Binding of IKe Gene 5 Protein to Polynucleotides. Fluorescence Binding Experiments of IKe Gene 5 Protein and Mutual Cooperativity of IKe and M13 Gene 5 Proteins

E. A. M. de Jong,[‡] B. J. M. Harmsen,[‡] R. N. H. Konings,[§] and C. W. Hilbers*[,‡]

Laboratories of Biophysical Chemistry and Molecular Biology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

Received July 11, 1986; Revised Manuscript Received November 18, 1986

ABSTRACT: Fluorescence studies of the binding of IKe gene 5 protein to various polynucleotides were performed to obtain insight into the question as to what extent the binding characteristics of the gene 5 proteins of the IKe and M13 phages resemble and/or differ from each other. The fluorescence of IKe gene 5 protein is quenched 60% upon binding to most polynucleotides. At moderate salt concentrations, i.e., below 1 M salt, the binding stoichiometry is 4.0 ± 0.5 nucleotides per IKe gene 5 protein monomer. The affinity of the protein for homopolynucleotides depends strongly on sugar and base type; in order of increasing affinities we find poly(rC) < poly(dA) < poly(rA) < poly(dI) < poly(rU) < poly(dU) < poly(dT). For most polynucleotides studied, the affinity depends linearly on the salt concentration: $[d \log (K_{int}\omega)]/(d \log [M^+]) = -3$. The binding is highly cooperative. The cooperativity parameter ω, as deduced from protein titration curves, is 300 ± 150 and appears independent of the type of polynucleotide studied. Estimation of this binding parameter from salt titrations of gene 5 protein–polynucleotide complexes results in systematically higher values. A comparison of the binding data of the IKe and M13 gene 5 proteins shows that the fluorescence quenching, stoichiometry, order of binding affinities, and cooperativity in the binding are similar for both proteins. From this it is concluded that at least the DNA binding grooves of both proteins must show a close resemblance. A method is presented by which the existence of mutual cooperativity between IKe and M13 gene 5 proteins can be studied. The experiments show that the mutual cooperativity factor between IKe and M13 gene 5 proteins upon binding to polynucleotides must be considerably lower than their individual cooperativity factors. From this it is concluded that the protein–protein interaction surfaces causing the cooperativity in the binding of both proteins diverged during evolution.

It is well-known that single-stranded DNA binding proteins play a fundamental role in the process of DNA replication. One of the most outstanding features of these proteins is the strong cooperativity exhibited upon binding to single-stranded polynucleotides. This property is thought to be an essential feature of the mechanism underlying the regulation of the DNA replication process and possibly gene regulation (von Hippel et al., 1982).

A typical representative of this class of proteins is the gene 5 protein encoded by the filamentous coliphage M13. The protein binds strongly and cooperatively to single-stranded DNA and is responsible for the fact that late in infection a transition from double-stranded replicative form DNA synthesis to asymmetric synthesis of progeny single-stranded viral DNA occurs (Saltstrom & Pratt, 1971; Alberts et al., 1972; Oey & Knippers, 1972; Ray, 1977; Mazur & Model, 1973). In this process the binding cooperativity plays a regulatory role; thanks to this property the protein can effect the transition within a limited concentration range after it has reached a certain threshold concentration. Physical–chemical studies have suggested that the cooperative binding behavior finds its origin in the interactions between the proteins bound on the DNA lattice. The best experimental evidence for this was obtained by Rasched and Pohl (1974). Their chemical cross-linking experiments showed that M13 gene 5 protein oligomers consisting of up to four dimers are formed upon addition of oligonucleotides as short as four nucleotides in length. Recently, Brayer and McPherson have attempted to specify these interactions on the basis of their X-ray crystal structure of the gene 5 protein of M13 (Brayer & McPherson, 1984a,b). A model was proposed for the intracellular gene 5 protein M13–DNA complex in which well-defined domains of the gene 5 protein are involved in protein–protein interactions, and it was assumed that it is these domains that are responsible for the binding cooperativity. Insight into this matter can be obtained experimentally by studying the binding behavior of gene 5 proteins, mutated in their amino acid composition at the protein–protein interaction surfaces. As a start to investigations in this field, we studied the polynucleotide binding properties of the gene 5 protein of the filamentous phage IKe alone and together with the gene 5 protein of M13.

The N-plasmid-specific filamentous single-stranded DNA phage IKe is distantly related to the F-plasmid-specific filamentous phage M13 (Peeters et al., 1983, 1985). Their life cycles are almost identical. The gene 5 proteins encoded by these phages exhibit an overall homology of 45% with respect to their amino acid sequences. Amino acid residues that are surmised to be involved in the interaction with DNA are conserved. Furthermore, complementation studies have demonstrated that recombinant plasmids carrying a cloned copy of M13 gene 5 are able to complement a lethal mutation in gene 5 of IKe, which indicates that the gene 5 proteins of IKe and M13 are exchangeable (Peeters, 1985). These results suggest that, despite the difference in the amino acid sequences, comparable three-dimensional structures are obtained for both proteins. This does not necessarily mean that the protein surfaces involved in interactions between different proteins in the DNA–protein complex have remained the same.

[‡] Laboratory of Biophysical Chemistry.
[§] Laboratory of Molecular Biology.

0006-2960/87/0426-2039$01.50/0

Indeed, in the present study we demonstrate that during evolution the amino acid sequences of these proteins have diverged to such an extent that their mutual binding cooperativity is greatly reduced. This is in contrast to the individual binding properties of these proteins, which appear to be similar.

Materials and Methods

Materials. IKe gene 5 protein was isolated from IKe-infected *Escherichia coli* cells as described (Peeters et al., 1983). Gene 5 protein concentrations were determined from the absorbance at 276 nm with a molar absorption coefficient of 7100 M^{-1} cm^{-1}. Poly(dA), poly(rA), poly(rU), and poly(rC) were obtained from Boehringer Mannheim. Poly(dI), poly(dU), and poly(dT) were purchased from P-L Biochemicals. The concentrations of the polynucleotide solutions [containing 1 mM sodium cacodylate (pH 6.8) and 50 mM NaCl] were determined from UV-absorption measurements with the following extinction coefficients: for poly(dA), 9300 M^{-1} cm^{-1} (260 nm); for poly(rA), 9500 M^{-1} cm^{-1} (260 nm); for poly(rC), 6500 M^{-1} cm^{-1} (267 nm); for poly(dU), 9200 M^{-1} cm^{-1} (260 nm); for poly(rU), 9200 M^{-1} cm^{-1} (260 nm); for poly(dI), 9400 M^{-1} cm^{-1} (260 nm); for poly(dT), 8700 M^{-1} cm^{-1} (265 nm) (Bulsink et al., 1985). KF (suprapure), KCl [suprapure and pro analysis (p.a.)], NaF (suprapure and p.a.), NaOAc (suprapure), and NaCl (suprapure and p.a.) were purchased from Merck. KF (p.a.) was obtained from Fluka AG. KOAc (p.a.) was purchased from J. T. Baker, Riedel-de Haën AG, or Merck.

