sample syringe when the freshly quenched solution reached the valve. (b) Control system to determine the efficiency of photo-cross-linking. The gene 5–fd DNA complex was preformed. (c) Another control system to monitor the quenching efficiency. The quencher was premixed with DNA.

Table II: Rapid Photo-Cross-Linking between Tritiated Gene 5 Protein and fd DNA at 88 ms after Mixing[a]

system	% gene 5 protein cosedimented with DNA in: Gdn·HCl and mercaptoethanol	% gene 5 protein cosedimented with DNA in: KCl and mercaptoethanol
a	7.24 (94)[b]	11.4 (83)
b	7.81 (100)	12.5 (100)
c	0.83 (0)	5.9 (0)

[a] The final mixture contained 10 mM 2-mercaptoethanol and either 3 M Gdn·HCl or 0.75 M KCl. The radioactivity corresponding to 1% was 45 dpm, and the counting error and the background were 2 and 70.4 dpm, respectively. [b] The values in parentheses are relative ones with 100 for system b and 0 for system c, and their estimated errors are 8.

at 88 ms after the mixing. In this condition, the relaxation of UV absorbance is almost complete (99%) at the stage of irradiation. The mixture was kept irradiated for 18 ms and further kept flowing for 44 ms. It was then mixed with a quenching solution to make the final output 3 M guanidine hydrochloride (Gdn·HCl) and 10 mM 2-mercaptoethanol or 0.75 M KCl and 10 mM 2-mercaptoethanol. The protein is completely denatured with 3 M Gdn·HCl (data not shown), while it cannot bind to DNA in 0.75 M KCl (Oey & Knippers, 1972). A control system to determine the efficiency of photo-cross-linking, system b, is explained in Figure 2b. The protein–DNA complex is preformed and then mixed with the buffer at the first mixing. Another control system, system c, determines the efficiency of quenching (Figure 2c). The output solutions (4500 dpm in 4.5 mL) were ultracentrifuged with the condition that fd DNA was pelleted but the gene 5 protein remained in the supernatant. The radioactivities of the gene 5 protein were counted in the supernatant and in the original mixture before centrifugation. The difference between these values gave the amount of the gene 5 protein pelleted.

The results are summarized in Table II. When the cross-linking was quenched with Gdn·HCl, the quenching was almost perfect (system c), and approximately the same amount of the protein was photo-cross-linked to DNA by rapid mixing (system a) as by preincubation (system b). The quenching with KCl was incomplete; system c produced the pelleted protein as much as 5.9%. This was due to the aggregated protein since the same amount of the protein was pelleted in

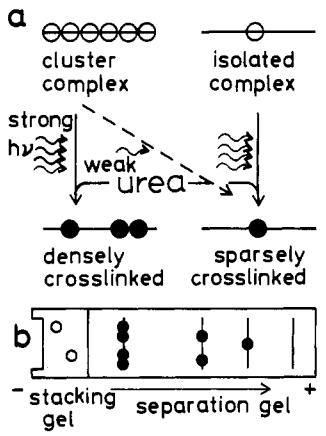

FIGURE 3: Assay of cluster formation of fd gene 5 protein on DNA. (a) Photo-cross-linking of the cluster complex and the isolated complex on a DNA chain produces a densely and a sparsely cross-linked complex, respectively. The protein noncovalently bound (O) is dissociated with urea, but that covalently bound (●) remains. The sparsely cross-linked complex is also produced from the cluster complex with a weaker irradiation. (b) The cross-linked complexes are separated according to the density of the cross-linked protein by urea–polyacrylamide gel electrophoresis. A DNA chain migrates from the left to the right, but the free protein hardly enters into the separation gel due to its positive charge.

