[12] Fluorescence Anisotropy Applied to Biomolecular Interactions

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Introduction

The appeal of fluorescence spectroscopy in the study of biomolecular systems lies in the characteristic time scale of the emission process, the sensitivity of the technique, and its ability to accommodate rapid and facile changes in the solvent milieu under conditions corresponding to thermodynamic equilibrium. The time scale of the emission process invites exploitation in two related manners. First, information on hydrodynamic aspects of the system is available from steady-state or time-resolved measurements. Second, detailed information on local dynamic processes within the biomolecular matrix may be derived. Information on hydrodynamic aspects of a macromolecular system may be used to study binding processes, that is, the association of small ligands with macromolecules or macromolecule–macromolecule interactions. In this chapter we focus on the latter applications of polarization or anisotropy data. We shall also try to clarify aspects of this area that our experience has shown to be occasionally misunderstood by initiates.

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Basic Principles

Polarized Emission and Rotational Diffusion

Further exposition on this topic requires that we clarify the definitions of polarization and anisotropy and describe the molecular events which determine their values. An excellent treatment of the origins of the polarization of fluorescence has been given by Weber.[1] Our treatment here will not enter into the same rigorous detail, and readers with a sustaining interest are referred to that article[1] and the references therein. On excitation of a fluorescent solution and observation of the emission at right angles both to the direction of propagation of the exciting light and to the direction of the electric vector, the polarization of the emission is defined as

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}) \tag{1}$$

where we understand $I_{\parallel}$ and $I_{\perp}$ to be the intensities of the emission viewed through parallel and perpendicularly arranged polarizers, respectively. Anisotropy (r), on the other hand, is defined as

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}) \tag{2}$$

We then note that

$$r = 2P/(3 - P) \qquad \text{or} \qquad r = 2/3(1/P - 1/3)^{-1} \tag{3}$$

The relations between polarization, fluorescence lifetime, and rotational diffusion were explored by F. Perrin,[2] no doubt inspired by the work of his father, J. Perrin, on the translational diffusion of macroscopic particles. The Perrin equation may be written

$$(1/P - 1/3) = (1/P_0 - 1/3)(1 + 3\tau/\rho) \tag{4}$$

where P_0 is the limiting or intrinsic polarization in the absence of depolarizing influences such as rotation or energy transfer,[3] τ is the excited state

[1] G. Weber, *in* "Fluorescence and Phosphorescence Analysis" (D. M. Hercules, ed.), p. 217. Wiley, New York, 1966.

[2] F. Perrin, *J. Phys. Radium* **7,** 390 (1926).

[3] The value of P_0 is determined by the angle (θ) between the absorption oscillator and the emission oscillator. For fluorophores randomly oriented in solution this value can, in principle, range between $+\frac{1}{2}$ and $-\frac{1}{3}$, its magnitude being given by the expression

$$(1/P_0 - 1/3) = (2/3)[(3\cos^2\theta - 1/2)/2]^{-1}$$

The positive limit, 0.5 (or 0.4 for anisotropy), is often assumed appropriate and, in fact, was a reasonable approximation in the days when only a few fluorophores based on naphthalene and fluorescein were utilized along with arc lamps/monochromator systems for excitation. Given the immense proliferation of probes currently available (see, e.g., a catalog from Molecular Probes, Eugene, OR) and the increasing use of lasers with

lifetime, and ρ the Debye rotational relaxation time. For a spherical molecule,

$$\rho = 3\eta V/RT \tag{5}$$

where η is the viscosity of the medium, V the molar volume monitored by the probe, R the gas constant, and T the absolute temperature. At this point we should draw attention to the fact that one often encounters in the literature the term τ_c, the rotational correlation time, and that[4]

$$\rho = 3\tau_c \tag{6}$$

Hence, when approaching the literature one must be very clear on this difference and on which definition is being utilized.

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various fixed outputs (such as 325 nm for helium–cadmium lasers), the fluorescence practitioner would do well to verify the P_0 value for the probe of choice given the particular excitation and emission wavelengths utilized. Pyrene, for example, exhibits a P_0 which is generally low (<0.2) and varies with both excitation and emission wavelength. This consideration also applies to intrinsic protein fluorescence: one often notices excitation in these cases at 295 nm, to minimize excitation of tyrosine, yet the highest P_0 of tryptophan (0.4) is not attained at excitation wavelengths below 300 nm.

[4] The rotational relaxation time was originally defined by Debye in relation to studies on dielectric phenomena. Dipolar molecules orient themselves in an electric field, and this preferential orientation decays and disappears on removal of the field. Because the orientation in which the molecular dipoles align antiparallel to the electric field is not equivalent to the orientation in which they lie parallel to it, the orientational distribution is a function of cos θ, the angle between the direction of the electric field and that of the dipole axis. The decay of the orientational polarizability at time t is then given by $\cos \theta(t) = \exp(-t/\rho)$ where ρ is the rotational relaxation time. For a spherical molecule Einstein's theory of molecular rotations gives $\rho = 3\eta v/kT$ where η is the solvent viscosity and v the molecular volume. In work on fluorescence depolarization by rotational diffusion Perrin used this definition by Debye as giving the characteristic time for the molecular rotations of spheres. Photoselection takes place because absorption of light is proportional to the cosine square of the angle between the transition moment in absorption and the electric vector of the exciting light, and molecules lying 180° apart in orientation have the same absorption probability. As a result the average cosine square of the angle of random molecular orientation and the electric vector of the light is not zero, as in the dielectric experiment, but rather 1/3. If instead of defining the decay of the orientation of the illumination according to the average angle of a preferred orientation, we were to do it according to the decay of the observable changes in polarization, the characteristic time for disorientation would appear to be three times shorter than for the previous case. This later point of view was adopted by Bloch in computing the decay in the nuclear polarization and was named by him the rotational correlation time. Various authors have followed Bloch and write the Perrin equation for the depolarization owing to molecular rotations as

$$r = r_0/(1 + \tau/\tau_c)$$

where $\tau_c = \rho/3$ (the authors thank Gregorio Weber for this historical insight).

