A SPIN PROBE APPROACH FOR MEASURING THE NUCLEIC ACID AFFINITY OF GENE 32 PROTEIN

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Summary: A novel and fast procedure for determining by electron spin resonance the affinity of proteins for nucleic acids is described. The assay makes use of nitroxide radicals which are covalently bound to various polynucleotides to the extent of one probe per 75 to 100 nucleotides. As a test example gene 32 protein was used, a protein known to interact with single stranded nucleic acids. Competition experiments with unlabeled nucleic acids made it feasible to directly monitor the gene 32 protein affinity for different nucleic acids. It was observed that DNA single strands are not necessarily favored by this protein for preferential binding. The experimental data also suggest that the difference in the binding constants for most of the complexes is remarkably large; for instance, $(dT)_n$ binds at least 3 to 4 orders of magnitude better to gene 32 protein than $(dA)_n$.

The discovery of T4 gene 32 protein, P32, made it possible to get some insight into the basic mechanism of DNA replication. It was observed that this protein binds cooperatively and preferentially to single stranded DNA, and only weakly, if at all, to double stranded DNA or RNA (1). Its specific affinity for single-stranded nucleic acids is believed to be responsible for DNA denaturation. Although the interaction forces in the P32·nucleic acid complex are not yet known in detail, it has been suggested that P32 contains a region of positively charged amino acids which interact with the nucleic acid phosphate (1).

In this communication we wish to report a novel approach for determining the affinity of a protein for various nucleic acids. Nitroxide labeled (spin

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Abbreviations: All abbreviations used for the homopolymeric nucleic acids are in accordance with the IUPAC-IUB recommendation 1970 (J. Biol. Chem. 245, 5171 (1970)); $(dUfl)_n$, homopolymeric 2'-fluoro-2'-deoxyuridylic acid; $\ell(N)_n$, a homopolymeric nucleic acid nitroxide labeled (spin labeled) with 4-(2-iodoacetamido)-2,2,6,6-tetra-methylpiperidineoxyl; P32, T4 gene 32 protein; fd DNA, circular single stranded DNA from bacteriophage fd.

labeled) nucleic acids are taken for monitoring their complex formation with P32 by electron spin resonance (ESR) spectroscopy. Subsequently, the spin labeled nucleic acid-protein complex is exposed to unlabeled nucleic acids, which will, depending upon their affinity for P32, completely release the nitroxide labeled nucleic acid. This event is directly monitored by ESR. Previously, nucleic acids labeled with such a probe had been used for following interactions among polynucleotides by ESR (2,3).

Materials and Method: P32 and fd DNA were gifts from Dr. B. Alberts (Princeton University). The homopolymeric nucleic acids were obtained from Miles Laboratories, Inc. The spin label 4-(2-iodoacetamido)-2,2,6,6-tetra-methylpiperidine-oxyl were incorporated into $(dUfl)_n$, $(dA)_n$, $(A)_n$ and $(U)_n$ according to (2). The spin label to nucleotide ratio was about one probe per 75 to 100 nucleotides. Competition experiments were done together with unlabeled fd DNA, $(dT)_n$, $(dUfl)_n$, $(dA)_n$, $(A)_n$ and $(U)_n$.

The concentrations of fd DNA, $(N)_n$, $\ell(N)_n$ and P32 were determined from known solution absorbancies.

The ESR spectra were measured on a Varian E-4 EPR spectrometer interfaced with a Fabri Tek 1074 signal averaging computer. A regular flat quartz cell was used for the measurement. All the studies were done in buffer B (1) (0.02M Tris-HCl, pH 8.1, 10% glycerol, 1mM Na_3EDTA, 1mM β-mercaptoethanol).

It was noticed that the presence of β-mercaptoethanol in buffer B reduces the radical concentration by about 10% during a period of 4 hours.

