Nucleic Acid Binding Affinity of fd Gene 5 Protein in the Cooperative Binding Mode*

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A sensitive ESR method which allows a direct quantitative determination of nucleic acid binding affinities of proteins under physiologically relevant conditions has been applied to the gene 5 protein of bacteriophage fd. This was achieved with two spin-labeled nucleic acids, (dT,dT)n, and (A,A)n, which served as macromolecular spin probes in ESR competition experiments. With the two different macromolecular spin probes, it was possible to determine the relative apparent affinity constants, Kapp, over a large affinity domain. In 20 mM Tris-HCl (pH 8.1), 1 mM sodium EDTA, 0.1 mM dithiothreitol, 10% (w/v) glycerol, 0.05% Triton, and 125 mM NaCl, the following affinity relationship was observed: 

\[
K_{\text{app}}^{\text{DNA}} = 2 \times 10^4 \quad K_{\text{app}}^{\text{RNA}} = 1.5 \times 10^5 \quad K_{\text{app}}^{\text{RNA}}
\]

Increasing the [NaCl] from 125 to 200 mM caused considerably less tight binding of gene 5 protein to (A,A)n, and a typical cooperative binding isotherm was observed, whereas at the lower [NaCl] used for the competition experiments, the binding was essentially stoichiometric. A computer fit of the experimental titration data at 200 mM NaCl gave an intrinsic binding constant, Kint, of 1300 m\(^{-1}\) and a cooperativity factor, \(\omega\), of 60 \((K_{\text{int}} = K_{\text{app}})\) for (A,A)n.

The gene 5 protein product of bacteriophage fd is essential for DNA replication during the life cycle of fd phage in Escherichia coli (for a review, see Refs. 1 and 2). Proposed structures for the fd DNA-gene 5 protein complex (3) and oligonucleotide-gene 5 protein complex (4, 5) recently have been given. By a variety of spectroscopic techniques, it was established that the gene 5 protein monomer binds to four nucleotide bases along the DNA lattice (6-9). The protein has a molecular weight of 9690 and exists primarily as a dimer in neutral buffers which are 0.15 M in NaCl (10, 11). It binds to single-stranded DNA of any sequence, and binding studies with defined oligodeoxynucleotides suggest that it has a much greater affinity for adenine-rich regions than for thymine-rich regions (8). Binding of the protein to single-stranded DNA is cooperative (12), and it was also shown that it forms a complex with an isolated tetranucleotide with a dissociation constant of \(\approx 10^{-6}\) M, whereas the complex with fd DNA has a dissociation constant of \(\approx 10^{-9}\) M (7). With respect to interaction specificity, it has been tacitly assumed that oligonucleotide binding to gene 5 protein approximates polynucleotide binding, although the cooperative protein-protein interactions may play an important role in binding specificity.

The high binding affinities often present in protein-nucleic acid interactions have caused serious problems when analyzing such interactions by conventional physical methods. In order to better understand the fundamental recognition process in molecular biology, it is necessary to design experimental methods which allow reliable characterization of binding specificity under physiologically relevant conditions. The DNase protection ("footprinting") method applied recently for estimating differential binding constants (13, 14) allows one to readily discriminate sequence specificity (differential binding constant) within at least one order of magnitude (13) by making some straightforward approximations for analyzing the digestion patterns. From data based on the DNase protection experiments, values for the cooperative interaction of the bacteriophage \(\lambda\) repressor with the \(\lambda\) right operator were determined which allowed the development of a model for gene regulation by the \(\lambda\) repressor (15). This laboratory put considerable effort in designing a sensitive ESR approach which allows a direct quantitative determination of relative nucleic acid binding affinities of proteins binding under physiologically relevant conditions to nucleic acids. The approach is based on competition experiments with macromolecular spin probes such as (dT,dT)n, and allows one to distinguish between small as well as large relative nucleic acid binding affinities. We had already shown qualitatively in 1975 that, for instance, the bacteriophage T4 gene 32 protein has a much greater affinity for (dT)n than for (dA)n (16). More recently, we described the quantitative ESR approach for determining the relative affinity of the gene 32 protein for various polynucleotides (9). Here, we use two macromolecular spin probes, (dT,dT)n, and (A,A)n, to determine the relative binding affinity of gene 5 protein for various polynucleotides. Our results confirm some qualitative observations made earlier with respect to the binding of gene 5 protein to fd DNA and R\(_{\text{I}}\) RNA (17), but we could find no evidence for the general conclusion that gene 5 protein has substantially greater affinity for adenine containing nucleotides than for thymine containing nucleotides (8).