The method of Perrin was later extended by Weber[5] to the case of nonspherical molecules such as ellipsoids of revolution. In addition to depolarization owing to overall or "global" rotational motion of the target macromolecule one must often take account of "local" probe motion. By "local" motion we understand any mobility of the probe in excess of that expected by the rotation of a rigid body to which it is attached. Hence, "local" motion includes internal or domain motions as well as specific movement of the probe molecule about its point of attachment to the macromolecule. The latter motion is dramatically reduced in cases of noncovalently bound probes, such as ANS, porphyrins, and NADH to name a few. In these cases the attainment of significant binding energies usually entails several points of interaction, thus reducing the probe mobility. In contradistinction covalent probes, including the intrinsic protein fluorophores such as tryptophan and tyrosine, often experience considerable movement limited only by the structural barriers imposed by the macromolecular framework.

Additivity of Polarization/Anisotropy

As stated earlier our goal here is not to expound on hydrodynamic information per se but rather to ascertain how the hydrodynamic (and dynamic) considerations may be utilized to study biomolecular interactions. The use of polarization/anisotropy to elucidate binding isotherms arose from the observations of Weber[5] on the additivity properties of polarization. Specifically, he demonstrated that (for polarized excitation)

$$(1/P_{\text{obs}} - 1/3)^{-1} = \Sigma f_i(1/P_i - 1/3)^{-1} \tag{7}$$

where P_{obs} is the actual polarization observed arising from i components and f_i represents the fractional contribution of the ith component to the total emission intensity. In terms of anisotropies then

$$r_{\text{obs}} = \Sigma f_i r_i \tag{8}$$

The motivation for considering the additivity function arose from the realization that a population of nonspherical proteins could give rise to a distribution of rotational rates depending on the orientation of the probe along the respective rotational axes. Hence, a clear understanding of the ways in which the individual contributions sum to the total signal was important. These considerations led immediately to the work of Laurence[6] who first described the application of polarization methods to follow the binding of small ligands to proteins. Dandliker and co-workers later applied

[5] G. Weber, *Biochem. J.* **51,** 145 (1952).
[6] D. J. R. Laurence, *Biochem. J.* **51,** 168 (1952).

these principles explicitly to the study of antibody–antigen interactions.[7,8] We may note at this point that Jablonski[9] introduced the term anisotropy, as defined above, and drew attention to the additive nature of the function and to the fact that the sum $(I_{\parallel} + 2I_{\perp})$ represents the total contribution to an emitting system viewed at right angle to the excitation. One must not, however, lose sight of the fact that anisotropy and polarization have the same information content, and the use of one or the other term is a matter of convenience not of substance.

Although conceptually straightforward, the utilization of polarization/anisotropy data to obtain binding isotherms can be subject to experimental subtleties. Once the f_i terms are determined the problem would seem virtually solved at least so far as having the information necessary to construct a binding isotherm (such as a Bjerrum or Scatchard plot[10]). The difficulty lies in the determination and significance of the f_i terms. One must realize that these terms represent, prima facia, the contribution of the ith component to the photocurrent. To convert the data to molar quantities assumes a thorough appreciation of the instrument response characteristics, including the detector and monochromator response to parallel and perpendicularly polarized light as a function of wavelength,[11] as well as the relative quantum yields of the free and bound probes.

The appeal of optical spectroscopy in the study of binding processes owes in large measure, as alluded to earlier, to the fact that these methods permit acquisition of data under conditions of thermodynamic equilibrium. Most other methods in fact rely on separation of free and bound materials, for example, by ultrafiltration, chromatography, or sedimentation. One must then clearly recognize the potential limits of these methods as regards the rapidity with which a new equilibrium may be established after the separation procedure and the possible perturbing effect of the method on the initial equilibrium (e.g., pressure effects during centrifugation). With spectroscopic methods one strives to obtain directly the concentrations of free and bound material by virtue of a variation in some observable parameter on complex formation. In the case of fluorescence the simplest case would be that in which the spectral maximum or quantum yield changes sensibly on binding. It may happen, however, that such changes are too slight to be experimentally resolvable (*vida infra*). In almost all such cases, though, be they small molecule–macromolecule or macromol-

[7] W. B. Dandliker and G. A. Feijen, *Biochem. Biophys. Res. Commun.* **5,** 299 (1961).
[8] W. B. Dandliker, M.-L. Hsu, J. Levin, and B. R. Rao, this series, Vol. 74, p. 3.
[9] A. Jablonski, *Acta Physiol. Pol.* **16,** 471 (1957).
[10] G. Weber, "Protein Interactions." Chapman & Hall, New York and London, 1992.
[11] D. M. Jameson, *in* "Fluorescein Hapten: An Immunological Probe" (E. Voss, Jr., ed.), p. 23. CRC Press, Boca Raton, Florida, 1984.