Fluorescence Titrations. Fluorescence measurements were performed with a Perkin-Elmer fluorescence spectrophotometer MPF-4 equipped with a 150-W xenon lamp. The instrument was used in the so-called ratio mode. Measured fluorescence intensities therefore are expressed in arbitrary units (it is noted that the fluorescence scales are linear in all figures presented). Excitation was at the absorption maximum (276 nm) of the tyrosyl residues, and the fluorescence was measured at the emission maximum of 303 nm.

The polynucleotide and protein solutions used in the experiments contained 1 mM sodium cacodylate (pH 6.8, unless mentioned otherwise) and various amounts of KCl or NaCl.

The binding characteristics of the protein were obtained by means of two types of titration experiments: (a) Titrations were carried out by adding a (concentrated) protein solution to a polynucleotide solution, both solutions being at the desired salt concentration; (b) solutions containing a low concentration of salt (20–50 mM NaCl or KCl) and a mixture of a polynucleotide and the IKe gene 5 protein (the first component being in excess, polynucleotide monomer to gene 5 protein monomer ratio of about 6) were titrated with a concentrated salt solution. We will refer to the former type of titration as "protein titration" and to the latter type as "salt titration".

The titrant additions were made with a microburet (Metrohm E475). Mixing was done by gently shaking the sample cell. The temperature of the microburet and the 5-mm cuvette were under thermostatic control. The fluorescence was corrected for dilution and for loss of exciting light as a result of absorption. The fluorescence intensity was insensitive to variations in KCl or NaCl concentration and turned out to be linearly dependent on the protein concentration, within the protein concentration range considered. In contrast, the fluorescence intensity of the protein depended strongly on the concentration of the fluoride and acetate salts tested (see Results).

Evaluation of Titration Curves. The analysis of both types of titration curves has been described previously (Alma et al., 1983; Bulsink et al., 1985). The titrations were interpreted by use of the model for binding of large ligands to "linear lattice", developed by McGhee and von Hippel (1974). The model provides a description for the binding in terms of the following parameters: (1) the intrinsic binding constant (K_{int}) for the binding of a ligand to an isolated site on the DNA, (2) the number of nucleotides covered by the ligand (n), and (3) the cooperativity parameter (ω), i.e., the factor by which K_{int} is multiplied if a ligand binds adjacent to an already occupied site.

In case of protein titrations an optimal set of values for these parameters was obtained by fitting calculated fluorescence intensities to the data points, with $K_{int}\omega$, K_{int}/ω, Q_{max}/n (Q_{max} is the fraction of protein fluorescence quenched upon binding), and n as adjustable parameters [see Alma et al. (1983)].

It has been demonstrated by McGhee and von Hippel (1974) that, at half-saturation of the polynucleotide, the effective binding constant, i.e., the product $K_{int}\omega$, is equal to the reciprocal of the corresponding free protein concentration, provided the value of ω is sufficiently high ($\omega \gg n$). This can be used (Bulsink et al., 1985) in the analysis of salt titration curves to obtain direct estimates of $K_{int}\omega$ for the salt concentration at half-saturation of the polynucleotide, provided the value of n is known. Following the procedure of Bulsink et al., we used the value of n determined from protein titration experiments.

In addition, a method to estimate ω from salt titration curves was derived by these authors. To this end theoretical salt titration curves were calculated by them, using the binding theory of McGhee and von Hippel combined with the relationship between the effective binding constant, $K_{int}\omega$, and the concentration of monovalent cations [M^+]. Studies of the binding of oligopeptides and proteins to RNA and DNA have shown that the effective binding constant conforms to

$$\log (K_{int}\omega) = c \log [M^+] + b \qquad (1)$$

where c and b are constants, for salt concentrations in the range 0.1–1 M NaCl (Helene & Lancelot, 1982; Record et al., 1981; Lohman et al., 1980; Kowalczykowski et al., 1981; Newport et al., 1981; Lonberg et al., 1981). Theoretical arguments for the validity of eq 1 were provided by Record et al. (1976, 1978). Like Bulsink et al., we estimated ω from the salt titration curves by fitting the theoretical fluorescence intensities to the experimental fluorescence intensities, using the experimentally available values for c, n, and $K_{int}\omega$ at half-saturation of the polynucleotide lattice in calculating the theoretical intensities.

Simulation of the Binding of Two Large Ligands to a Linear Lattice. For simulations of binding isotherms of IKe gene 5 protein in the presence of a preadded amount of M13 gene 5 protein (and for the reverse experiments as well), we use the theory of Schwartz and Stankowski (1979). According to this theory the simultaneous binding of two large different ligands (gene 5 proteins) to an infinite (polynucleotide) lattice can be described by

$$\omega_{11}X_{00}^{-n_1/S_1}X_{01} = 1 + (\omega_{11} - 1)X_{01} + (\omega_{12} - 1)X_{02} \qquad (2a)$$

$$\omega_{22}X_{00}^{-n_2/S_2}X_{02} = 1 + (\omega_{21} - 1)X_{01} + (\omega_{22} - 1)X_{02} \qquad (2b)$$

In these expressions $S_1 = \omega_{11}K_1L_1$ and $S_2 = \omega_{22}K_2L_2$ are the effective binding constants of ligand 1 or 2 times the free (unbound) ligand concentration L_1 or L_2 when ligand 1 or 2 is binding separately to the lattice. Thus, ω_{11} is the cooperativity parameter for interactions between ligands 1 and K_1 is the intrinsic binding constant for ligand 1; the parameters K_2 and ω_{22} have analogous meanings for ligand 2. n_1 and n_2 are the number of contacts (nucleotides) covered by ligands

1 and 2; ω_{12} and ω_{21} stand for the mutual cooperativity factors between ligands 1 and 2.

The conditional probability X_{ij} represents the (relative) chance of finding, adjacent to the last contact (nucleotide) on the lattice bound to a ligand in mode i, a lattice contact (nucleotide) bound to a ligand in mode j. In our case i,j = 0, 1, or 2, which means that unoccupied lattice contacts indicated by $i,j = 0$ are incorporated in the definition. From the definition it follows that the sum of the conditional probabilities

$$X_{00} + X_{01} + X_{02} - 1 = 0 \qquad (2c)$$

For a given set of values for ω_{11}, ω_{22}, ω_{12} (ω_{21}), K_1, K_2, L_1, and L_2 the values of X_{00}, X_{01}, and X_{02} can be obtained from the conditions in eq 2a–c. This was done by expressing X_{01} and X_{02} in terms of X_{00} (using eq 2a and 2b) and varying X_{00} until eq 2c was fullfilled. If it is assumed that $\omega_{12} = \omega_{21}$, the fraction of contacts θ_i occupied by ligand in binding mode i is (Schwartz & Stankowski, 1979)

$$\theta_i = \phi_i/(\phi_1 + \phi_2 + 1) \qquad (3a)$$

$$\phi_i = \theta_i/\theta_0 = n_i(X_{0i}/X_{00})[1 + (\omega_{i1} - 1)X_{01} + (\omega_{i2} - 1)X_{02}] \qquad (3b)$$

where θ_0 is the fraction of unoccupied contacts. Thus $\phi_i = \theta_i/\theta_0$ represents an apparent equilibrium constant for occupying vacant contacts in mode i.