The formation of the P32·$\ell(N)_n$ complex was followed by monitoring the ratios of high field (h_{-1}) and low field (h_1) to center field (ho) hyperfine components ($\frac{h_{-1}}{ho}$, $\frac{h_1}{ho}$), which show within the error limits the same characteristic values for all the different complexes. The time elapsed between mixing P32 and $\ell(N)_n$ and the final ESR recording was usually 5-8 minutes. Since the ESR spectrum did not change its line shape any further after the first recording, it is concluded that the attainment of equilibrium is not a slow process in this system. Full release of $\ell(N)_n$ from its protein complex

caused by a different unlabeled $(N)_n$ was inferred when the characteristic ESR line shape of $\ell(N)_n$ would reappear.

Results and Discussion: The strategy of determining the affinity binding of P32 for various labeled and unlabeled polynucleotides, $\ell(N_A)_n$ and $(N_B)_n$, is summarized in the scheme shown below, which takes into account the observation of the cooperative binding of P32 to $(N)_n$ (1). Saturation binding levels obtained by titrating the various $\ell(N_A)_n$ with P32 were taken into account for the ESR assay; the binding levels were noticed to vary to a small extent for the different nucleic acids (unpublished result) and therefore the variables a,b,c,d,i, and j were introduced into the scheme. The experiments were designed in such a way that an excess of $\ell(N_A)_n$ would always be present. Thus, upon adding of a $(N_B)_n$ with strong P32 affinity (route A) in an amount which equals that of the $\ell(N_A)_n$, complete release of the $\ell(N_A)_n$ could be observed

The ESR line shape characteristics of $\ell(N)_n$, $\ell(N_A)_n \cdot (N_B)_n$ and $\ell(N)_n \cdot P32$ are tabulated in Table 1. h_1/ho and h_{-1}/ho are the ratios defined in the

SCHEME

$$\ell(N_A)_n + (1 - a)\cdot i(P32) \downarrow$$

$$(1 - a)\cdot\ell(N_A)_n(P32)_i + a\cdot\ell(N_A)_n$$

$$+ (N_B)_n$$

A ← | → B

$$\ell(N_A)_n + (1 - b)\cdot(N_B)_n(P32)_j + b(N_B)_n \qquad (N_B)_n + (1 - a)\cdot\ell(N_A)_n(P32)_i + a\cdot\ell(N_A)_n$$

C ↓

$$c\cdot(N_B)_n + (1-c)\cdot(N_B)_n(P32)_j + d\cdot\ell(N_A)_n + (1 - d)\cdot\ell(N_A)_n(P32)_i$$

Scheme for cooperative binding of P32 to $\ell(N_A)_n$ without saturating all $\ell(N_A)_n$ and subsequent addition of $(N_B)_n$. Route A: $(N_B)_n$ has a higher affinity towards P32 than $(N_A)_n$; Route B: $(N_A)_n$ has a higher affinity than $(N_B)_n$; Route C: no substantial difference exists in the affinity between $(N_A)_n$ and $(N_B)_n$ for P32; (a,b,c and d are the mole fractions of the polynucleotides saturated with P32; i and j can be different numbers of P32 covering the same number of residues in $(N_A)_n$ and $(N_B)_n$ since the saturation binding levels are not always the same for both nucleic acids).

materials and method part. These ratios allow to get qualitative characteristics of the degree of the nitroxide mobility. Previously, the motion of the nitroxide probe in $\ell(U)_n$, $\ell(dUfl)_n$, $\ell(U)_n \cdot (A)_n$, and $\ell(dUfl)_n \cdot (A)_n$ was analyzed by a more vigorous approach, namely, by computer simulating the ESR spectra according to the theory of Freed (5). As is obvious from table 1 the ratio h_1/ho and h_{-1}/ho do not differ strongly within a particular group. This is especially the case for the complexes of the $\ell(N)_n \cdot P32$ set, whose nitroxide labels are all more immobilized than those present in $\ell(N)_n$ and $\ell(N_A)_n \cdot (N_B)_n$.