MATERIALS AND METHODS

Gene 5 protein was a generous gift of Loren A. Day (Public Health Research Institute of the City of New York). It was stored in 0.05 M NaCl, 0.02 M Tris-HCl (pH 8.1), 1 mM mercaptoethanol, and 10% (w/v) glycerol at \(-60^\circ\) C. Its concentration was determined from solution absorbance using \(E_{280}=0.73\) mg\(^{-1}\) cm\(^{-1}\) with a correction for

* The abbreviations used are: (dT,dT)n, (dA)n, chemically spin-labeled with \(4-(\alpha\text{-iodoacetamido})-2,2,6,6\text{-tetramethylpiperidino}-1\text{-oxy}\); (dT)n, polythymidylic acid; (dA)n, polydeoxyadenosine acid; (A,A)n, (A), chemically spin-labeled with \(4-(\alpha\text{-iodoacetamido})-2,2,6,6\text{-tetramethylpiperidino}-1\text{-oxy}\); (A), polyriboadenosinic acid; F, fraction of spectral ESR array arising from the fully saturated spin-labeled polynucleotide used in the linear combination.

† This work was supported in part by National Institutes of Health Grant GM-27002. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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light scattering (6). All homopolymers were purchased from P-L Biochemicals, whereas fd DNA and a mixture of (40%) 16 S (60%) 23 S ribosomal RNA were bought from Miles Laboratories, Inc. R17 EDA was prepared and purified as described earlier (18).

Preparation of Spin-labeled Polynucleotides—(dT,dT)₉₀, (IA,A)₉₀, and (dA,dA)₉₀, were prepared by chemical modification of (dT), and (A)n, respectively, with 4-(α-iodoacetamide)-2,2,6,6-tetramethylpiperidine-1-oxyl (Eastman Organic Chemicals) by using a previously published procedure. The spin-labeled and unlabeled polynucleotides were all purified through Sephacryl S-200 (1.5 x 80 cm) eluted with 0.04 M ammonium bicarbonate. Disc gel electrophoresis according to standard procedures was used to check the homogeneity and weight-average molecular weight of the nucleic acids. All spin-labeled and unlabeled synthetic polynucleotides had molecular weights of 100,000-200,000. The nitroblue-labeled nucleotide to unlabeled nucleotide ratio is given in the figure legend and was determined according to standard procedures.

Nucleic Acid Concentrations—Nucleic acid concentrations were calculated from solution absorbances with the following extinction coefficients (x10⁻³ M⁻¹ cm⁻¹): ε₂₅₇ = 8.7 for (dT), and (dT,dT),. ε₂₅₇ = 10.1 for (A), and (IA,A)₉₀. ε₂₅₉ = 8.6 for (dA), and (dA,dA)₉₀. ε₂₅₉ = 7370 for fd DNA; ε₂₅₉ = 7500 for R17 RNA; ε₂₅₉ = 7400 for 16 S + 23 S ribosomal RNA.

ESR Measurements—All ESR spectra were obtained with a Varian E-104A Century Series Spectrometer which was interfaced with an Apple II plus microcomputer. Customized software was developed in our laboratory to provide for computer control of scan range, scan time, averaging of data, and ESR recorder operation. Each 100 G data point consists of 1000 data points at a digital resolution of 12 bits and the digitized spectral arrays are stored on floppy discs. For the titration and competition studies, an E-238 cavity was used along with an E-238-3 quartz flat ESR cell onto which a paramagnetic Cr³⁺ standard was externally fixed.