Knowing the total nucleotide concentration N_t (expressed in mononucleotides), we may directly calculate from the θ_i values the concentration of protein bound in each mode P_1 and P_2, respectively, because

$$P_1 = \theta_1 N_t/n_1 \quad \text{and} \quad P_2 = \theta_2 N_t/n_2 \qquad (4)$$

Throughout the competition binding experiments the total protein concentrations $P_{t,i}$ are known. Binding curves can be calculated by a second iterative procedure, minimalizing

$$P_1 + L_1 - P_{t,1} = \delta_1 \quad \text{and} \quad P_2 + L_2 - P_{t,2} = \delta_2 \qquad (5)$$

in which δ is the minimalization criterion.

RESULTS

Stoichiometry of the Binding of IKe Gene 5 Protein to Polynucleotides. As a first characterization of this binding the stoichiometry n, i.e., the number of nucleotides covered by one protein monomer, and the fluorescence quenching factor Q_{max} were determined. To this end titrations of different polynucleotide solutions with the protein were performed at salt concentrations where the determination of these parameters is most accurate; i.e., titrations were performed at salt concentrations where it is not necessary to build up a threshold concentration of protein before binding to the polynucleotide lattice starts (Alma et al., 1983). At salt concentrations below 0.75–1 M NaCl (or KCl) the number of nucleotides covered by a protein monomer turned out to be 4.0 ± 0.5 for all polynucleotides studied. On the average Q_{max} was 0.6 and constant, within the salt concentration range mentioned above, for all polynucleotides except poly(dT). For this polynucleotide the value of the fluorescence quenching factor is 0.7 at salt concentrations below 1 M NaCl. These values are concurrent with those found for the M13 gene 5 protein (Alma et al., 1983; Bulsink et al., 1985; Pörschke & Rauh, 1983). In the range from 1 to 4 M salt both values alter. At those salt concentrations the binding of IKe gene 5 protein to most polynucleotides is relatively weak. The only exception in this respect is formed by poly(dT): even at 4 M NaCl, binding of IKe gene 5 protein to this polynucleotide occurs without

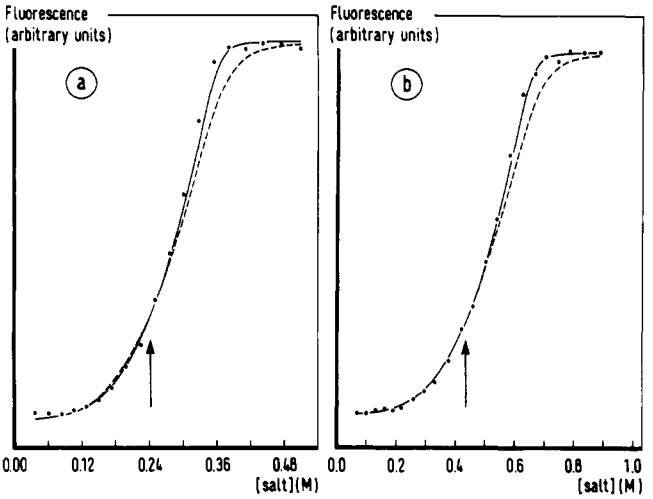

FIGURE 1: Change in IKe gene 5 protein fluorescence as a function of KCl concentration, at 3.5 °C, upon dissociation from poly(rA). The initial protein concentration and initial polynucleotide (mononucleotide) concentration are (a) 5.2 and 30 μM, respectively; (b) 41 and 240 μM, respectively. The curves were calculated according to the procedure presented under Materials and Methods. The dashed and solid lines correspond with values for ω equal to 300 and 2000, respectively. For each of these curves c (see eq 1) was taken equal to −3.3. The arrows indicate the salt concentration at half-saturation of the polynucleotide.

first building up a measurable free-protein pool. From titrations of poly(dT) with IKe gene 5 protein above 1 M salt, n values of about 5 were calculated, while Q_{max} appeared to decrease from 0.7 to 0.6 within the salt concentration range from 1 to 4 M NaCl.

Effective Binding Constant. The effective binding constant, $K_{int}\omega$, was measured by means of protein titrations and salt titrations. Titrations of IKe gene 5 protein polynucleotide mixtures with a concentrated KCl solution were carried out for various ribo- and deoxyribohomopolynucleotides. At low ionic strength the protein is bound to the polynucleotide; increasing the KCl concentration results in a dissociation of the protein from the polynucleotide lattice, which is accompanied by an enhancement of the protein fluorescence. Titration curves obtained for the dissociation of the poly(rA)–IKe gene 5 protein complex are presented as an example in Figure 1. These curves were obtained in experiments in which the total protein concentrations differed by a factor of ~8. The polynucleotide/protein ratio was chosen so as to ensure that at low salt concentration the polynucleotide was covered to a large extent. From these titrations one can easily derive $K_{int}\omega$ at half-saturation of the polynucleotide lattice (Bulsink et al., 1985). By variation of the total protein concentration, $K_{int}\omega$ was determined as a function of salt concentration for a number of different polynucleotides. The results are collected in Figure 2, where log ($K_{int}\omega$) is plotted as a function of log [M^+]. For poly(dA) results derived from titrations of the polynucleotide with the protein at a fixed salt concentration are given as well. The data are in good agreement with those obtained from the salt titrations.

For poly(dA) and poly(rA), titrations were carried out at different temperatures as well. Within experimental accuracy the binding constants were independent of temperature. This means that within the limited temperature range considered the binding enthalpy is close to 0. Consequently, for these polynucleotides the stability of the complex is mainly determined by the binding entropy.