TABLE 1. ESR Characteristics of $\ell(N)_n$, $\ell(N_A)_n \cdot (N_B)_n$ and $\ell(N)_n \cdot P32$

System	Buffer	h_1/ho	h_{-1}/ho
$\ell(A)_n$	B	.90±.01	.33±.01
$\ell(dA)_n$	B	.90±.01	.32±.01
$\ell(U)_n$	B	.98±.01	.47±.01
$\ell(dUfl)_n$	B	.97±.01	.44±.01
$\ell(A)_n \cdot (dT)_n$	B	.72±.01	.30±.01
$\ell(A)_n \cdot (dUfl)_n$	B	.72±.01	.30±.01
$\ell(dA)_n \cdot (dT)_n$	B	.74±.01	.27±.01
$\ell(U)_n \cdot (A)_n$	B+0.02MNaCl	.75±.01	.24±.01
$\ell(A)_n \cdot P32$	B	.60±.05	.20±.05
$\ell(dA)_n \cdot P32$	B	.60±.05	.20±.05
$\ell(U)_n \cdot P32$	B	.60±.05	.20±.05
$\ell(dUfl)_n \cdot P32$	B	.55±.05	.20±.05

Figure 1 gives some examples of ESR results obtained from P32 and polynucleotides which are not complementary to each other. Spectrum a shows uncomplexed $\ell(U)_n$, whereas spectrum b reveals a mixture consisting of $\ell(U)_n$ saturated with P32 and some uncomplexed $\ell(U)_n$. Upon addition of $(dUfl)_n$ to the mixture of the ESR line shape characteristics of $\ell(U)_n$ are recovered (spectrum c) This observation is indicative of a complete release of $\ell(U)_n$ from its P32 complex. It should be pointed out that the overall signal intensity of the recovered $\ell(U)_n$ ESR spectrum is smaller. Such a loss in intensity was noted for all released $\ell(N)_n$ and is attributed to a partial destruction of the nitroxide probe by buffer B (see Materials and Method) and possibly also

to a small extent by P32. Spectra d and e show uncomplexed $\ell(dUfl)_n$, and a mixture of $\ell(dUfl)_n$ saturated with P32 plus uncomplexed $\ell(dUfl)_n$, respectively. No release of single stranded spin labeled $(dUfl)_n$ is observed upon addition of $(U)_n$ (spectrum f). Based on these two different experiments, it can be unequivocally derived that P32 has a considerably greater affinity for $(dUfl)_n$ than for $(U)_n$. This method of determining the affinity binding of P32 for various nucleic acids was repeated for all possible combinations with a

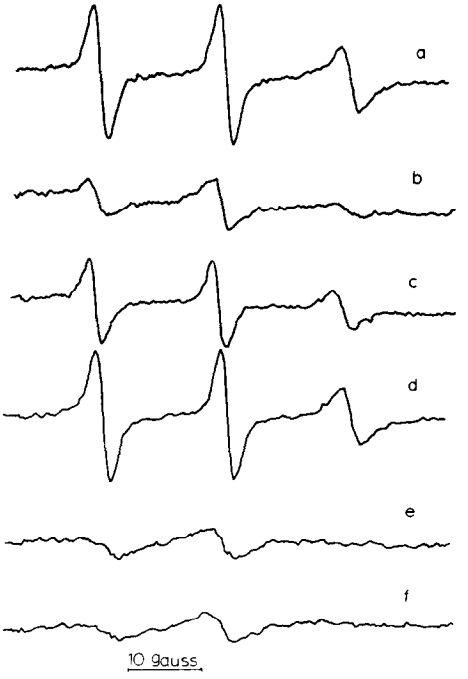

Figure 1. ESR experiments for determining the P32 affinity for $(U)_n$ and $(dUfl)_n$ in buffer B_o. Spectrum a: 10 nmole $\ell(U)_n$; spectrum b: upon addition of 1 nmole P32 to 10 nmole $\ell(U)_n$ (representing a mixture consisting of $\ell(U)_n$ saturated with P32 and uncomplexed $\ell(U)_n$); spectrum c: after the addition of 10 nmole $(dUfl)_n$ to the mixture shown in spectrum b; spectrum d: 10 nmole $\ell(dUfl)_n$; spectrum e: upon addition of 1 nmole P32 to 10 nmole $\ell(dUfl)_n$ (representing a mixture consisting of $\ell(dUfl)_n$ saturated with P32 and uncomplexed $\ell(dUfl)_n$); spectrum f: after the addition of 10 nmole $(dUfl)_n$ to the mixture shown in spectrum e.

labeled and an unlabeled nucleic acid, neither of which was complementary to each other. With this approach the following relationship could be established: $(dT)_n > (dUfl)_n$; $(dT)_n > (U)_n$; $(dUfl)_n > (U)_n$; $(A)_n > (dA)_n$.