ESR Data Analysis—The ESR spectra were analyzed in terms of a two-state model using a published algorithm (19). The program to determine the fraction of saturation, F, of the macromolecular spin probe is now written in BASIC and the analysis can be done directly with the Apple II plus. The paramagnetic Cr³⁺ standard to align the spectra was made by first preparing pure magnesium oxide which was dissolved in dilute HCl solution. To this solution, a known amount of chromium nitrate was added and subsequently the solution was precipitated with ammonium hydroxide. After washing the precipitate, it was dried at 400°C to yield magnesium oxide containing chromium as lattice impurity. This mixture was mixed with polyethylene powder in different proportions and hot-rolled between Teflon sheets to give a Cr³⁺ standard which is stable and convenient to handle.

RESULTS

We reported earlier the ESR line shapes of (dT,dT), in the presence and absence of gene 5 protein (9) as well as plots of F, the fraction of complexed (dT,dT), versus [protein]/[dT] based on ESR titrations of the macromolecular spin probe (dT,dT), with the protein. In Fig. 1, we show the line shape change of (IA,A)₉₀ in the absence and presence of gene 5 protein. As in the case of (dT,dT),, the binding of the protein ligand to (IA,A)₉₀ gives rise to a significant change in the ESR line shape. Complexation broadens the spectrum considerably, and the overall line shapes of the (dT,dT),, gene 5 protein complex (9) and the (IA,A)₉₀, gene 5 protein complex look similar. The substantial difference between spectral array A and B in Fig. 1 allows the monitoring of the binding of the gene 5 protein to (IA,A)₉₀. By utilizing the entire digitized ESR spectral arrays measured during the titration of (IA,A)₉₀ with gene 5 protein, it was found that a two-component analysis method can be used to determine the fraction F of complexed spin-labeled polynucleotides as was the case for bacteriophage T4 gene 32 protein and (dT,dT), or gene 5 protein and (dT,dT), (9).

A typical analysis of titration results obtained with gene 5 protein and (IA,A)₉₀ is shown in Fig. 2, where F is plotted versus [protein]/[IA]. The titrations were carried out at different NaCl concentrations and it is apparent that the binding is very salt-sensitive. At relatively low salt (e.g. in the presence of 50 and 125 mM NaCl) the binding is essentially stoichiometric, which allows one to determine the binding stoichiometry, since virtually all the protein added is bound. For that purpose, the initial slope which is the same for both salt concentrations, is extrapolated to F = 1, and the projected value of the abscissa is taken for the [protein]/[IA] ratio. It follows that gene 5 protein covers about 4 nucleotide residues, a value which is in good agreement with titration results obtained earlier with (dT,dT),, as well as with other spectroscopic techniques. At a higher salt concentration (e.g. in the presence of 200 mM NaCl), binding becomes less tight and a typical "cooperative" binding isotherm is observed. This binding isotherm is characterized by a lag phase at low protein concentration, where very little binding is seen, followed by a sharp rise in binding. It is apparent from Fig. 2 that the high binding affinity of gene 5 protein at 50 and 125 mM NaCl...
does not allow the determination of apparent binding constants, \( K_{\text{app}} \), at \( F = 0.5 \) by standard procedures. However, for binding isotherms as observed in 200 mM NaCl, the value of free protein concentration can be determined as shown earlier (21), provided that the cooperativity factor, \( \omega \), is much larger than the binding site size, \( n \). Since this condition is partially met in the case of gene 5 protein and \((1A,A)_n\) (see subsequent paragraph for the value of \( \omega \)), one can calculate the amount of free protein (ligand) at \( F = 0.5 \) by subtracting the amount of bound protein (using for that purpose the stoichiometry, \( s = 4 \)) from the total amount of protein added (free protein = 7.75 \( \mu \)M; bound protein = 6.25 \( \mu \)M). A calculation of \( K_{\text{app}} \) in this way gives a value of \( 1.3 \times 10^3 \text{ M}^{-1} \) for the binding of gene 5 protein to \((1A,A)_n\) in 200 mM NaCl. A more quantitative determination of \( K_{\text{app}} \), where \( K_{\text{app}} = K_{\text{mst}} \), \( \omega \) and \( K_{\text{mst}} \) corresponds to the intrinsic binding constant, is achieved by fitting theoretical titration curves to experimental ones as was done in the case of poly(rA) and gene 32 protein (21). Fig. 3 shows a computer “fit” of the experimental titration data obtained in 200 mM NaCl. The simulation was obtained by using the equation developed for the cooperative binding of large ligands to an infinite lattice (20) and setting \( s \), the protein binding site size, equal to 4. The “best fit” theoretical titration isotherm for gene 5 protein and \((1A,A)_n\) was obtained with an intrinsic binding constant of 1300 M\(^{-1}\) and \( \omega \) of 60.