ecule–macromolecule interactions, changes in rotational rates do occur on complex formation and hence indicate the utility of a method based on molecular rotational rates, such as polarization/anisotropy methods. Another limitation with intensity measurements may be encountered if the binding is sufficiently weak that appreciable concentrations of the molecular species involved must be utilized to assure complex formation. In such cases the optical densities of the solution may be unavoidably high (even allowing for smaller cuvette or front-face geometries), and the requisite inner filter corrections may lower confidence in the binding data. Polarization/anisotropy measurements, like lifetime measurements, are intensive quantities and as such are not subject to first-order inner filter corrections.

Applications

Small Molecule–Protein Interactions

Kasprzak and Villafranca[12] characterized the binding of the fluorescent nucleotide 1,N^6-ethenoadenosine 5′-monophosphate (εAMP) to *Escherichia coli* carbamoyl-phosphate synthase (CPS) using fluorescence anisotropy titrations. This approach was particularly relevant in this system since the binding of εAMP to CPS is not accompanied by alterations in the quantum yield or emission maximum of the probe. To ascertain the anisotropy of the bound εAMP (r_b) they first titrated a small concentration of probe with excess enzyme until the anisotropy approached a limiting value (Fig. 1a). Next, CPS, at a constant concentration, was titrated with εAMP, and a Scatchard plot was constructed as shown in Fig. 1b. The fraction of bound εAMP, f_b, at any given probe/CPS ratio, was calculated from the observed anisotropy, r_{obs}, by

$$f_b = (r_{obs} - r_{in})/(r_b - r_{in}) \tag{9}$$

where r_{in} is the initial anisotropy. In this case, because the quantum yield does not change on binding, additional corrections as discussed earlier are not required.

The above procedure applies well to situations in which the ligand is the source of the fluorescence signal. If the acceptor is the source of the fluorescence signal then a slightly different procedure is recommended as illustrated in the section on intrinsic protein fluorescence in protein–DNA interactions. An extensive treatment of the application of these methods to antigen–antibody interactions has been given by Dandliker *et al.*[8] Other

[12] A. A. Kasprzak and J. F. Villafranca, *Biochemistry* **27,** 8050 (1988).

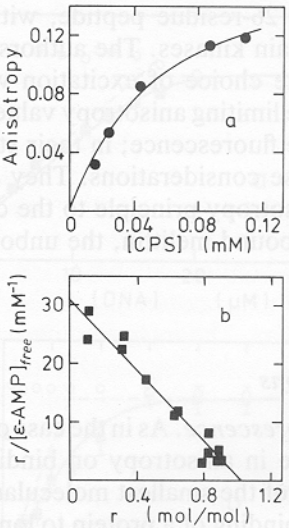

FIG. 1. (a) Anisotropy titration of a constant amount of εATP (15 μM) with carbamoylphosphate synthase. The solid line corresponds to $r_b = 0.176$ and $K_d = 0.04$ mM. (b) Scatchard plot calculated from data obtained from the titration of a constant amount of enzyme (51.5 μM) with εATP. The solid line corresponds to $K_d = 0.033$ mM and $n = 1$. (From Kasprzak and Villafranca.[12])

authors who have explicitly considered the application of polarization methods to the characterization of small molecule–protein interactions include Rajkowski and Cittanova,[13] Teichberg and Shinitsky,[14] and Deranleau *et al.*[15]

Peptide–Protein Interactions

Malencik and Anderson have described a series of studies, using various probe strategies, on the interaction of peptides with calmodulin.[16] Because calmodulin does not contain tryptophan, they were able to observe the increase in the anisotropy of tryptophan in various peptides on binding. For example, the anisotropy from the tryptophan of *Polistes* mastparan, a toxic peptide from the social wasp, on excitation at 294 nm, increased from around 0.02 to 0.13 on binding to calmodulin. In another study Malencik and Anderson[17] characterized the association of melittin,

[13] K. M. Rajkowski and N. Cittanova, *J. Theor. Biol.* **93,** 691 (1981).
[14] V. I. Teichberg and M. Shinitsky, *J. Mol. Biol.* **74,** 519 (1973).
[15] D. A. Deranleau, T. Binkert, and P. Bally, *J. Theor. Biol.* **86,** 477 (1980).
[16] D. A. Malencik and S. R. Anderson, *Biochem. Biophys. Res. Commun.* **114,** 50 (1983).
[17] D. A. Malencik and S. R. Anderson, *Biochemistry* **27,** 1941 (1988).

a tryptophan-containing 26-residue peptide, with calmodulin and three types of myosin light chain kinases. The authors make the point that, in such protein studies, the choice of excitation wavelength represents a compromise between the limiting anisotropy value, the sensitivity, and the minimization of tyrosine fluorescence; in their studies 294 nm excitation generally optimized these considerations. They also explicitly extended the additivity of the anisotropy principle to the case of three fluorescent species, namely, the unbound melittin, the unbound light chain, and the complex.