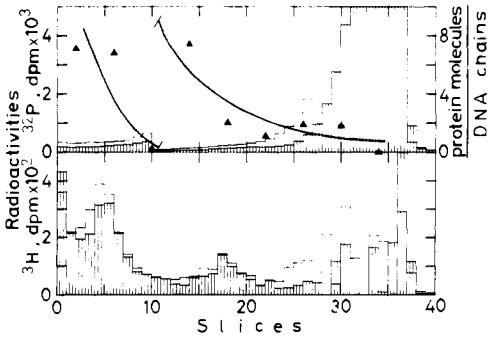

FIGURE 4: Separation of the photo-cross-linked complex according to the density of the protein cross-linked on a DNA chain. 660 pmol of the tritiated gene 5 protein (65.2 dpm/pmol) and 17.6 pmol of the 5′ end labeled DNA chans (2.09×10^4 dpm/pmol) were mixed and irradiated. After the addition of the sample solution including urea, the mixture and the nonirradiated control were loaded on the gel. The gel was cut into 40 slices. The first slice includes the boundary between the stacking gel and the separation gel. The dye front was at position 37. (Upper panel) Distribution of DNA chains in the separation gel. The distribution of the irradiated sample is shown with open bars and the nonirradiated sample with the striped bars. The scale for the radioactivities from slices 11–40 is reduced one-tenth. The number of protein molecules per DNA chain was calculated for every four slices from both the radioactivities of ^{32}P and ^3H (▲). The scale for the number of protein molecules from slices 1–10 is reduced one-tenth. (Lower panel) Distribution of the tritiated protein.

a nonirradiated control of system a. In any case, the amount of the photo-cross-linked protein in system a was more than 80% of that in system b. Therefore, the fraction of the photo-cross-linked protein was almost the same for both the preformed complex and the complex formed by rapid mixing, irrespective of the quenching method. Thus, it is concluded that the complex formation is complete when the UV relaxation is just finished. This indicates that the observed UV relaxation is in fact due to the complex formation.

The photo-cross-linking of gene 5 and fd DNA was further confirmed by SDS–polyacrylamide gel electrophoresis. The same samples as used in ultracentrifugation were analyzed as described under Materials and Methods. The photo-cross-linked protein is expected to migrate less than the 10-kdalton protein. In comparison to system c, systems a and b yielded 10% and 8% more radioactivity, respectively, which migrated less than the 14-kdalton marker protein.

Assay of Cluster Formation by Urea–Polyacrylamide Gel Electrophoresis of Photo-Cross-Linked Gene 5 Protein. We devised a qualitative assay of cluster formation as follows. When we irradiate an isolated complex, the cross-linked complex may have few protein molecules on a DNA chain (Figure 3a). The same irradiation may convert a cluster complex into a densely cross-linked product in the presence of urea. Therefore, the degree of clustering determines the density of the protein covalently cross-linked. Because of the positive charges on the protein and the increased steric hindrance of the "beaded" DNA chains, the photo-cross-linked products migrate at different velocities in urea–polyacrylamide gel electrophoresis (Figure 3b).

Tritiated gene 5 was incubated in a quartz cell with short single-stranded DNA with an average length of 350 bases and was labeled at the 5′ end with ^{32}P. Since most gene 5 was clustered at equilibrium, we produced a sparsely cross-linked complex by weakly irradiating the cluster complex (Figure 3a). The light beam was made slightly out of focus so that the irradiation produced various densities of the cross-linked complexes. On the average 3.5% of the protein was photo-cross-linked. Figure 4 shows the distribution of radioactivities in the gel thus prepared and the number of gene 5 molecules cross-linked to a DNA chain. With no irradiation, most DNA chains migrated further than position 31. The irradiation significantly increased the radioactivity of ^{32}P in slices 1–36. Since we had to use a conventional rectangular well with gel electrophoresis (see below) and to photo-cross-link the protein with a lower efficiency, a somewhat larger amount of ^3H counts (7%) was observed in the gel. However, the counts in slices 2–29 were never diminished as expected by the irradiation. If the counting error of 5 dpm was taken into account, a significant increase in ^3H counts was observed in slices 3–6, 13–17, and 24–32. From these increases, we calculated the number of protein molecules per DNA chain. The number was several tens near the top of the gel and decreased with increasing migration as expected. Even a couple of protein molecules cross-linked hindered the migration of DNA. These results show that the assay of clustering works at least for qualitative estimates; more tritium counts are found near the top of the gel with the cluster formation. The large numbers observed near the top of the gel might be partly due to long DNA molecules slightly contained in the DNA preparation or to the presence of the cluster complex with higher efficiency for photo-cross-linking.

Panels a, b, c, and d, respectively, of Figure 5 show the results of rapid photo-cross-linking experiments using no DNA, fd DNA, and two short single-stranded DNAs with lengths from 700 to 2000 nucleotides and from 200 to 300 nucleotides. The use of a sample well shaped like a prism in gel electrophoresis diminished the amount of un-cross-linked protein that entered the gel (Figure 5a). However, the well is not suitable when we need to carefully compare the results of different lanes because the migration slightly varied from lane to lane. The DNAs were mixed with the protein and irradiated 5 min after the mixings. The results confirmed that the new assay correctly detected the cluster complex formed at equilibrium. The formation was independent of the length of DNA at least in the range tested. If the isolated complex was transiently formed before cluster formation, it should be detected by the new assay method. The gene 5 protein was mixed with a 700-fold excess of short single-stranded DNA with an average length of 300 nucleotides. It was not fractionated in size. Panels e and f of Figure 5 show the results of rapid photo-