Subsequently, the same strategy was used with nucleic acids which are complementary to each other for studying their affinity towards P32. Possible formation of the $\ell(N_A)_n \cdot (N_B)_n$ duplexes shown in Table 1 as well as their rate of formation was studied in buffer B before carrying out the P32 affinity experiments. It was noticed that the duplex formation was a relatively slow process in this buffer for all systems studied. To get constant ESR lineshape characteristics of $\ell(dUfl)_n \cdot (A)_n$, $\ell(dA)_n \cdot (dT)_n$, and $\ell(A)_n \cdot (dT)_n$ shown in Table 1, it took approximately half an hour, one hour and two hours, respectively. Even after 18 hours a mixture of equimolar amounts of $\ell(U)_n$ and $(A)_n$ would not give an ESR spectrum with a constant lineshape. However, the rate of duplex formation was considerably increased upon adding NaCl into buffer B. The ESR spectra recorded within 5 to 8 minutes after mixing remained constant. The ESR characteristics of $\ell(U)_n \cdot (A)_n$ are reported in buffer B containing 0.02 M NaCl (Table 1). No interaction was noted to take place between an equimolar mixture of $(dA)_n$ and $\ell(U)_n$ in buffer B. It is known that the latter system forms triple strands under appropriate conditions (4). Taking into account the slow ESR lineshape changes for the different systems, the formation of 1:1 complexes in buffer B was established in the cases of $\ell(dUfl)_n \cdot (A)_n$; $\ell(dA)_n \cdot (dT)_n$ and $\ell(A)_n \cdot (dT)_n$ by adding small portions of $(N_B)_n$ of known concentration to a known amount of $\ell(N_A)_n$ and monitoring the lineshape changes upon each addition. This approach had been previously taken to titrate $(A)_n$ with $\ell(U)_n$ or $\ell(dUfl)_n$ in a buffer of higher ionic strength where the duplex formation was rapid (5).

As was pointed out earlier P32 binding to single stranded nucleic acids cannot be a slow process, since the ESR spectrum remains constant after the first recording which is obtained 5 to 8 minutes after mixings. It was observed with all the complementary nucleic acids used in this assay that the formation

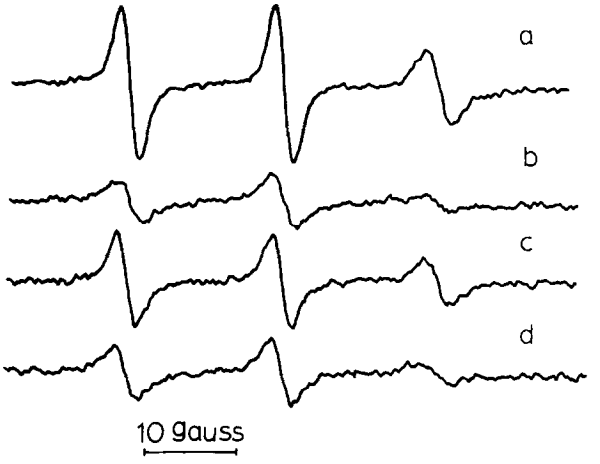

Figure 2. ESR experiments for determining the P32 affinity for $\ell(U)_n$ and $(A)_n$ in buffer B (spectra a, b, c) and for demonstrating the formation of $\ell(U)_n \cdot (A)_n$ in the presence of P32 upon addition of NaCl (spectrum d). Spectrum a: 10 nmole $\ell(U)_n$; spectrum b: upon addition of 1 nmole P32 to 10 nmole $\ell(U)_n$ (representing a mixture consisting of $\ell(U)_n$ saturated with P32 and uncomplexed $\ell(U)_n$); spectrum c: after the addition of 10 nmole $(A)_n$ to the mixture shown in spectrum b; spectrum d: after the addition of 2×10^{-6} mole of NaCl to the mixture of spectrum c.