Protein–DNA Interactions

Intrinsic Protein Fluorescence. As in the case of protein–ligand interactions, the largest change in anisotropy on binding will occur when the fluorescence anisotropy of the smallest molecular weight species is being followed. Thus, for the binding of a protein to long DNA the fluorescence of the protein should be observed. On the other hand, for the binding of a protein to a short recognition sequence of DNA, such as a 20 base pair (bp) operator sequence recognized by a repressor protein, it may be more beneficial to label the DNA.

It is important to appreciate the interplay between steady-state anisotropy and the lifetime of the fluorophore. The short lifetime (1–6 nsec) of tryptophan in proteins does not necessarily preclude its use for monitoring protein–DNA interactions. The correlation time for the global tumbling of the protein may be long relative to the lifetime of tryptophan such that the anisotropy of the protein in free solution is high and may increase little on binding DNA. However, it is often observed that the indole side chains of tryptophan have substantial mobility owing to rotation about the C_α–C_β bond such that the anisotropy of the protein in free solution is small. In these cases there might be a useful increase in anisotropy on binding to DNA. For example, the tryptophan anisotropy of the Ada protein, a DNA repair enzyme from *E. coli*, increases on binding nonspecifically to DNA,[18] as shown in Fig. 2. In this case, the anisotropy of the free Ada protein is relatively large, indicating restricted motion of the tryptophan residues. Nevertheless, the small increase in anisotropy (~0.02 units) was sufficient for analysis of the binding equilibria. Corrections for the inner filter effect were not necessary. The stoichiometry was confirmed by a reverse titration in which the Ada protein was added to a constant amount of DNA.

[18] M. Takahashi, K. Sakumi, and M. Sekiguchi, *Biochemistry* **29,** 3431 (1990).

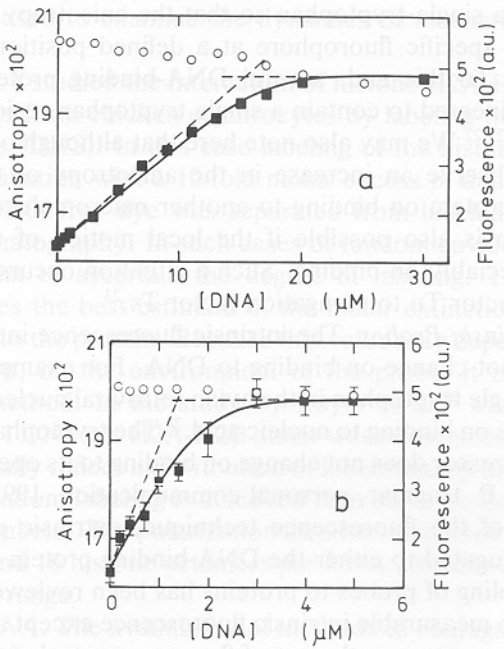

FIG. 2. The tryptophan anisotropy of the Ada protein (■) on interaction with DNA at two protein concentrations: (a) 2.0 *M*; (b) 0.2 *M*. No change was observed in the steady-state fluorescence signal (○). The dashed lines are extrapolations to estimate the site size. The solid line is the theoretical curve for an association constant of $4 \times 10^7\ M^{-1}$ and a site size of 7 bp assuming no site–site interactions. (From Takahashi *et al.*[18])

The measurement of the tryptophan anisotropy has the additional advantage that it can be used to establish the polymeric state of the protein. In the case of the Ada protein, the anisotropy was independent of protein concentration, consistent with the observation that the protein remains in a monomeric state over a wide range of protein concentrations. An increase in the steady-state tryptophan anisotropy of the *trp* repressor on binding a 26 bp operator sequence has also been observed.[19]

Besides quantitatively reflecting the binding of a protein to DNA, studies of time-resolved anisotropy provide information about the dynamics of the tryptophan chromophore. In favorable circumstances, the fluorescence emitted from two tryptophan residues in a protein can be resolved.[20] However, it is desirable that studies be carried out on proteins

[19] T. Fernando and C. A. Royer, *Biochemistry* **31,** 3429 (1992).

[20] C. A. Royer, J. A. Gardner, J. M. Beechem, J.-C. Brochon, and K. S. Mathews, *Biophys. J.* **58,** 363 (1990).

which contain a single tryptophan so that the anisotropy decays can be attributed to a specific fluorophore at a defined position in the amino acid sequence. To this end, several DNA-binding proteins have been genetically engineered to contain a single tryptophan residue for fluorescence analysis.[21,22] We may also note here that although one expects and usually does observe an increase in the anisotropy of the tryptophan emission of a protein on binding to another macromolecule, a decrease in this function is also possible if the local motion of the tryptophan increases appreciably on binding. Such a situation occurs in the binding of elongation factor Tu to elongation factor Ts.[23]

Use of Extrinsic Probes. The intrinsic fluorescence intensity of some proteins does not change on binding to DNA. For example, the fluorescence of the single tryptophan in the avian retroviral nucleocapsid protein does not change on binding to nucleic acid.[24] The tryptophan fluorescence of the TyrR repressor does not change on binding to its operator sequence (M. Bailey and P. Hagmar, personal communication, 1993). To increase the versatility of the fluorescence technique, extrinsic probes may be covalently conjugated to either the DNA-binding protein or to the DNA itself. The coupling of probes to proteins has been reviewed elsewhere.[25]