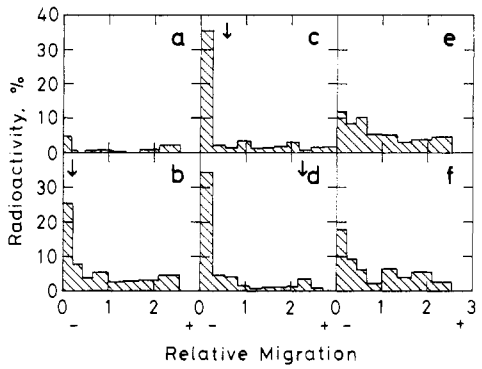

FIGURE 5: Examination of cluster formation by urea–polyacrylamide gel electrophoresis. The distribution of tritiated gene 5 was analyzed by electrophoresis after photo-cross-linking (a) without DNA, (b) with fd DNA, (c) with single-stranded DNA with lengths from 700 to 2000 nucleotides, (d) with single-stranded DNA with lengths from 200 to 300 nucleotides, and (e and f) with a 700 times excess amount of the sonicated single-stranded DNA, unfractionated in size. Cross-linking was made at 5 min after the mixing except for (e) where the cross-linking was made at 2 s. The arrows in (b–d) indicate the center of the band for free DNA without the photo-cross-linking. Approximately 2×10^4 dpm was applied in a slot, and from 5% to 9% of the radioactivity migrated into the gel except for (a) (1%). The radioactivity of each slice is normalized to the total radioactivity of each lane for (b–f). In the case of (a), it is normalized to the average of the total radioactivities for lanes in (b–f).

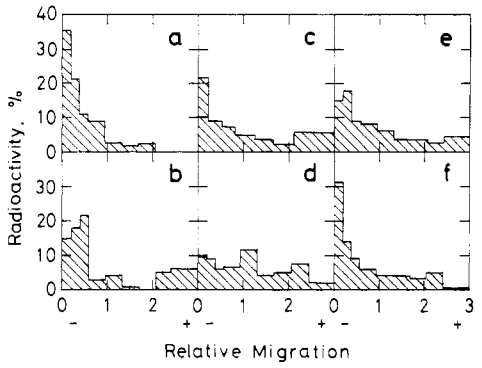

FIGURE 6: Breakdown of the gene 5 cluster on fd DNA monitored by trapping the dissociated gene 5 with short single-stranded DNA. UV irradiation was made at (a) 0.2, (b) 0.6, (c) 1.8, (d) 5, (e) 30, and (f) 300 s after mixing of the tritiated gene 5–fd DNA complex with a 700-fold excess amount of the sonicated single-stranded DNA. Transfer of gene 5 from fd DNA to short DNA is shown from (a) to (d) and re-formation of clusters on the short DNA is shown in (d) and (f).

cross-linking at 2 s and 5 min, respectively, after mixing with the DNA. It is clearly seen by comparing the two results that isolated complexes or clusters of small sizes exist more at 2 s, whereas the cluster complexes dominate at 5 min. The amounts of the photo-cross-linked protein at 2 s and 5 min were 7% and 9% of the total, respectively. Therefore, the change in the distribution indicates the conversion of the isolated complex into the cluster complex rather than cluster formation de novo.

Dissociation of the Gene 5–fd DNA Complex As Monitored with Rapid Photo-Cross-Linking. In the case of the gene 5 protein, its affinity to single-stranded DNA is too strong to allow the coexistence of measurable amounts of free gene 5, DNA, and the complex. In order to quantitate the affinity, it is therefore essential to determine the rate of dissociation of the protein from the cluster. For this purpose, we must invent a method to trap the dissociated protein molecules, which would otherwise bind back to the cluster. Here, we successfully trapped them by photo-cross-linking after addition of a large excess amount of short DNA. The gene 5–fd DNA complex was preformed, and then 700-fold excess amounts of short single-stranded DNA were mixed. Photo-cross-linking was made after a certain period, i.e., the reaction time. The photo-cross-linked complex was analyzed by the same method as described above.