For most polynucleotides studied, log ($K_{int}\omega$) varies almost linearly with log[M^+]; the only exception is formed by poly-

Table I: Binding Affinities for Binding of IKe Gene 5 Protein to Different Polynucleotides Obtained from Salt Titrations

	polynucleotide							
	poly(rC)	poly(dA)	poly(rA)				poly(dI)	poly(rU)
[-d log ($K_{int}\omega$)]/(d log [M^+])	2.6	2.7	3.0	3.5	3.1	3.4	3.3	2.6
type of salt	KCl	KCl	KCl	KCl	KCl	NaCl	NaCl	NaCl
temperature (°C)	3.4	3.7	3.4	12.5	19.3	12.5	3.3	4.3
$K_{int}\omega$ (M^{-1}) at 0.20 M salt	8.3×10^4	1.9×10^5	1.3×10^6	1.9×10^6	1.2×10^6	6.8×10^5	2.8×10^7	2.5×10^7

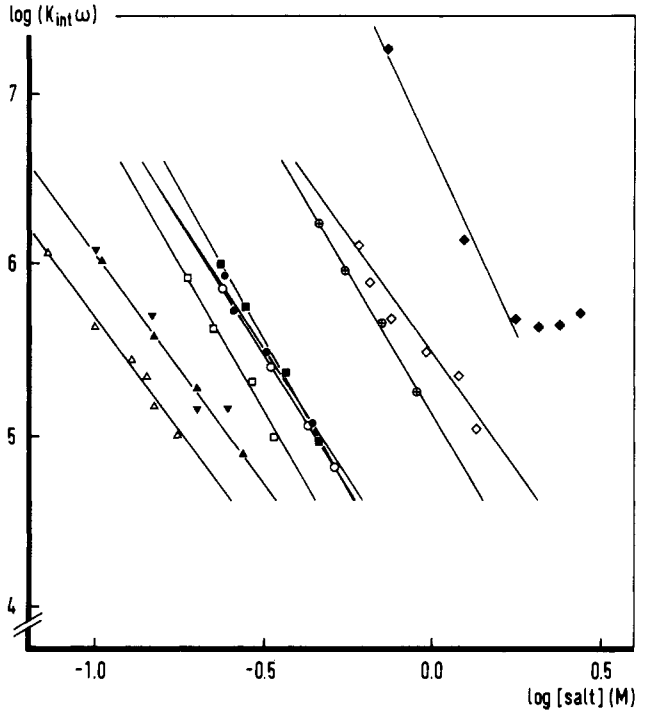

FIGURE 2: Salt dependency at pH 6.8 of effective binding constant, $K_{int}\omega$, for IKe gene 5 protein–polynucleotide complexes as derived from salt titrations of the complex (see Figure 1) unless mentioned otherwise. Titrations were carried out with KCl or NaCl at various temperatures: poly(rC), 3.4 °C, KCl (△); poly(dA), 3.7 °C, KCl (▲); poly(dA), 22.0 °C, KCl (▼) (from binding isotherms); poly(rA), 12.5 °C, NaCl (□); poly(rA), 3.4 °C, KCl (○); poly(rA), 12.5 °C, KCl (■); poly(rA), 19.3 °C, KCl (●); poly(dI), 3.3 °C, NaCl (⊕); poly(rU), 4.3 °C, NaCl (◇); poly(dU), 22.0 °C, KCl (◆) (from binding isotherms).

(dU). The average value found for the slope of the plots of log ($K_{int}\omega$) vs. log [M^+] for the different polynucleotides (see Table I) is

$$[\text{d log } (K_{int}\omega)]/(\text{d log } [M^+]) = -3.0$$

Although the dependency of $K_{int}\omega$ on the salt concentration is similar for all polynucleotides studied, this is not true for the value of $K_{int}\omega$ at a fixed salt concentration. We find the following order in affinity of the IKe gene 5 protein for different polynucleotides: poly(dT) > poly(dU) > poly(rU) > poly(dI) > poly(rA) > poly(dA) > poly(rC). In addition, the magnitude of $K_{int}\omega$ is influenced by the type of cation used as counterion in the polynucleotide solutions. This is clearly demonstrated by the titrations of the poly(rA)–protein complexes performed at 12.5 °C with NaCl and KCl, respectively. However, the difference is not large; the effective binding constants differ by a factor of 2.5. The fact that NaCl is somewhat more effective in lowering the binding constant than KCl must be due to the difference in ionic radii.

To test whether there is anion release in the binding of the IKe gene 5 protein to polynucleotides, we started to study the binding in the presence of NaF, KF, NaOAc (OAc, acetate), and KOAc. It is known that in the presence of fluoride and acetate ions binding parameters may differ dramatically from those in the presence of chloride ions, if there is net anion release from the proteins in the binding to polynucleotides (Kowalczykowski et al., 1981).

For the IKe gene 5 protein such experiments were marred by two problems. (1) Test experiments revealed a strong dependence of the protein fluorescence on the concentration of the salts mentioned above. This fluorescence quenching (in the order of 50% and more at about 1 M concentration of the fluoride and acetate salts) was nonspecific because the fluorescence of *N*-tyrosine ethyl ester (at micromolar concentrations, pH 7, in the presence and absence of buffer components) was influenced to the same extent. Fluoride and acetate salts of analytical grade and even suprapure quality, obtained from different manufacturers, gave equal results. (2) The solubility of the IKe gene 5 protein in the presence of fluoride and acetate salts decreased by about 1 order of magnitude relative to that in KCl solutions of comparable concentration. This limited solubility of the protein in the fluoride and acetate solutions prohibited the performance of protein titrations. The salt titrations of IKe gene 5 protein–poly(dA) mixtures performed with those salts were difficult to interpret due to the increased quenching of free protein fluorescence upon raising the salt concentration.

Is Polynucleotide Binding of IKe Gene 5 Protein and M13 Gene 5 Protein Mutually Cooperative? One of the outstanding features of the binding of the gene 5 proteins of IKe and M13 to DNA and RNA is the binding cooperativity. This means that the binding constant for binding of a protein molecule to an already bound protein is roughly a few hundred times higher than for binding to a naked lattice. This effect becomes particularly pronounced in titration curves obtained for polynucleotide–protein complex formation at an ionic strength that requires the accumulation of a protein threshold concentration before binding to the polynucleotide lattice can take place (Alma et al., 1983). An example is provided in Figure 3b, where poly(rC) is titrated with the IKe gene 5 protein at a salt concentration of 0.13 M KCl. This situation should be contrasted with the titration of poly(rC) with M13 gene 5 protein (Figure 3a) in which only a low concentration of free protein needs to accumulate before binding to the lattice takes place. This shows that the intrinsic binding constant of the M13 gene 5 protein for complexation to poly(rC) is somewhat higher than that of the IKe gene 5 protein.

