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Micro and nanosecond detection of biomolecular dynamics by polarized luminescence

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Abstract- Dynamic processes in proteins and cell membranes, whose rates span the range 10^3 - 10^{12} s^{-1}, can be tracked by means of time-resolved two-step photoselection, which entails irradiating the specimen by a plane-polarized flash and observing the absorption or emission of light polarized parallel and perpendicular to the exciting flash. The technique is illustrated by discussing the ns and μs dynamics of proteins (soluble and membranous) and platelet lipid bilayers involved in haemostasis.

1. INTRODUCTION

Though time-resolved polarized luminiscence spectroscopy can be used to unravel a host of biophysical phenomena, this lecture will focus on a single topic: the elucidation of the orientational dynamics in proteins and cell membranes.

Since long polypeptide chains, made up from strong covalent bonds, are common to all proteins, the complex structure of the proteins and the large diversity of their chemical and physical properties should be due to differences in the amino acid side chains. Steric interactions between the side chains determine the secondary structure, while S-S and weaker H-bonds stabilize the three-dimensional architecture of the biomolecule. A well-defined overall structure does not however preclude internal motions of the amino acid side chains and of large chain segments (1). To take a specific example, viz. isoleucine side chains in a α-helix of myoglobin (1): while rotation about the first link of the chain was severely restricted, the rotational relaxation times around the second C-C bond were from 12 to 20 ps, and those of the terminal methyl group were in the range of 1-5 ps. Similarly, rates of rotational motion of tyrosine and phenylalanine in a relatively stiff protein (bovine pancreatic trypsin inhibitor) range from those typical of a fluid solution of the free amino acid (ps) to values in the ms range (2). These examples indicate the variability expected in the internal constraints upon neutral side chain rotation between particular domains of the same polypeptide. In addition, charged side chains such as those of lysines may have reorientational times as short as 70 ps (3). This implies that electrostatic interactions, so conducive to the stability of the protein, may be fluctuating with GHz frequencies!.

Movements of large fragments of a protein, often called *segmental* flexibility, may span several tens of ns. This aspect of *conformational* dynamics has been studied extensively in immunoglobulins; reorientational times of 30-60 ns have been ascribed to wiggling of the Fab arms (4). Finally, the overall, field-free rotation of rigid molecules of the size of proteins occurs within a range of approximately 5-500 ns.

Processes in cell membranes display an even richer variety of orientational motions. This part of the cell contains a lamellar superstructure, made up of a diacyl-lipid bilayer forming a (mostly) fluid interface. In

addition, the bilayer contains a large fraction (up to 50% w/w) of glycosylated proteins. The array of internal motions of these integral membrane proteins is similar to that described above for soluble enzymes. On the other hand, the overall motions of membrane polypeptides are severely constricted on account of their amphipatic structure, which makes rotational diffusion across the bilayer plane very unlikely. Therefore, the angular reorientations take the form of uniaxial rotation (around the normal to the bilayer) or librational motions. This anisotropic rotational dynamics is observed usually (5-6) in the μs time range.

The lipidic component of the membrane might contain up to 200 different lipids, including sterols. Popular depictions of the cell bilayer often overlook the fact that in many eucaryotic cells (as those of the higher organisms) one of the sterols (cholesterol) is present in a molar ratio as high as 1:1!. Lipid molecules also display a highly anisotropic global rotation, due to the average equilibrium orientation of the lipids perpendicular to the bilayer plane. The rigid-body, cone-shaped angular fluctuations around that preferred orientation occur in the range of 1-10ns (7). Finally, the lipid acyl chains may show very complex internal conformational fluctuations (e.g. *trans-gauche* isomerizations) that, at room temperature, appear in the ps-ns time-range.

Optical spectroscopic techniques owe their success in tackling so many dynamic phenomena in these specific biosystems to the diversity of photophysical processes that can be exploited for the task at hand; the optical methods provide an extremely flexible observation window, from ps to seconds in width, that can be moved along the time-axis so as to circumscribe the dynamic process of interest. This feature, together with the additional advantage of single quantum detection sensitivity makes polarized spectroscopy such a powerful tool. On the other hand, the most obvious disadvantages are those which bedevil any experimental method based on probe molecules and the lack of spectral resolution in dealing with heterogeneous emitting species.