When the mixture of the cluster complex and the short DNA was irradiated at 0.2 s after mixing, most of the gene 5 protein still stayed on fd DNA (Figure 6a). The cross-linked gene 5–fd DNA complex showed a similar distribution as in the absence of short DNA (Figure 5b). However, a significant amount of gene 5 was observed to have been trapped by the short DNA as isolated complexes at 0.6 and 1.8 s (panels b and c, respectively, of Figure 6). We concluded therefore that dissociation of the complex occurred at a rate on the order of 1 s^{-1}. From this overall dissociation rate constant and the second-order rate constant for association obtained by the stopped-flow UV absorption measurements, the dissociation constant of the protein for the cluster complex was calculated to be on the order of 10^{-10} M. In the time interval from 5 to 300 s, the protein once trapped as isolated complexes again started to form clusters as shown in Figure 6d,e. It should be remarked that the amount of the tritiated protein that migrated into the separation gel was similar. Thus, the dissociated protein was first trapped as isolated complexes by the abundant short DNA chains, and then the protein repeated dissociation and association until the protein cluster was again formed on the short DNA. The protein cluster is in fact the most stable complex for this protein at equilibrium.

Discussion

Pulsed Photo-Cross-Linking and Its Efficiency. The light dose we used for the photo-cross-linking may be compared with that used in studies of stationary irradiations. Paradiso et al. (1979) reported a similar efficiency of cross-linking with the same order of dose. A higher efficiency was reported with much less dose in the cross-linking in vivo (Lica & Ray, 1977), but a conflicting result has been reported (Paradiso & Konigsberg, 1982). Since the UV light not only cross-links the complex but also slowly breaks it (Paradiso et al., 1979), a significant comparison between our pulsed method and the conventional method is difficult. The differences in the light source, the time period for irradiation, the filtering effect by samples, and the absolute intensity of the light all affect the efficiency.

Diffusion-Controlled Association of fd Gene 5 Protein to fd DNA. The native form of the gene 5 protein is a dimer, and each dimer has two binding sites for DNA (Oey & Knippers, 1972; Pretorius et al., 1975; Cavalieri et al., 1976). It might bind to DNA with either of the sites in the presence of an excess amount of the protein compared to DNA. This situation might cause difficulty in interpreting the results. In the present study, therefore, most of the experiments were carried out in the presence of an excess amount of DNA.

The rate constant of association obtained by stopped-flow UV absorption is 1.6×10^{10} M^{-1} s^{-1}, which is one of the largest association rate constants ever obtained among protein–DNA interactions. For example, RNA polymerase binds to poly(dA-dT) with a rate constant of 10^8 M^{-1} s^{-1} (Hincle & Chamberlin, 1972), *lac* repressor binds to *lac* operator with a rate constant of 10^{10} M^{-1} s^{-1} (Riggs et al., 1970; von Hippel, 1979), and the T4 gene 32 binds to synthetic polynucleotides with a rate constant of 10^9 M^{-1} s^{-1} (Lohman & Kowalczykowski, 1981). These values are considered to be the diffusion-control limits. It is therefore interesting to examine if the binding process of the gene 5 protein to fd DNA is another such case.

The diffusion-controlled bimolecular rate constant, k_{dif}, was estimated from the simplified Debye–Smoluchowski equation:

$$k_{dif} = 4\pi r(D_1 + D_2)(N_A/1000) \quad (3)$$

where D_1 and D_2 are the diffusion coefficients of DNA and the gene 5 protein, respectively, r is the encounter radius, and N_A is Avogadro's number. The freely diffusing DNA and protein molecules are assumed to form a complex when the two molecules approach each other within the encounter radius.

One can calculate, by using the Svedberg equation, the diffusion coefficients for fd DNA and the gene 5 protein as 7×10^{-8} and 6.4×10^{-7} cm^2 s^{-1}, respectively, from their values of the sedimentation coefficients, 23.1 and 1.3 S, respectively (Ikehara et al., 1975; Oey & Knippers, 1972). One cannot directly determine the encounter radius but only estimate it. The lowest limit may be the Stokes radius since fluctuating DNA molecules may catch the protein beyond the hydrodynamic radius. On the other hand, the encounter radius may be much smaller than half the contour length of fd DNA. These considerations lead to the following estimation for r

$$350 \text{ Å} < r << 5000 \text{ Å} \quad (4)$$

or for the diffusion-controlled bimolecular rate constant

$$2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} < k_{dif} << 3 \times 10^{11} \text{ M}^{-1} \text{ s}^{-1} \quad (5)$$

In the above estimate, the electrostatic interaction between DNA and the protein is neglected. Its contribution depends on the strength of electrostatic attraction between the two molecules outside the encounter radius. However, the attraction becomes effective only within a range much smaller than the radius in the presence of enough salt. In addition, it may be compensated for by the orientational restriction for encounter. Therefore, we neglected its contribution in the present rough estimate of the limit. Von Hippel (1979) made a similar but more detailed discussion on the *lac* repressor. The reasonable upper limit of the association rate constant may thus be on the order of 10^{10} M^{-1} s^{-1}. The obtained rate constant is close to this theoretical upper limit.