Poly(rC) forms an excellent system to test whether mutual cooperativity between the proteins does occur. To answer this question, M13 gene 5 protein was added to a poly(rC) solution to such a concentration that cooperative binding is manifest (indicated by the arrow in Figure 3a). Subsequently, the titration was continued with IKe gene 5 protein. The result is given in Figure 3c. Comparison with the titration curve of poly(rC) with IKe gene 5 protein without the preaddition of M13 gene 5 protein (Figure 3b) shows that both titration curves are virtually identical. The only difference is that in Figure 3c the curve starts at a higher fluorescence intensity arising from the previously added M13 gene 5 protein. Hence, before binding of the IKe gene 5 protein to the polynucleotide can take place, a threshold concentration of this protein has

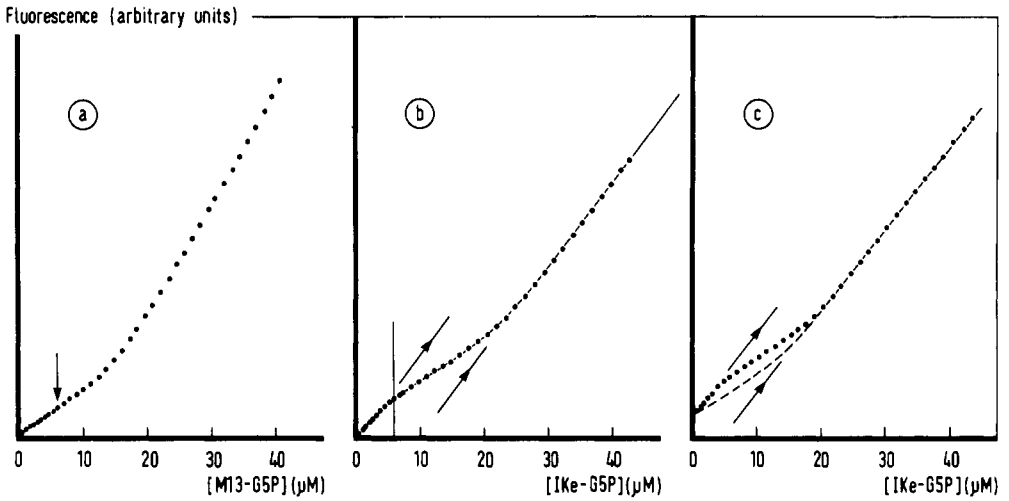

FIGURE 3: Fluorescence titrations of poly(rC) in 1 mM sodium cacodylate and 0.13 M KCl at 5 °C and pH 6.5: (a) with M13 gene 5 protein, [poly(rC)] = 64 μM; (b) with IKe gene 5 protein, [poly(rC)] = 70 μM; (c) with IKe gene 5 protein after preaddition of 6 μM M13 gene 5 protein, [poly(rC)] = 70 μM. In (b) the line through the data points represents the theoretical binding isotherm forming the best fit to the experimental data points. It was calculated by using the following parameters: $K_{int}\omega = 2.6 \times 10^5$ M^{-1}, $\omega = 2.8 \times 10^2$, $n = 3.9$, and $Q_{max} = 0.59$. The arrows in (b) and (c) indicate the slopes of the fluorescence changes at the beginning and the end of the titration with IKe gene 5 protein. The arrow in (a) indicates the degree of saturation of poly(rC) with M13 gene 5 protein at which in (c) the titration with IKe gene 5 protein is started. The dashed line in (c) is the titration curve expected for IKe gene 5 protein if a 6 μM concentration of this protein was preadded instead of 6 μM M13 gene 5 protein.

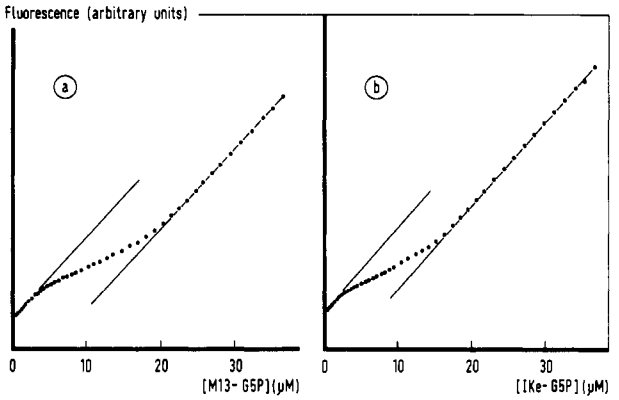

FIGURE 4: Fluorescence titrations of a 60 μM solution of poly(dA) in 1 mM sodium cacodylate and 0.175 M KCl at 22 °C and pH 6.5. (a) Titration with M13 gene 5 protein after preaddition of 5 μM IKe gene 5 protein. (b) Titration with IKe gene 5 protein after preaddition of 5 μM M13 gene 5 protein.

to accumulate as if there was no gene 5 protein present on the lattice. Therefore, the conclusion must be that there is no or little cooperativity between bound M13 gene 5 protein molecules and the added IKe gene 5 protein molecules. The reverse experiment can be performed as well. A representative example, in which both experiments are compared, is provided by the titration curves in Figure 4. A solution of poly(dA) (salt concentration of 0.175 M KCl) was titrated with M13 gene 5 protein after preaddition of IKe gene 5 protein up to a concentration of 5 μM (Figure 4a), or the titration was performed with IKe gene 5 protein after preaddition of 5 μM M13 gene 5 protein (Figure 4b). For both proteins, the protein titration curves of poly(dA) without any preaddition, performed at 0.175 M KCl, looked similar to the titration curve of poly(rC) titrated with IKe gene 5 protein presented in Figure 3b. From those titrations it was inferred that with the addition of 5 μM M13 (or IKe) gene 5 protein the system is in a stage where cooperative binding becomes manifest. The binding curves in Figure 4 show, however, that new threshold concentrations of unbound molecules (either M13 gene 5 protein or IKe gene 5 protein) have to be formed before binding of these molecules to the polynucleotide takes place. A more quantitative analysis of these observations is presented under Discussion.

Discussion

Background. IKe gene 5 protein consists of 88 amino acids, which is one amino acid more than the sequence of the M13 gene 5 protein. If the glutamine at position 21 in the IKe gene 5 protein is considered as an "extra" residue, the IKe and the M13 gene 5 proteins can be aligned such that 39 amino acids have identical positions in both proteins (Peeters et al., 1983). Hence, the amino acid sequences exhibit an overall homology of 45%. This suggests that the proteins are folded into the same three-dimensional structure. Indeed, a number of observations indicate the general correctness of this suggestion. NMR experiments have shown that in the case of M13 gene 5 protein at least one tyrosyl residue and one phenylalanyl residue are involved in the interaction with single-stranded DNA (Alma et al., 1981). Subsequent NMR experiments for IKe gene 5 protein indicate that most likely the same residues are involved in these interactions (de Jong et al., unpublished experiments). The most telling example is probably the earlier mentioned observation that in a complementation experiment the two proteins are interchangeable. It is therefore important to see to what extent the proteins have retained a common set of binding characteristics, despite the progressed divergence of the amino acid sequences.