In order to determine quantitative \( K_{\text{app}} \) values under tight binding conditions, i.e. under conditions where the binding is essentially stoichiometric and where it follows from the component concentrations that \( K_{\text{app}} \) is \( >10^3 \text{ M}^{-1} \), the competition formalism developed recently for gene 32 protein (9) can be applied. As pointed out earlier, the experiments are conveniently carried out by first establishing the relationship existing between \( F \) and the empirical peak height ratio \( h_{11}/h_0 \) for a particular protein-macromolecular spin probe system. In Fig. 4, this relationship is shown for gene 5 protein and \((1A,A)_n\) or \((dT,dT)_n\). It is apparent that the empirical peak height ratio changes considerably for both macromolecular spin probes as a function of lattice saturation. With Fig. 4, the fraction of saturated spin-labeled nucleic acid upon addition of some competitive ligands is readily determined by calculating the peak height ratios of the experimental ESR spectra measured in the course of competition experiments.

With Figs. 5 and 6, typical competition plots with the two macromolecular spin probes \((1A,A)_n\) and \((dT,dT)_n\), respectively, are shown with gene 5 protein as the protein-ligand. Fig. 5 shows the effect of adding unlabeled nucleic acid competitors to \((1A,A)_n\), complexed with gene 5 protein to reach an initial fraction of saturation, \( F_i \), between 0.64 and 0.74.

**Fig. 3.** Titration of 9.9 nmol of \((1A,A)_n\) \((1A/A = 0.03 \pm 0.003)\) with gene 5 protein under the conditions described in Fig. 1 with 200 mM NaCl. +, experimental values; O, theoretical curve calculated with \( K_{\text{mst}} = 1300 \text{ M}^{-1}, \omega = 60 \) \((K_{\text{app}} = K_{\text{app}})\), and \( s = 4 \).

**Fig. 4.** Plots of \( F \), the fraction of complexed \((1A,A)_n\) \((1A/A = 0.03 \pm 0.003) \) and \((dT,dT)_n\) \((dT/dT = 0.025 \pm 0.003) \) versus the \( h_{11}/h_0 \) peak height ratios determined during the ESR titration with gene 5 protein under the buffer conditions described in Fig. 1 with 125 mM NaCl.

**Fig. 5.** Plots of \( F \), the fraction of complexed \((1A,A)_n\) \((1A/A = 0.03 \pm 0.003) \) covered with 1.3 nmol of gene 5 protein, versus the amount of competitive nucleotides required to release gene 5 protein from its gene 5 protein \((1A,A)_n\) complex. The unlabeled nucleic acids used in competition with \((1A,A)_n\), for gene 5 protein were as follows: fd DNA ( ), \((A)_n\) (O), rRNA ( ), and \( R_{17} \) RNA ( ). The competitions were done under the buffer conditions described in Fig. 1 with 125 mM NaCl.