The rapid association was confirmed by the photo-cross-linking experiment, where the cross-linking efficiencies for the isolated and cluster complexes were assumed equal. This assumption is likely because a similar amount of the protein was cross-linked irrespective of the reaction time as shown in Figures 5e,f and 6. The binding process, therefore, is diffusion controlled like the above three examples. This is in contrast to the complex formation between enzymes and small ligands, which is often characterized by much smaller association rates.

What enables the gene 5 protein to bind to DNA so effectively? The diffusion-controlled association means that the encounter complex is readily converted into the real complex with specific site–site interactions which demand exact orientations and conformations of interacting molecules. The answer to the question might be found in a three-dimensional model of the gene 5 protein presented by McPherson et al. (1979); the putative DNA binding site was widely opened, and hydrophobic side chains were exposed on the binding site. It may be suggested that the hydrophobic interaction between DNA and the wide binding site is effective to some extent in converting the encounter complex into the real one without very strict orientations and conformations. An asymmetric distribution of basic residues on one side of the protein may play the roles of a pilot and an anchor.

Sliding Mechanism and the Limitation of Analytics in Kinetic Analyses. The kinetics of the cooperative binding of proteins has been analyzed by the nucleation–elongation model

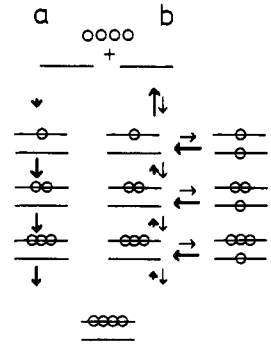

FIGURE 7: Two types of strongly cooperative binding: (a) association-controlled cooperativity; (b) dissociation-controlled cooperativity. The thick arrows indicate the process responsible for the cooperativity. Short arrows denote processes slower than those denoted by long arrows. See the text for details.

(Lohman & Kowalczykowski, 1981; Schneider & Wetmur, 1982), which is usually expressed by the following equations:

nucleation

$$\text{free protein} + \text{DNA} \rightleftarrows \text{isolated complex} \quad (6)$$

elongation

$$\text{free protein} + \text{cluster of size } n \rightleftarrows \text{cluster of size } n + 1 \quad (7)$$

However, many lines of evidence have been accumulated which show the "sliding" or "hopping" of protein molecules along the DNA chain (Park et al., 1982; Winter et al., 1981; Lohman & Kowalczykowski, 1981). These processes are very effective in elongation. Therefore, more exact treatment of elongation may need another equation related to the rearrangement:

$$\text{isolated complex} + \text{cluster of size } n \rightleftarrows \text{cluster of size } n + 1 \quad (8)$$

The set of the two or three kinetic equations described above gives a set of nonlinear differential equations which are impossible to solve analytically without drastic simplifications.

Two Extreme Kinetic Mechanisms in Strongly Cooperative Binding. Here we approach this problem by asking whether the association step or the dissociation step is responsible for the strong cooperativity. We begin with a qualitative discussion of two extreme cases. In the first case (Figure 7a), the protein molecule has a larger association rate constant for the formation of the cluster complex than for the isolated one. The cooperativity originates from the association process, and it may be termed association-controlled cooperativity. This type of cooperativity may occur in the following case: A protein molecule, which has already been bound to DNA, opens up new binding sites in its direct neighbor that are easily accessible to free protein molecules. In this case, the association rate constant for the isolated complex should be much smaller than the diffusion-controlled limit; otherwise, the rate of cluster formation would exceed the upper limit. If the isolated complex is unstable and dissociates or slides at a much larger rate than the cluster complex, the cluster complex will dominate the system at equilibrium. This type of cooperativity, which would be called dissociation controlled, results from a mechanism in which the protein molecules in a cluster are stabilized by the contacts with the neighboring molecules (Figure 7b). This cooperativity is not directly related to the association process, and the association rate constant for the isolated complex could be as large as the diffusion-controlled limit. Actually, this is the case that was valid for the binding of the fd gene 5 protein to fd DNA.