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of $\ell(N_A)_n \cdot$P32 or $(N_B)_n \cdot$P32 was strongly favored in buffer B and that a $\ell(N_A)_n \cdot (N_B)_n$ duplex would only form under special circumstances. For instance, the addition of some NaCl to buffer B weakens a $\ell(N_A)_n \cdot$P32 complex and stabilizes a $\ell(N_A)_n \cdot (N_B)_n$ duplex. Such a situation is encountered in Fig. 2, where the first three spectra from the top are used to establish the P32 affinity of $(U)_n$ versus $(A)_n$ and spectrum d shows the formation of an $(A)_n \cdot (U)_n$ duplex upon addition of NaCl. Considerable formation of $\ell(N_A)_n \cdot (N_B)_n$ could also occur according to the scheme shown earlier, if the mole fraction is too large. For that reason the mole fraction a was kept small so that only a negligible amount of duplex would form, if at all.

Thus, affinity binding with two complementary strands can give similar ESR

patterns as shown in Fig. 1. For instance, in Fig. 2, ESR results are shown with P32 and the complementary polynucleotides $\ell(U)_n$ and $(A)_n$. Addition of $(A)_n$ to a mixture of $\ell(U)_n \cdot$P32 and $\ell(U)_n$ causes fast and complete release of $\ell(U)_n$, as can be concluded from the ESR spectrum which has the characteristics of $\ell(U)_n$ (spectrum c in Fig. 2). A complete uncomplexed $\ell(U)_n$ spectrum is observed, because the mole fraction of the excess of $(A)_n$ was kept small and in addition, the formation of the $(A)_n \cdot (U)_n$ complex is a very slow process in buffer B, as was mentioned previously. As a control $(U)_n$ was added to a mixture of $\ell(A)_n \cdot$P32 and $\ell(A)_n$, and as expected this addition caused no change in ESR lineshapes (not shown). Therefore, it can be concluded that P32 has a greater affinity for $(A)_n$ than for $(U)_n$. This strategy was repeated with the remaining $\ell(N_A)_n$ and $(N_B)_n$ reported in materials and method considering only combinations of polynucleotides which are complementary to each other. The following P32 affinity relationship was found: $(dT)_n > (dA)_n$; $(dT)_n > (A)_n$; $(dUfl)_n > (A)_n$; $(A)_n > (U)_n$; $(U)_n \approx (dA)_n$.

In addition, fd DNA was included in this study by checking its ability for releasing $\ell(N_A)_n$ in the $\ell(N_A)_n \cdot$P32 complexes. It was noticed that this DNA would cause full release of the spin labeled polynucleotides in all the complexes, which in terms of affinity means that: fdDNA > $(dUfl)_n$, $(A)_n$, $(U)_n$, $(dA)_n$.

Combining the conclusions obtained with the various polynucleotide pairs permits to establish the following P32 affinity relationship for some polyribo- as well as polydeoxyribonucleotides:

$$(dT)_n > (dUfl)_n > (A)_n > (U)_n \approx (dA)_n$$

Thus, single stranded DNA is not necessarily favored for preferential binding by P32. The experimental data also suggest that the association constants of the various $(N)_n \cdot$P32 complexes must be remarkably different from each other in most cases. It is for instance inferred that the association constant of $(dT)_n$ for P32 is at least 3 to 4 orders of magnitude larger than that of $(U)_n$ or $(dA)_n$. Large differences in association constants have already been noted

before in the lac repressor-operator system, where an association constant of 1×10^{13} M^{-1} was observed for the operator site, a value which is by several orders of magnitude greater than those found for other sites (6).

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