DNA has no measurable intrinsic fluorescence except at low temperatures,[26] and for many years the use of fluorescence techniques to monitor DNA–protein interactions has not been possible owing to the absence of methods to attach fluorescent probes covalently at particular points in the base sequence. Intercalating probes such as ethidium bromide and the acridine dyes have been used extensively but have the disadvantage that they bind almost nonspecifically along the DNA helix. Moreover, the lengthening of the DNA helix that results from the intercalation may severely interfere with the binding of proteins. An alternative approach is to incorporate fluorescent nucleotide analoges into synthetic oligonucleotides. The fluorescence of poly(1,N^6-ethenoadenylic acid) has been used to examine the nonspecific binding of recA protein and eukaryotic polypeptide chain initiation factors,[27,28] and the adenine analog 2-aminopu-

[21] T. Hard, M. H. Sayre, E. P. Geiduschek, and D. R. Kearns, *Biochemistry* **28,** 2813 (1989).
[22] C. R. Guest, R. A. Hochstrasser, C. G. Dupuy, D. J. Allen, S. J. Benkovic, and D. P. Millar, *Biochemistry* **30,** 8759 (1991).
[23] D. M. Jameson, E. Gratton, and J. F. Eccleston, *Biochemistry* **26,** 3894 (1987).
[24] J. Secnik, Q. Wang, C.-M. Chang, and J. E. Jentoft, *Biochemistry* **29,** 7991 (1990).
[25] R. P. Haugland, *in* "Excited States of Biopolymers" (R. F. Steiner, ed.), p. 29. Plenum, New York, 1983.
[26] J. Eisinger and A. A. Lamola, *in* "Excited States of Proteins and Nucleic Acids" (R. F. Steiner and I. Weinryb, eds.), p. 107. Plenum, New York, 1971.
[27] M. Chabbert, C. Cazenave, and C. Helene, *Biochemistry* **26,** 2218 (1987).
[28] D. J. Goss, C. L. Woodley, and A. J. Wahba, *Biochemistry* **26,** 1551 (1987).

rine has been used to examine the dynamics of mismatched base pairs in DNA.[29]

Royer *et al.*[30] studied the interaction of histone H2A-H2B with 200 bp DNA fragments from chicken erythrocytes by labeling the histones with dansyl chloride (DNS). In this case labeling of the histones was accomplished by incubation with a 10-fold molar excess of dansyl chloride for 10 min at pH 8.0; free dye was separated from labeled protein by gel filtration chromatography. In such cases of random covalent labeling it is often of interest to ascertain the degree of labeling. To this end one typically utilizes the best estimate of the molar extinction coefficient of the dye bound to the protein. Because this value may depend, to a greater or lesser extent, on the environment of the probe, it is often only an approximate method. In the study by Royer *et al.*[30] a labeling ratio of approximately 1 DNS per H2A-H2B dimer was determined (average labeling ratios typically reflect a distribution of labels among the target population; if truly random labeling is achieved then one may assume a Poisson distribution of labels). The polarization titrations indicated stoichiometries of between 4 and 16 histone octamers per DNA 200-mer with affinities in the nanomolar range.

Labeling DNA. The availability of methods to conjugate fluorophores to oligonucleotides has greatly widened the scope for fluorescence analysis of DNA–protein interactions. Strategies based on fluorescence quenching and resonance energy transfer, as well as anisotropy, are now possible but as yet have been infrequently applied. A number of methods of specifically labeling synthetic oligonucleotides is now available. The most common derivatives are as follows (Fig. 3).

1. *Terminal labeling:* A primary aliphatic amine can be conjugated to the 5′-phosphate group of an oligonucleotide using standard phosphoramidite chemistry. Labeled products are commercially available from Applied Biosystems (Foster City, CA) and Clontech (Palo Alto, CA) and come with spacers of 2, 3, 6, or 12 carbon atoms between the 5′ oxygen and the protected amino group. 5′-Thiol modifiers are also available. Labeling at the 3′ terminus is now possible with an amino-modified controlled pore glass suitable for use in oligonucleotide synthesizers (Applied Biosystems and Clontech). After cleavage from the column and deprotection, a primary amino group is available for conjugation at the 3′ terminus of the oligonucleotide. An alternative strategy for labeling the 5′ terminus is to prepare a 5′-phosphorylated derivative of the oligonucleotide with T4

[29] C. R. Guest, R. A. Hochstrasser, L. C. Sowers, and D. P. Millar, *Biochemistry* **30,** 3271 (1991).

[30] C. A. Royer, T. Ropp, and S. F. Scarlata, *Biophys. Chem.* **43,** 197 (1992).