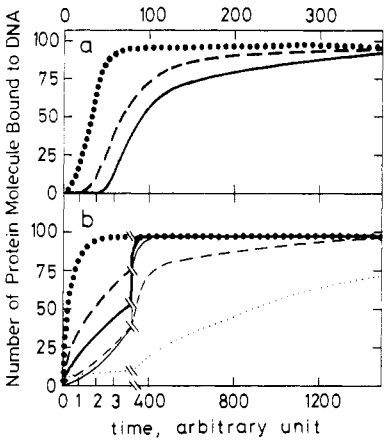

FIGURE 8: Simulation of the theoretical model for association-controlled cooperativity (a) and dissociation-controlled cooperativity (b). The thick lines represent the time course of total bindings and the thin ones that of cluster formation. The thin lines in (a) almost overlap the solid ones. The solid, broken, and dotted lines show the binding of 100 protein molecules to 100, 200, and 800 binding sites, respectively. See Materials and Methods for details.

In order to clarify the difference between the two types of cooperativity, we made numerical simulations of binding time courses. It is particularly interesting to investigate the effects of DNA concentration on cluster formation. In this simulation, we neglected the end effects of DNA molecules for simplicity and arranged the binding sites as a linear sequence. The total number of binding sites corresponds to the DNA concentration in the actual experiments. Let 100 protein molecules bind to these sites. In the association-controlled cooperative binding, the association rate for the cluster complex is assumed to be 2000 times as large as that for the isolated one. This is an observed value for the binding between the gene 5 protein and fd DNA (Dunker, 1975). Figure 8a shows the results obtained from the binding time course for three numbers of binding sites. Since the binding starts with the formation of an isolated complex at a slower rate, there is clearly seen a time lag that is proportional to the number of binding sites. Once appreciable binding occurs, most of the complex is formed as the cluster. The rate of binding does not depend very much on the number of binding sites because the rate of cluster formation depends on the number of cluster binding sites rather than the total number of binding sites. The relative times required for half-saturation of 100, 200, and 800 binding sites are 3.3, 2.4, and 1, respectively. The corresponding numbers are 1.2, 1.1, and 1 if the lag time is neglected. This result indicates that, in the association-controlled cooperativity, the cluster formation is slightly accelerated with increasing DNA concentration. This behavior is independent of the magnitude of the dissociation rate (data not shown).

In sharp contrast, the binding time course for the dissociation-controlled cooperativity exhibits a strong dependence on the number of binding sites (Figure 8b). The initial velocity of the total complex formation is proportional to DNA concentration, and no time lag is seen. The binding curve looks exponential at large DNA concentrations and shifts to the biomolecular reaction curve at small DNA concentrations. On the other hand, the cluster formation slows down with increasing DNA concentration as shown by the results for long reaction times. The relative times required for half-cluster formation are 1, 1.1, and 200 for 100, 200, and 800 DNA binding sites, respectively. The isolated complexes have to redistribute themselves to form clusters, and more trials are required at higher DNA concentrations. In addition, the redistribution slows down when the dissociation rate of the isolated complex becomes small.

Table III: Behavior of Three Types of Cooperative Binding[a]

cooperativity	step producing cooperativity	effect of excess DNA on cluster formation	regulation by cluster formation
strong	association	accelerated	positive feedback
strong	dissociation	decelerated	negative feedback
weak	both	impeded	negative feedback

[a] See the text for details.

To summarize the important findings from the model simulation, the dissociation-controlled cooperativity decelerates the cluster formation in the presence of excess amounts of DNA, but the association-controlled cooperativity accelerates it. The behavior is summarized in Table III for the typical types of cooperative bindings.

Mechanism of Cooperative Binding of fd Gene 5 Protein. There are three lines of evidence that conclude that the cooperativity of binding of gene 5 to DNA is dissociation controlled: (1) the relaxation rate is proportional to the DNA concentration, and no time lag is found; (2) the value obtained for the association rate constant is close to the diffusion-controlled limit, and no further acceleration is possible; (3) rearrangement of the presumptive isolated complex is observed.

The results obtained by stopped-flow UV absorption measurements suggest the involvement of the sliding or hopping mechanism of the gene 5 protein as follows. Since the ratio of the dissociation rate constant of the isolated complex to that of the cluster complex (about 1 s^{-1}) is the cooperativity constant itself, the dissociation rate constant of the isolated complex is estimated to be 10^2–10^3 s^{-1}. On the other hand, the constant obtained by stopped-flow UV absorption is much smaller.