**Fig. 6.** Plots of \( F \), the fraction of complexed \((dT,dT)_n\) \((dT/dT = 0.025 \pm 0.003) \) covered with 1.5 nmol of gene 5 protein, versus the amount of competitive nucleotides required to release gene 5 protein from its gene 5 protein \((dT,dT)_n\) complex. The unlabeled nucleic acids used in competition with \((dT,dT)_n\), for gene 5 protein were \((dT)_n\) ( ), and fd DNA ( ). The competitions were done under the buffer conditions described in Fig. 1 with 125 mM NaCl.

Removing the ligand from the macromolecular probe with increasing amounts of fd DNA, \((A)_n\), \( R_{17} \) RNA, or rRNA results in different linear relationships. It is also apparent that considerably different amounts of competitors are needed to reach a final fraction of saturation, \( F_f \), of e.g. \( F_f = 0 \). Using the relationship derived earlier (9), it is possible to calculate the ratio \( K_{\text{app}}/K_{\text{app}} \), where \( K_{\text{app}} \) and \( K_{\text{app}} \) are the affinity constants of the protein ligand for the nucleic acids A and B.
respectively.

\[
K_{\text{app}} / K_{\text{app}} = \frac{S_h - P_s (1 - F_s)}{S_h - P_s (1 - F_s)}
\]

(1)

\(S_h\) and \(S_p\) are calculated from the known amount of competing nucleotides \(N_h\) and \(N_p\) and the binding stoichiometry \(s = 4\) by using the relationship \(S = N/s. P\) corresponds to the total amount of protein present. The nanomoles, \(N\), of fd DNA and \(N\), needed to reach \(F_1 = 0\) in Fig. 5 are 8 and 85, respectively, which gives a ratio \(K_{\text{app}}^{\text{DNA}} / K_{\text{app}}^{\text{RNA}} = 20 \pm 2\), whereas considerably larger amounts of rRNA and R17 RNA were needed to “pull off” the gene 5 protein from the (\(IA_A\)) lattice. Using \(dA\), as competitor would not have been meaningful, because the addition of gene 5 protein to the competition buffer solution containing \(dA\), or \((lA_A, dA)\), caused the formation of turbidity. With \((lA_A, dA)\) as macromolecular spin probe, it was possible to establish the relative affinity relationship between \((dT)\), and fd DNA (Fig. 6). In that case, 9 nmol of \((dT)\), and about 3000 nmol of fd DNA were needed to reach \(F_1 = 0\). This results in a value of about 1000 for the \(K_{\text{app}}^{\text{DNA}} / K_{\text{app}}^{\text{RNA}}\) ratio. Also, we established that fd DNA has no effect on the ESR spectra of \((lA_A)\), and \((lA_A, dT)\), (data not shown) and therefore does not seem to interact with the two macromolecular spin probes. With the above shown competition data, the following affinity relationship was established: \(K_{\text{app}}^{\text{DNA}} = 10^3 K_{\text{app}}^{\text{RNA}} = 2 \times 10^5 K_{\text{app}}^{\text{RNA}} = 6.6 \times 10^4 K_{\text{app}}^{\text{RNA}} = 1.5 \times 10^6 K_{\text{app}}^{\text{RNA}}\). Thus, under physiologically relevant conditions, \((dT)\), displays an affinity for the gene 5 protein which is several orders of magnitude greater than that of fd DNA or \((lA_A)\), and the data also demonstrate that \((lA_A)\), has a greater affinity for the protein than RNA or R17 RNA.

**DISCUSSION**

The primary aim of this study was to apply the ESR competition assay to a different nucleic acid binding protein and to further expand the affinity range of the assay by using an additional macromolecular spin probe. The choice of the additional probe to evaluate quantitatively relative affinity constants was dictated by its protein affinity and ESR properties. In order to be useful, it had to display a considerably lower affinity for gene 5 protein than, for instance, \((lA_A, dT)\), and its ESR properties had to be such that the fraction of saturation of the probe could be conveniently monitored by using, for instance, some internal standardization as displayed in Fig. 4. Also, the new probe was expected, as the probe \((lA_A, dT)\), not to interact with an unlabeled competitor such as fd DNA, so that fd DNA could be used to make cross-comparisons with results obtained using \((lA_A)\), and \((lA_A, dT)\), as probes. We found that \((lA_A)\), met all these criteria. On the other hand, both \((dA)\), and \((lA_A, dA)\), could not be included in this study because they formed a precipitate with gene 5 protein in the competition buffer. No such precipitation was noted with all other nucleic acids. The reason for the observed precipitation is presently not known, and we noticed no such problems with \((dA)\), or \((lA_A, dA)\), and gene 32 protein.