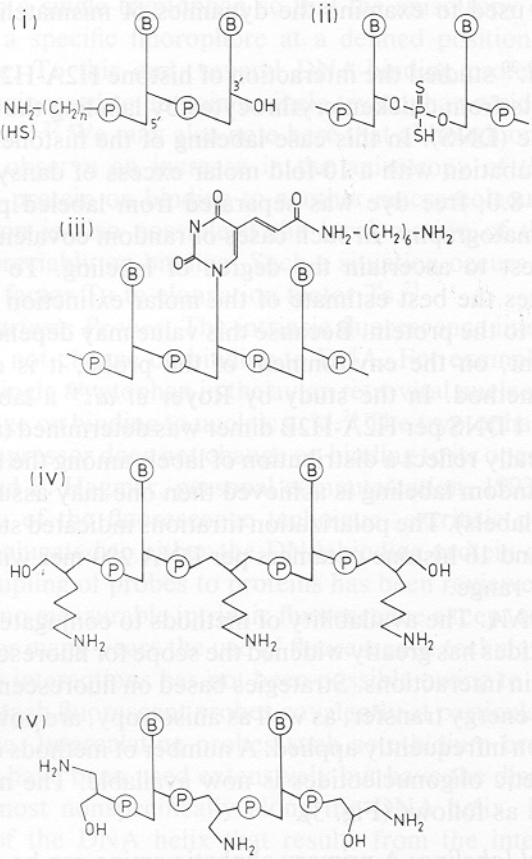

FIG. 3. Modified oligonucleotides for the conjugation of amino or thiol reactive fluorescent probes to DNA. (i) Labeling at the 5′ terminus (Applied Biosystems, Clontech); (ii) labeling at the internucleotide phosphate using a phosphorodithioate derivative; (iii) labeling of a modified base: an amino-modified thymidine (Applied Biosystems); (iv) internucleotide labeling using a 3-carbon bridge with an aminobutyl side arm [the reagent can also be placed at the 3′ and 5′ ends of the oligonucleotide (Clontech)]; and (v) internucleotide labeling with a 2-carbon bridge having an aminomethyl side arm; [the reagent can also be placed at the 3′ and 5′ ends of the oligonucleotide (Clontech)].

polynucleotide kinase and to couple cystamine using a carbodiimide reaction: reduction of the disulfide bond with dithiothreitol provides a thiol group that can be linked to a maleimide or iodoacetate probe derivative.[31]

[31] T. Heyduk and J. C. Lee, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 1744 (1990).

2. *Labeling at internucleotide phosphorus:* Synthesis of deoxynucleotide 3′-phosphorothioamidites, incorporation into DNA using commercially available oligonucleotide synthesizers, and subsequent alkylation have been reviewed by Caruthers *et al.*[32]

3. *Labeling modified base:* Deoxyuridines substituted at C-5 carrying a blocked primary aliphatic amino group have been prepared by Haralambidis *et al.*[33–35] The chemistry allows for the incorporation of spacer arms of various lengths. Fluorescent derivatives of 5-(aminopropyl)deoxyuridine have been reported by Gibson and Benkovic[36] and used to study the interaction of the Klenow fragment of *E. coli* DNA polymerase I with DNA.[36–38] An amino-modified thymidine phosphoamidite with an 11 atom spacer is now available from Applied Biosystems.

4. *Labeling between nucleotides:* Replacement of a nucleoside with a 3 carbon bridge between the 3′-phosphate of one nucleotide and the 5′-phosphate of the next is said to conserve the interphosphate distance. The bridge carries an aminobutyl side arm which can be conjugated to a fluorescent probe. This method has the disadvantage that a nucleotide is replaced by a foreign bridging group. Standard phosphoamidite chemistry can be used, and a reagent known as Uni-Link AminoModifier is available from Clontech.

In our experience, labeling the 5′ terminus with the dansyl fluorophore connected via a 6 carbon spacer does not affect the melting point of the duplex and the degree of hypochromicity observed. Nor does the melting affect the fluorescence characteristics of the dansyl group (M. Bailey and W. H. Sawyer, unpublished observations, 1993). These observations suggest that end labeling of oligonucleotides does not interfere with duplex formation and that the dansyl label on its spacer is oriented away from the helix and does not fold back to interact with the helical barrel. Labeling of short oligonucleotides at the 3′ or 5′ terminus may be sufficient to permit the monitoring of the binding equilibria using anisotropy and has the advantage that the fluorophore cannot interfere directly with the protein–DNA interaction site. The length of a spacer arm between the DNA helix and the probe may be crucial. If the fluorophore is attached via a

[32] M. H. Caruthers, G. Beaton, J. V. Wu, and W. Wiesler, this series, Vol. 211, p. 3.
[33] J. Haralambidis, M. Chai, and G. W. Tregear, *Nucleic Acids Res.* **15,** 4857 (1987).
[34] J. Haralambidis, L. Duncan, K. Angus, and G. W. Tregear, *Nucleic Acids Res.* **18,** 493 (1990).
[35] J. Haralambidis, K. Angus, S. Pownall, L. Duncan, M. Chai, and G. W. Tregear, *Nucleic Acids Res.* **18,** 501 (1990).
[36] K. J. Gibson and S. J. Benkovic, *Nucleic Acids Res.* **15,** 6455 (1987).
[37] D. J. Allen, P. L. Darke, and S. J. Benkovic, *Biochemistry* **28,** 4601 (1989).
[38] D. J. Allen and S. J. Benkovic, *Biochemistry* **28,** 9586 (1989).

flexible extension, it may retain its flexibility on binding to the protein such that the anisotropy is unaffected. Thus short spacer arms are desirable in that rapid depolarizing motions of the probe at the point of attachment are limited and the anisotropy will more accurately reflect the global motion of the DNA and the DNA–protein complex. The possibility that the DNA-binding protein interacts with the fluorophore as well as or instead of the double helix should be carefully examined. Determining whether the protein binds the fluorescent probe or a low molecular weight fluorescent conjugate (e.g., dansyl lysine) is one way to test this possibility.