The exact expression of k in eq 1 is

$$k = k_{sec}[\text{DNA}] + k_d \qquad (9)$$

where k_d is the dissociation rate constant. The fact that the value of $k/[\text{DNA}]$ is independent of DNA concentration indicates that k_d is small, 10 s^{-1} at the most. This value is much smaller than the above estimate. The discrepancy is solved if one takes into account the sliding mechanism. The isolated complex formed in the early stage of binding may translocate by sliding or hopping at a rate constant of 10^3 s^{-1}. The protein is still in the anionic cage around DNA, and the entire shape of the complex remains the same, leading to little change in UV absorption and scattering.

In conclusion, the most probable binding mechanism is as follows. The protein binds to single-stranded DNA with the diffusion-controlled association rate of 2×10^{10} M^{-1} s^{-1}, and the isolated complex is formed first. This complex usually translocates within a DNA molecular domain but rarely dissociates into the free protein and DNA; the dissociation rate constant would be less than 10 s^{-1}. The rearrangement of the isolated complex continues until an occasional cluster formation occurs. The cluster complex of a large size is very stable and dissociates into the free protein with a rate constant of about 1 s^{-1}.

Possible Role of Dissociation-Controlled Cooperativity. Although the biological function of the gene 5 protein is not fully understood, there is enough evidence to believe that the gene 5 protein–DNA complex is involved in fd DNA synthesis (Pratt & Erdahl, 1968; Salstrom & Pratt, 1971). A destruction of gene 5 activity results in the production of fd RF DNA, suggesting that the coverage of the product, single-stranded DNA, with gene 5 prevents the synthesis of the

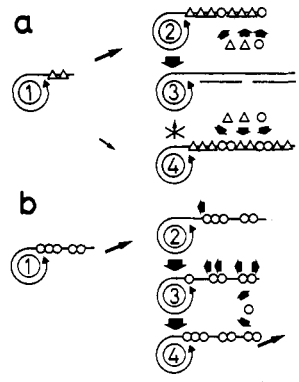

FIGURE 9: Dynamic model for the role of gene 5 protein in fd replication. Associations and slidings of proteins are indicated by small and blunt arrows, and elongation of DNA is denoted by long and sharp arrows. (a) At an early stage of infection, there is the host helix-destabilizing protein, SSB (△), but the gene 5 protein (○) is not accumulated yet. The leading strands may be produced by "rolling circle intermediates" (1). The gene 5 protein and SSB are associated with the leading strand and share the strand according to their populations (2). The phage protein molecules are distributed sparsely, and the rapid elongation of DNA allows little time for the rearrangements of gene 5 proteins to form cluster complexes of large sizes. On most strands, priming takes place, and the complementary strands are synthesized. This is the predominant RF synthesis (3). On the contrary, randomly distributed gene 5 molecules occasionally form clusters of small sizes among clusters of SSB (4). This complex may inhibit the priming, and single-stranded DNA synthesis may occur even at an early stage of infection. (b) After the accumulation of gene 5 protein molecules, they are predominantly bound to the replication intermediates (1), and RF is hardly synthesized. However, rapid elongation of the single-stranded DNA does not allow cluster complex formation of large sizes. Therefore, there may be uncovered portions on the DNA synthesized (2). A limited rearrangement of bound gene 5 takes place rapidly (3), and free gene 5 molecules are also trapped. The rearrangement may produce at the replication fork a cluster of a size appropriate for single-stranded DNA synthesis (4), and the elongation may resume. Free gene 5 protein molecules may seldom hit the fork directly, because the host replication should otherwise be seriously injured.

lagging strand (Mazur & Model, 1973; Mazur & Zinder, 1975). In addition to prevention, a positive role of the protein has been suggested (Staudenbauer & Hofschneider, 1972). In fact, no synthesis of fd DNA was observed in the reconstituted system without a helix-destabilizing factor (Meyer & Geider, 1982).

The switching in the synthesis of fd DNA from RF to the viral strand may occur when the gene 5 protein replaces the host helix-destabilizing protein (SSB) in the binding with the single-stranded DNA product. Geider (1978) showed from static experiments of competitive binding reactions of the two proteins that the exchange took place cooperatively in an all or none fashion. A numerical simulation by Alma et al. (1983) also showed that the displacement occurred in a very narrow concentration region of gene 5. However, the switching in vivo seems to take place more gradually; there is a fair amount of viral DNA synthesis at 4 min after infection when the switching occurs about 15 min after infection (Forsheit et al., 1971).