Comparison of Binding Characteristics of IKe and M13 Gene 5 Proteins. From the fluorescence measurements discussed above, it follows that upon binding to polynucleotides the IKe gene 5 protein covers four nucleotides under physiological conditions. This result concurs with the data obtained for the M13 gene 5 protein, although we find a larger spread in n values, i.e., 4 $\pm$ 0.5, for the IKe than for the M13 gene 5 protein (see Table II). The fluorescence quenching, Q_{max}, amounts to about 60% as found for the M13 gene 5 protein. Both results, i.e., an n value of 4 and a Q_{max} of $\simeq$60%, are expected if both proteins have the same three-dimensional structure and if the same tyrosyl residues are involved in the interaction with the polynucleotide as was suggested above.

In line with these observations we find that at about 0.2 M salt concentrations, i.e., salt concentrations comparable to

Table II: Comparison of Binding Properties of M13 Gene 5 Protein (Bulsink et al., 1985; Alma et al., 1983) and IKe Gene 5 Protein to DNA and RNA

	M13 gene 5 protein	IKe gene 5 protein
stoichiometry (n)	4.0 ± 0.3	4.0 ± 0.5
$K_{int}\omega$ decreases (same salt concn)[a]	poly(dT)	poly(dT)
	poly(dC)	
	poly(dU)	poly(dU), poly(rU) (2.5×10^7), poly(dI) (2.8×10^7)
	poly(dI) (5.1×10^6), poly(rI), poly(rU) (3.1×10^6)	
		poly(rA) (6.8×10^5)
	poly(rA) (2.9×10^5), poly(dA), poly(rC)	poly(dA), poly(rC)
Q_{max}	0.65 ± 0.05 [0.80 for poly(dT)]	0.62 ± 0.04 [0.70 for poly(dT)]
$[-d \log (K_{int}\omega)]/(d \log [M^+])$	4.0 ± 0.8	3.0 ± 0.4
cooperativity, as deduced from protein titrations	150 ± 100	300 ± 150

[a] Values of $K_{int}\omega$ (M^{-1}), determined at pH 7 and 3–5 °C in 0.2 M NaCl for the binding of IKe gene 5 protein and M13 gene 5 protein, that differ to a significant extent are given within parentheses after the corresponding polynucleotides.

cellular conditions (Kao-Huang et al., 1977), the effective binding constants of the proteins are grossly similar. There are some interesting differences in the affinities of the proteins toward different polynucleotides, but binding constants are equal within 1 order of magnitude (see Table II).

It has been generally assumed that single-stranded DNA binding proteins bind more strongly to DNA than to RNA. We have demonstrated that this does not hold for the M13 gene 5 protein (Bulsink et al., 1985) and the same is true for the IKe gene 5 protein. For the M13 gene 5 protein it was found, however, that their binding to polyribonucleotides was weaker than (or equal to) that to the polydeoxyribonucleotide homologues. This simple rule does not even apply to the IKe gene 5 protein; this protein binds more strongly to poly(rA) than to poly(dA), albeit by only a factor of ~3. As for the M13 gene 5 protein, we cannot at this point offer an explanation for the binding preferences of the IKe gene 5 protein (Bulsink et al., 1985). We want to mention, however, that the relatively weak binding to poly(dA) and poly(rA) arises (in part) from the stacking of the adenine bases in the free polynucleotides. As for the M13 protein, we find for the IKe gene 5 protein that the binding to poly(rA) is temperature independent, at least for the limited temperature range considered. This means that the binding enthalpy is close to 0; the enthalpy needed for the disruption of the base–base stacking is compensated for by the gain in enthalpy upon stacking of the aromatic rings of protein amino acid residues upon the adenine rings (Alma et al., 1983).

A difference between the binding parameters of the M13 and IKe gene 5 proteins that deserves separate attention is the different salt dependence of the effective binding constants of both proteins. The linear salt dependence of log ($K_{int}\omega$) can be described by the model proposed by Record et al. (1976, 1978):

$$[-\partial \log (K_{int}\omega)]/(\partial \log [\text{salt}]) = m'\psi + a$$

where $m'\psi$ is the number of monovalent cations (in our case Na^+ or K^+) released from the polynucleotide while a is the number of chloride ions expelled from the protein upon binding to the polynucleotide. The parameter ψ is somewhat dependent on the type of polynucleotide; for poly(rU) and poly(rA) the values for ψ have been estimated to be 0.68 and 0.78, respectively (Record et al., 1978). When the protein covers four nucleotides ($n = 4$), the maximum value for m', the number of ion pairs formed, will be equal to 4. Theoretical and experimental values for the salt dependence of log ($K_{int}\omega$) appear to coincide, if it is assumed that $a = 0$ and $m' = 4$ [i.e., $m'\psi$[poly(rU)] = 2.7; $m'\psi$[poly(rA)] = 3.1; Table I]. The salt dependency of the binding of the IKe gene 5 protein can therefore be explained on the basis of just cation release; there is no need to assume any anion release from the protein. This is in contrast to the result obtained for M13 gene 5 protein (Alma et al., 1983; Bulsink et al., 1985). In that case the average salt dependence [d log ($K_{int}\omega$)]/(d log [Na^+]) = −4 and it was concluded that at least one chloride ion was liberated from the protein. The most simple explanation for this difference is that due to changes in the amino acid sequence a chloride binding site present in M13 gene 5 protein has been lost in the IKe gene 5 protein. Where in the M13 gene 5 protein such a chloride binding site is located remains to be determined.

As discussed under Results, a thorough evaluation of the hypothesis that there is no net release of anions upon binding of the IKe gene 5 protein to polynucleotides was not feasible by considering the effect of different anions on the binding.

Binding Cooperativity of the IKe Gene 5 Protein. As for the M13 gene 5 protein, excellent fits can be obtained between IKe gene 5 protein titration curves and theoretical protein titration curves calculated according to the linear lattice binding theory of McGhee and von Hippel (1974) (see, for example, Figure 3b). The values for the cooperativity factor, ω, obtained from these fits did not show a polynucleotide-type or salt concentration dependency. The average cooperativity factor estimated from the protein titration curves was 300 ± 150. Similar results were obtained for the M13 gene 5 protein (Alma et al., 1983; Bulsink et al., 1985). For this protein an average value for ω of 150 ± 50 was estimated from protein titrations.

In contrast to this, estimates of the value for ω obtained by analyzing salt titration curves (Bulsink et al., 1985) depend on the polynucleotide type and salt concentration. In general they were higher than the values obtained from protein titration curves. By use of the individual values for the dependency of the effective binding constant on the monovalent cation concentration (listed in Table I), i.e., the parameter c defined in eq 1, ω ranged from 150 to about 500 for poly(rU) and poly(dI). For poly(rC) and poly(rA), values in the order of 2000 or even higher were obtained in this way (see, for example, Figure 1). Optimal fits for salt titration curves of poly(dA) complexes were obtained for ω values in the range from 750 to 1500. For each of the polynucleotide types studied, the estimated ω values decreased as a function of the salt concentration at half-saturation of the polynucleotide (about a factor of 2–3 over the salt concentration range considered for each of the polynucleotides). A similar picture was found for the gene 5 protein of M13 (Bulsink et al., 1985).