The binding of the *E. coli* cAMP receptor protein to a 32 bp fragment of the *lac* promoter provides another example of this strategy.[31] The DNA was labeled with a coumarin derivative, and the binding stoichiometry was determined by titration of the ligand into a fixed concentration of acceptor under conditions of stoichiometric binding, that is, when all added ligand is bound. Stoichiometric binding can be realized if the acceptor concentration, or more precisely the concentration of binding sites, is at least 10-fold greater than the K_d. A plot of the anisotropy versus the molar ratio of ligand to acceptor should then have a clear inflection point (as illustrated in Fig. 4a), indicating the binding stoichiometry. A second titration, carried out under conditions of equilibrium binding in which the acceptor concentration is approximately equal to the K_d, then yields values

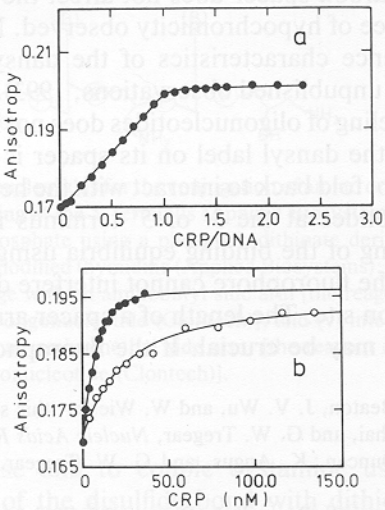

FIG. 4. (a) Stoichiometric titration of a coumarin-labeled 32 bp DNA fragment of the *lac* promoter (32-bp-CPM) with the cAMP receptor protein (CRP). The concentration of 32-bp-CPM was fixed at 98.5 n*M*. (b) Titration of 32-bp-CPM (11.1 n*M*) with CRP in the presence of 0.5 μM cAMP (○) and 500 μM cAMP (●). (From Heyduk and Lee.[31])

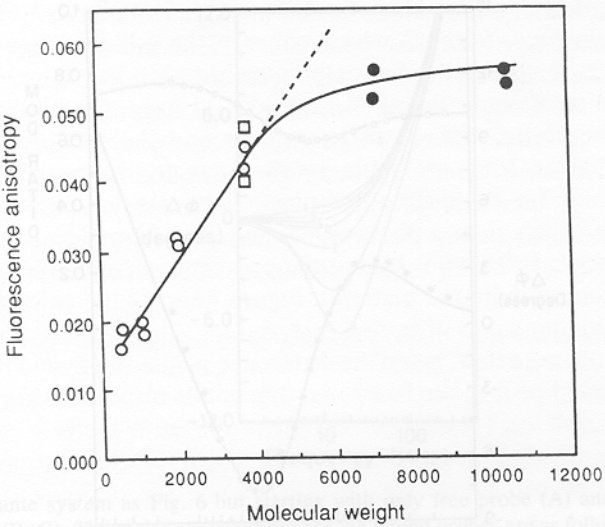

FIG. 5. Fluorescence anisotropy of oligonucleotides covalently labeled with fluorescein at the internucleotide phosphate. The open symbols are for oligothymidine molecules containing 2, 5, or 11 bases. The filled symbols are for 11-mers complexed with the complementary dA strand; [fluorescent probe] = [oligonucleotide] = 0.1 *M*; 0.1 *M* phosphate buffer (pH 7.0), 20°. (From Murakami *et al.*[39])

of the fractional saturation which, together with the site number determined from the first titration, provides the information necessary for construction of a binding plot (Fig. 4b).

An example of steady-state anisotropy measurements of a series of fluorescein-labeled oligothymidylates is shown in Fig. 5.[39] The anisotropy increases linearly with chain length (2–11 nucleotides) and therefore with molecular weight. Increasing the molecular weight by formation of a duplex is less effective in increasing the anisotropy compared to increasing the chain length. In this system, the steady-state anisotropy is determined by the angles between the absorption and emission transition moments of the dye and the axis of rotation and by three depolarizing rotations: (i) end-to-end tumbling, (ii) rotation about the long axis of the molecule or helix, and (iii) motion of the probe at the point of attachment. Time-resolved anisotropy measurements (*vida infra*) are useful for resolving some of these contributions. For example, for a 5′-mansyl-labeled 42-mer the independent motion of the probe at the point of attachment has a

[39] A. Murakami, M. Nakaura, Y. Nakatsuji, S. Nagahara, Q. Tran-Cong, and K. Makino, *Nucleic Acids Res.* **19,** 4097 (1991).