The discrepancy may be explained by the dynamic characteristics of the binding of gene 5 elucidated in the present study (Figure 9). In an early phase of infection, new leading strands are kept synthesized from the rolling circles by the binding of SSB at the replication fork. The gene 5 population is still small, and DNA synthesis is faster than cluster complex formation (see below). In this condition, few cluster complexes of large size are formed. Instead, most gene 5 protein may exist in the form of the isolated complex, while the cluster complex of small size is occasionally generated. The complexes are distributed evenly over the product single-stranded DNA molecules by repeating dissociation and association. Now we assume that the RF synthesis is prohibited by the cluster complexes of small sizes. Then the DNA molecules with such complexes are used for the viral DNA synthesis. The single-stranded synthesis could thus become significant when the cluster complex formed at equilibrium is negligible.

Next we consider the viral DNA synthesis at a stationary state where the number of rolling circles is constant and the RF synthesis is negligible. This situation could be maintained by the fact that the accumulated gene 5 proteins inhibit the expression of gene 2 which is required for the synthesis of the rolling circles (Model et al., 1982; Yen & Webster, 1982). It should be remarked here that the cluster complex of large size, as shown in the present study, is formed much slower than the DNA synthesized by DNA polymerase III holoenzyme, 10^3 nucleotides/s in vivo (Kornberg, 1982). In other words, the viral DNA synthesis should be treated dynamically but not statically. To keep up with the relatively rapid single-stranded synthesis, replication forks need an efficient supply of gene 5 protein. Two possible pathways for the supply are the direct association from the bulk solution and the sliding from the isolated complex or the cluster complex of the small size on the preformed single-stranded DNA. Neither of these mechanisms totally explains why the host replication is not seriously injured. We only suggest here that the latter sliding mechanism is more likely because it seems very difficult to protect the forks of the host replication from the direct attacking of the abundant phage protein. In the sliding mechanism, more gene 5 protein is trapped on the single-stranded viral DNA as the synthesis proceeds, providing a good source for both the supply and storage of the protein. In this connection, we may remark that the gene 5 molecules counted as soluble proteins in a cell lysate in vitro (Pratt et al., 1974) may have originated at least partly from the isolated complex.

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Direct Observation of Complexes of ssb and recA Proteins with a Fluorescent Single-Stranded Deoxyribonucleic Acid Derivative†

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ABSTRACT: Evidence is presented from fluorescence and kinetic experiments that ssb binds to a recA–ssDNA–ATP complex causing a major structural change in which some 40% of the bound recA is released. On addition of ssb to recA–ϵDNA–ATP (containing the fluorescent analogue of ssDNA ϵDNA), there is a slow first-order decrease in fluorescence ($t_{1/2} \sim 3$ min). This is accompanied by a loss in the ATPase activity of recA protein. The resultant complex does not exchange ϵDNA for added ssDNA. Measurement of the DNA-stimulated ATPase activity on addition of excess ssDNA reveals that 40% of the previously bound recA has been released. The stoichiometry of recA bound to ϵDNA thus changes from 1 mol per six nucleotides to 1 per 10 on addition of ssb. Formation of the ssb–recA–ϵDNA complex is dependent on ATP, and the rate varies with the concentration of ssb.

General recombination and repair of DNA damage in *Escherichia coli* require the presence of the recA gene product (Clark, 1973; Radding, 1978; Radman, 1975; Witkin, 1976). This is a protein of M_r 37 800 (Horii et al., 1980; Sancar et al., 1980) that has a large number of activities in vitro. It is a ssDNA[1] and dsDNA-dependent ATPase (Ogawa et al., 1979; Roberts et al., 1979; Weinstock et al., 1979; McEntee et al., 1979; Shibata et al., 1979a,b; West et al., 1980); it catalyzes D loop formation and strand exchange (Cassuto et al., 1981; Cox & Lehman, 1981; DasGupta et al., 1981; West et al., 1981) and has also been shown to possess an ssDNA- and ATP-dependent protease activity for certain repressor proteins (Roberts et al., 1979; Craig & Roberts, 1980).

Recent studies have indicated that interactions with other proteins affect the activity of recA. The interaction with the ssDNA binding protein (ssb) from *E. coli* has been shown to be important both in vitro and in vivo. A combination of recA and ssb will catalyze D loop formation, strand exchange, and the protease reaction far more efficiently than recA protein alone, although the ATPase reaction is inhibited (Shibata et al., 1980; McEntee et al., 1980; Resnick & Sussman, 1982; Cox & Lehman, 1982). Cox & Lehman (1982) have suggested that this may be related to a tightening up of the recA–ssDNA complex, but, as yet, attempts to isolate a

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); NTP, nucleotide triphosphate; ϵDNA, product obtained by treating ssDNA with chloroacetaldehyde, which contains 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine residues; ssb, ssDNA binding protein; Tris, tris(hydroxymethyl)aminomethane.

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