An evaluation of the curve-fitting procedures demonstrated that the discrepancy in the values for ω derived from both types of titrations could not be explained on the basis of statistical errors in the parameters defining the shape of the binding

curves. For example, the value of c influences the steepness of the salt titration curves as does ω. For instance, to obtain reasonable fits for the salt titrations of poly(rA) complexes, $-c$ has to be larger than 4 when values for ω in the order of 300 are used. Experimentally, an average value of 3.3 ± 0.3 is obtained for $-c$ from the four series of poly(rA), which were performed under different circumstances (see Table I).

It was already noted by Bulsink et al. (1985) that the shape of the calculated salt titration curve at low salt concentration is rather insensitive to the value of ω, whereas at high salt concentration, i.e., at a low degree of saturation of the polynucleotide, the shape of the curve is much more affected by ω. This is in contrast to the calculated protein titration curves; their sensitivity toward ω is almost the same at low and high degrees of saturation. It is possible that the discrepancy in the values for ω observed for protein and salt titration fits is linked up with this difference. In both cases the McGhee and von Hippel model forms the basis for the definition of ω. That we find different values for the cooperativity factor can be explained on the basis of a change in the value of the intrinsic binding constant or the cooperativity factor as a function of the degree of saturation. The physical basis for such a phenomenon can be an electrostatic contribution in the course of the binding which may lead to a cooperativity effect not incorporated in that formalism (Bulsink et al., 1985). Other effects such as long range protein–protein interactions and polynucleotide strand folding [from electron microscopy studies it is known that rodlike particles are formed upon binding of the gene 5 protein of M13 to circular DNA (Alberts & Frey, 1972)] may contribute to the differences as well. The accuracy of the measurements, however, is considered to be insufficient for a further evaluation of these arguments.

Mutual Cooperativity between IKe and M13 Gene 5 Proteins Is Small. The similarity of the binding characteristics of the IKe and M13 gene 5 proteins points to a close resemblance of the three-dimensional structures of the proteins, especially for the RNA and DNA binding grooves. On the other hand, the difference observed between the mutual cooperativity and the individual cooperativities in the binding of M13 and IKe gene 5 proteins indicates a difference in the interactions causing the cooperativity in the binding of both proteins.

To obtain a quantitative picture about the effect of a diminished mutual cooperativity upon the fluorescence titration curves when a polynucleotide is titrated with the IKe protein as well as the M13 protein, we have simulated such curves for different conditions. To this purpose we employed the theory of Schwartz and Stankowski (1979), which allows for the description of the simultaneous binding of two (or even more) large different ligands to an infinite lattice (see Materials and Methods). The results of the application of this formalism are present in Figure 5 for different values of the mutual cooperativity. The cooperativity parameters ω_{11} and ω_{22} (i.e., the cooperativity parameters of the individual proteins) were taken equal to 300, while the number of nucleotides covered by one protein monomer $n_1 = n_2 = 4$ was introduced. These values may be considered representative for the binding of the M13 and IKe gene 5 proteins to poly(dA) at ~0.2 M salt. Also, values for K_1 and K_2 (the intrinsic binding constants) were chosen to be representative of the complex formation between the proteins and poly(dA) at this salt concentration. Thus, the simulated curves are expected to exhibit a close correspondence to the results presented in Figure 4.

In Figure 5, left of the vertical line, the fluorescence change is calculated when only protein 1 (P_1) is added to the polynucleotide sample. This means that this part of the simulated curve corresponds with the fluorescence binding curve of pure protein 1. The competition between the binding of the two proteins is represented on the right-hand side of the vertical line. For different values of ω_{12} the fluorescence binding curves are calculated when protein 2 (P_2) is added to the mixture of the polynucleotide and protein 1 (represented by the position of the vertical line). As expected, when $\omega_{12} = \omega_{11} = \omega_{22} = 300$, the calculated curve is identical with the one obtained for binding of only one of the proteins to poly(dA). However, upon reduction of ω_{12} by a factor of 3, a discontinuity is observed in the simulated curve at the point at which addition of protein 2 was started. This discontinuity becomes more pronounced upon further reduction of ω_{12}, and the increase of the fluorescence at this point reflects the formation of a pool of unbound protein 2 molecules before cooperative binding of this molecule occurs. From a comparison of the simulated fluorescence binding curves in Figure 5 and the measured curves in Figure 4, it may be concluded that the mutual cooperativity ω_{12} is at least 5–10 times smaller than the individual cooperativity parameters ω_{11} and ω_{22}, which characterize the binding to poly(dA) of the M13 and IKe gene 5 proteins, respectively. Similar calculations can be performed for the situation that the intrinsic binding constants of the proteins are not the same.

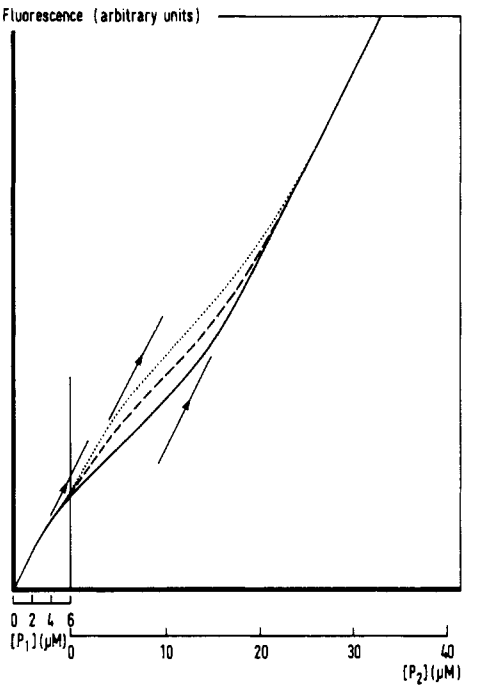

FIGURE 5: Theoretical curves for titration of a 60 μM polynucleotide solution with two different binding proteins P_1 and P_2. The titration with protein 2 (P_2) started after preaddition of 6 μM protein 1 (P_1). The individual binding parameters were assumed to be equal for both proteins and were taken as follows: $(K_{int}\omega)_1 = (K_{int}\omega)_2 = 2 \times 10^5$; $\omega_{11} = \omega_{22} = 300$; $Q_{max,1} = Q_{max,2} = 0.60$. The curves obtained after preaddition of protein 1 were calculated for different values of the mutual cooperativity: $\omega_{12} = \omega_{21} = 300$ (—), 100 (- - -), or 1 (···).