The spin assay to determine the affinity of nucleic acid binding proteins offers many advantages as outlined earlier (9, 22). Binding can be observed directly in solution under physiological conditions without isolating the protein-nucleic acid complex as is necessary with centrifugation or radiolabeling approaches. The measurements can be carried out with nanomole quantities of proteins and nucleic acids, which is considerably less than that required by other direct methods such as UV, fluorescence, or CD. The method of analysis is straightforward and does not depend on any “adjustment of parameters.” It should be noted that the absolute affinity of the protein for the macromolecular probe does not affect the \(K_{\text{app}}^{\text{DNA}} / K_{\text{app}}^{\text{RNA}}\) ratio as follows from the derivation of Equation (9). Thus, any potential perturbation introduced by the nitroxide radical in the macromolecular probe will not affect the relative affinity of the unlabeled nucleic acid competitors for the protein.

Although valuable information can usually be obtained in simple model systems by determining some parameters as a function of salt concentration or temperature, it is often necessary to make assumptions which might not be valid with macromolecules such as proteins and nucleic acids. For instance, there is some uncertainty about the ionic strength effect on the dimer formation of gene 5 protein, and therefore it would be extremely difficult to determine some binding characteristics with a ligand which changes its state as a function of salt concentration. It has been claimed that gene 5 protein remains a dimer under various conditions such as changing the ionic strength of the solution from 0 to 0.5 M KCl or changing the pH from 5.0 to 11.0 or varying the temperature from 5 to 20°C (11). On the other hand, it has been shown (10) that the monomer-dimer equilibrium is a strong function of salt type and concentration. It was noted that increasing the [NaCl] beyond 0.15 M had a definite effect on the sedimentation behavior of gene 5 protein, and it was suggested that the transition from dimers to monomers changes neither the CD nor the tyrosyl fluorescence of the protein (10). The observed decrease of binding affinity of gene 5 protein for the macromolecular probe \((lA_A)\), when increasing the ionic strength from 125 to 200 mM NaCl, could well reflect a shift in the monomer-dimer equilibrium, which in that case would mean an increased monomer concentration. It is reasonable to assume that, in the absence of unfavorable entropic or steric constraints, a gene 5 dimer would display greater affinity for nucleic acids than the monomer as was shown to be the case for bis(methidium)sperrmine (23). The absolute \(K_{\text{app}}^{\text{DNA}}\) calculated for \((lA_A)\), in 200 mM NaCl with two different formalisms was in the order of \(10^4 M^{-1}\) and \(\omega = 60\). It is noteworthy that, based on sucrose gradient experiments and a straightforward simple model, it was already suggested in 1972 that the affinity of gene 5 protein for a contiguous site must be at least sixty times greater than its affinity for an isolated site (12).

In view of the above given monomer-dimer equilibrium problem, no attempt was made to systematically study the binding process with gene 5 protein as a function of salt concentration and to interpret the salt effects in terms of ionic interactions and release of structured water involved in the binding process (24, 25). We selected a NaCl concentration for the competition studies which results in very tight binding so that the approximation of no free protein in solution made for the derivation of the competition formalism (9) is valid with both macromolecular spin probes. The affinity relationship observed under these tight binding conditions reveals, as in the case of gene 32 protein, differences in binding affinities of several orders of magnitude. Both proteins have by far the largest affinity for \((dT)\), and significant differences in affinity exist for all other nucleic acids tested.

**Acknowledgment**—We wish to thank Dr. Loren Day for a generous gift of gene 5 protein.

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J. Biol. Chem. 1984, 259:2130-2134.

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