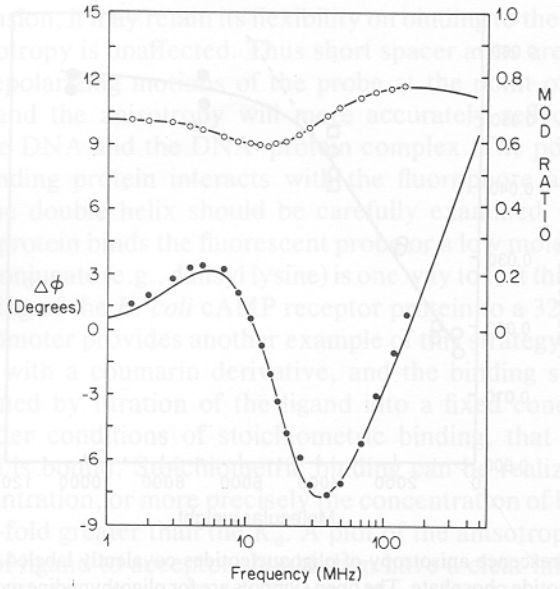

FIG. 6. Example of anomalous phase delay resulting from associative decay. Differential phase (●) and modulation (○) data and a two-component fit (solid lines) are shown for a system containing free ethidium bromide and ethidium bromide bound to Phe-tRNA^{Phe}. The lifetimes of free and bound ethidium bromide were 1.86 and 26 nsec, respectively, and the associated rotational relaxation times were 0.54 and 136 nsec, respectively.

correlation time of 300–400 psec compared to global motion of the chain at 4–5 nsec (D. P. Millar, M. Bailey, and W. H. Sawyer, unpublished observations, 1992). Correlation times of 240–590 psec for a dansyl probe attached to a C-5-modified uridine base within an oligonucleotide have also been ascribed to independent motion of the probe. A longer correlation time, attributed to the global tumbling of the DNA helix, was shown to increase substantially on binding of the Klenow fragment of DNA polymerase I.[22] In this case, changes in the steady-state fluorescence intensity were used to measure the binding of the protein to the duplex.

Time-Resolved Methods

Time-resolved fluorescence methods are discussed elsewhere in this volume, and we shall not enter into lengthy descriptions of the technique but rather will point out some interesting aspects of time-resolved anisotropy methods as they apply to binding studies. The two principal time-resolved methodologies presently utilized are the impulse–response

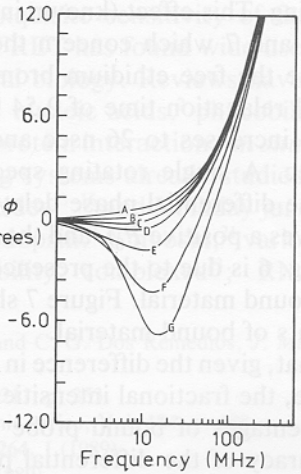

FIG. 7. Same system as Fig. 6 but starting with only free probe (A) and additions of bound probe (B–G). The molar contributions for the bound probes are as follows: (A) 0.0%, (B) 0.18%, (C) 0.36%, (D) 0.54%, (E) 1.1%, (F) 2.1%, and (G) 3.2%.

method and the harmonic response method. In the first technique[40] the direct time decay of the emission, following a brief excitation pulse, is recorded, whereas in the latter technique the excited state decay parameters are deduced from the response of the emitting system to sinusoidally modulated excitation.[41] In the harmonic response method one can resolve rotational modalities of the emitting system by monitoring the phase delay between the perpendicular and parallel components of the emission, on excitation by parallel polarized light.[42] One is thus able, in favorable circumstances, to separate "global" from "local" motions of bound fluorophores.

In principle, one can also resolve free from bound probes, and, in fact, an interesting phenomenon occurs in these types of measurements which can be used to determine binding isotherms. Namely, if the lifetime of the fluorophore increases on binding, in addition to its rotational rate, then one may observe, at particular modulation frequencies, anomalous phase delays, that is, negative values for the phase delay between the parallel and perpendicular components, which provide a sensitive measure

[40] J. Yguerabide and E. E. Yguerabide, *in* "Optical Techniques in Biological Research" (D. L. Rousseu, ed.), p. 181. Academic Press, Orlando, Florida, 1984.

[41] T. L. Hazlett and D. M. Jameson, *Proc. Soc. Photo-Opt. Instrum. Eng.* **909,** 412 (1988).

[42] D. M. Jameson and T. L. Hazlett, *in* "Biophysical and Biochemical Aspects of Fluorescence Spectroscopy" (G. Dewey, ed.), p. 105. Plenum, New York, 1991.

of the extent of binding. This effect (known informally as "Chip dip") is illustrated in Figs. 6 and 7 which concern the ethidium bromide/tRNA system.[41] In this case the free ethidium bromide has a lifetime of 1.86 nsec and a rotational relaxation time of 0.54 nsec. On binding the Phe-$tRNA^{Phe}$ the lifetime increases to 26 nsec and the rotational relaxation increases to 136 nsec. A single rotating species cannot give rise to a negative value for the differential phase delay (assuming excitation at a wavelength which gives a positive P_0), and the negative excursion for this function shown in Fig. 6 is due to the presence of free ethidium bromide in equilibrium with bound material. Figure 7 shows the calculated effects for increasing amounts of bound material.

We should note that, given the difference in quantum yield between the free and bound probe, the fractional intensities utilized in Fig. 7 actually represent small percentages of bound probe on a molar basis. In fact, considering the accuracy of the differential phase measurement (better than 0.1°) one can detect, in this system, on the order of 0.1% bound probe. This phenomenon also occurs in time-domain measurements. Specifically, if one monitors the anisotropy decay of a system which displays multiple lifetimes associated with multiple rotational diffusion rates then one may observe a decline at short times of the anisotropy followed by a rise at latter times and subsequent decrease. This "dip and rise" effect has been observed by Millar and co-workers[22] in studies on protein–DNA interactions, specifically in the case of the interaction of a fluorescent DNA duplex with the Klenow fragment of DNA polymerase.

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