An interesting situation is obtained when the weakest binding protein is first bound to the polynucleotide lattice until about half-saturation and subsequently the strongest binding protein is added. This will result in extra binding of the weakest binding protein if strong mutual cooperativity exists. This effect leads to a decrease of the observed fluorescence intensity (see Figure 6).

In general, we can conclude from these experiments that already bound M13 or IKe gene 5 proteins are not able to stimulate the binding of IKe or M13 gene 5 proteins, re-

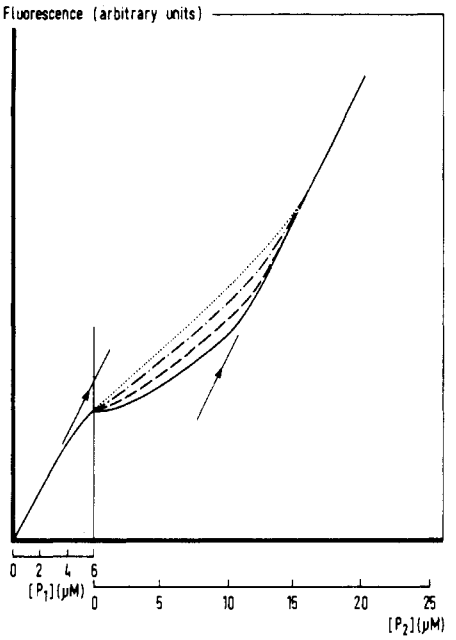

FIGURE 6: Theoretical curves for titration of a 60 μM polynucleotide solution with two different binding proteins P_1 and P_2, assuming that protein 2 (P_2) has a higher affinity for the polynucleotide than protein 1 (P_1). The titration with protein 2 started after preaddition of 6 μM of protein 1. For the binding parameters the following values were used $(K_{int}\omega)_1 = 2 \times 10^5$; $(K_{int}\omega)_2 = 2 \times 10^7$; $\omega_{11} = \omega_{22} = 300$; $Q_{max,1} = Q_{max,2} = 0.60$. The curves obtained after preaddition of protein 1 were calculated for different values of the mutual cooperativity: $\omega_{12} = \omega_{21} = 300$ (—), 150 (- - -), 50 (-·-), or 1 (···).

spectively. This implies that the binding cooperativity is protein-specific for both proteins, which underlines that the origin of the cooperativity must lie in protein–protein interactions. The absence of (or at least low) mutual cooperativity between the M13 and IKe proteins must be connected with mismatches at the protein interaction surfaces, due to changes in the amino acid composition of these surfaces during the evolution of the IKe and M13 phages.

ACKNOWLEDGMENTS

H. Nijhuis and W. Boerhof are thanked for their assistance with the fluorescence experiments and with the calculations of the theoretical titration curves. The assistance of C. W. J. M. Prinse in the preparation of the gene 5 proteins is gratefully acknowledged. P. G. M. Kersten-Piepenbrock is thanked for typing the manuscript.

Registry No. Poly(rC), 30811-80-4; poly(dA), 25191-20-2; poly(rA), 24937-83-5; poly(dI), 27732-54-3; poly(rU), 27416-86-0; poly(dU), 35297-30-4; poly(dT), 25086-81-1.

REFERENCES

Alberts, B., Frey, L., & Delius, H. (1972) *J. Mol. Biol. 68*, 139–152.

Alma, N. C. M., Harmsen, B. J. M., Hull, W. E., van der Marel, G., van Boom, J. H., & Hilbers, C. W. (1981) *Biochemistry 20*, 4419–4428.

Alma, N. C. M., Harmsen, B. J. M., de Jong, E. A. M., van de Ven, J., & Hilbers, C. W. (1983) *J. Mol. Biol. 163*, 47–62.

Brayer, G. D., & McPherson, A. (1984a) *Biochemistry 23*, 340–349.

Brayer, G. D., & McPherson, A. (1984b) *J. Biomol. Struct. Dyn. 2*, 495–510.

Bulsink, H., Harmsen, B. J. M., & Hilbers, C. W. (1985) *J. Biomol. Struct. Dyn. 3*, 227–247.

Garssen, G. J., Hilbers, C. W., Schoenmakers, J. G. G., & van Boom, J. H. (1977) *Eur. J. Biochem. 81*, 453–463.

Helene, C., & Lancelot, G. (1982) *Prog. Biophys. Mol. Biol. 39*, 1–68.

Kao-Huang, Y., Revzin, A., Butler, A. P., O'Connor, P., Noble, D. W., & von Hippel, P. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4228–4232.

Kowalczykowski, S. C., Lonberg, N., Newport, J. W., & von Hippel, P. H. (1981) *J. Mol. Biol. 145*, 75–104.

Lohman, T. M., De Haseth, P., & Record, M. T. (1980) *Biochemistry 19*, 3522–3530.

Lonberg, N., Kowalczykowski, S. C., Paul, L. S., & von Hippel, P. H. (1981) *J. Mol. Biol. 145*, 123–138.

Mazur, B. J., & Model, P. (1973) *J. Mol. Biol. 78*, 285–300.

McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469–489.

Newport, J. W., Lonberg, N., Kowalczykowski, S. C., & von Hippel, P. H. (1981) *J. Mol. Biol. 145*, 105–121.

Oey, J. L., & Knippers, R. (1972) *J. Mol. Biol. 68*, 125–138.

Peeters, B. P. H. (1985) Ph.D. Thesis, Chapter VII, University of Nijmegen, Nijmegen, The Netherlands.

Peeters, B. P. H., Konings, R. N. H., & Schoenmakers, J. G. G. (1983) *J. Mol. Biol. 169*, 197–215.

Peeters, B. P. H., Peters, R. M., Schoenmakers, J. G. G., & Konings, R. N. H. (1985) *J. Mol. Biol. 181*, 27–39.

Pörschke, D., & Rauh, H. (1983) *Biochemistry 22*, 4737–4745.

Rasched, I., & Pohl, F. M. (1974) *FEBS Lett. 46*, 115–118.

Ray, D. S. (1977) *Compr. Virol. 7*, 105–178.

Record, M. T., Lohman, T. M., & De Haseth, P. (1976) *J. Mol. Biol. 107*, 145–158.

Record, M. T., Anderson, C. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 11*, 103–178.

Record, M. T., Mazur, S. J., Melancon, P., Roe, J.-H., Shaner, S. L., & Unger, L. (1981) *Annu. Rev. Biochem. 50*, 997–1024.

Saltstrom, J. S., & Pratt, D. (1971) *J. Mol. Biol. 61*, 489–501.

Schwartz, G., & Stankowski, S. (1979) *Biophys. Chem. 10*, 173–181.

von Hippel, P. H., Kowalczykowski, S. C., Lonberg, N., Newport, J. W., Paul, L. S., Stormo, G. D., & Gold, L. (1982) *J. Mol. Biol. 162*, 795–818.