2. PHOTOSELECTION AND ROTATIONAL DIFFUSION

The probability that a molecular electronic transition moment (a) will absorb a *Z*-polarized photon from a light pulse (assumed to be of infinitesimal duration) is proportional to a^2_z, the square of the projection of a (Fig.1) on the direction of the exciting electric vector. This anisotropy in the photoabsorption process causes preferential excitation (photoselection) of those molecules whose absorbing transition moments are inclined towards *Z*. The anisotropy in the orientation of excited oscillators in the photoselected ensemble will be maximum at t=0 (Fig.1). If we write $I_\parallel$ and $I_\perp$ for the intensities of the emission polarized along the *Z* and *X* axes respectively, then the *emission anisotropy* is defined by:

$$r(t)=\frac{I_\parallel(t)-I_\perp(t)}{I_\parallel(t)+2I_\perp(t)} \qquad (1)$$

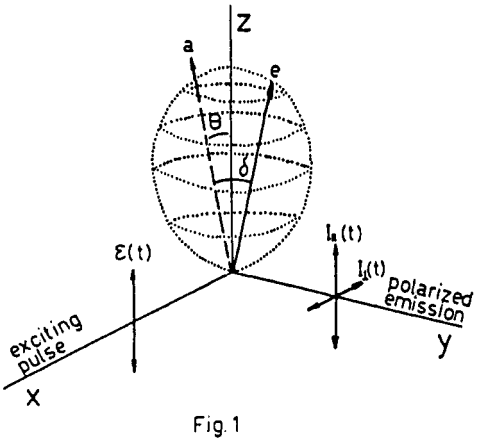

Fig.1

If the same transition moment is involved in the absorption and emission steps the emitted light would be strongly polarized; for a sample which is initially isotropic, $I_\parallel(0) = 3I_\perp(0)$. Since random excursions of the photoselected molecules would tend to equalize $I_\parallel(t)$ and $I_\perp(t)$, accurate quantification of r(t) provides a convenient means for following the diverse dynamical phenomena mentioned above.Since the pertinent instrumental techniques and the underlying theory have been described several times (see e.g. refs.7-11) it will be sufficient to recall here only the most useful

expressions. The theoretical value of r(0) for an isotropic solution of non interacting absorbers depends in general on the relative orientation (δ) of the excitation and emission transition moments:

$$r(0)=\frac{2}{5}\left(\frac{3\cos^2\delta-1}{2}\right) \qquad (2)$$

from which one sees that

$$-0.2 \leq r(0) \leq 0.4 \qquad (3)$$

In the absence of an orienting force (i.e. for free rotation), $r_\infty \equiv r(t\to\infty) = 0$, which is a statement of the fact that eventually isotropy is restored. But in an anisotropic system, the limiting value will (in general) be finite and determined by the equilibrium orientation of the photoselected molecules (which may or may not the same as that of ground state species); the asymptotic (or residual) anisotropy and the rate at wich r(0) approaches r_∞ are analogous, respectively, to the equilibrium constant and the relaxation constat of the reaction $A \rightleftharpoons B$.

Field-free rotation

The overall free rotation of proteins in aqueous solution is approximated by the behaviour of a rigid body (sphere or ellipsoid of revolution) obeying the rotational diffusion equation (which involves three diffusion coefficients) and low Reynolds number hydrodynamics. For a sphere, all three diffusion coefficients are equal (to D, say) and r(t) decays with a single constant $\phi = (6D)^{-1}$. For a symmetric top, two diffusion coefficients ($D_\parallel$ and $D_\perp$) characterize the rotation, and the decay of anisotropy is controlled (8) by three relaxation constants : $\phi_1 = (4D_\parallel + 2D_\perp)^{-1}$, $\phi_2 = (D_\parallel + 5D_\perp)^{-1}$ and $\phi_3 = (6D_\perp)^{-1}$. As the determination of three relaxation constants from the observed r(t) is fraught with problems, it has been pointed out (12) that the initial slope of r(t) can be combined with hydrodynamic data to disentangle $D_\parallel$ and $D_\perp$. A new method for the determination of the initial slope has recently been developed (13), and is expected to prove useful for handling noisy anisotropy curves.

Restricted rotation

Even if the sample is isotropic (on the bulk), but rotational motions are locally hindered - as in the fast oscillation of an amino acid side-chain, the wobbling of a lipid molecule or the overall reorientation of an integral membrane protein in a suspension of membrane fragments - the emission anisotropy of the reporting chromophore decays, for reasons explained above, from r(0) to a residual value, $r_\infty \neq 0$. The asymptotic value r_∞ would only be actually observed if the hindered motion occurs on a much shorter time scale than that pertaining to the overall tumbling of the supporting frame. The extraction of dynamic and structural molecular parameters from the experimentally recorded decay of r(t) requires now a much heavier mathematical armament than in the previous case of free rigid-body rotation. Nevertheless, in the study of fast (ps-ns) local motions in proteins and rotational dynamics of lipids in bilayers it is often assumed that the hindered depolarizing motions of a chromophore may be represented by free Brownian movement over a (radially symmetric) restricted part of the unit sphere, as in the "wobbling-in-cone" model (7). The monoexponential approximation to this model can be visualized as a three-component cyclic reaction scheme (10,14) :
$X \rightleftharpoons Y \rightleftharpoons Z \rightleftharpoons X$, in which X and Y can be interchanged (but Z is unique) and the forward rate constant for $X \rightleftharpoons Z$ or $Y \rightleftharpoons Z$ does not equal the backward rate constant. The hindered, rigid-body uniaxial reorientation of integral membrane proteins embedded in the lipid bilayer may be analyzed by an anisotropy function r(t) that decays with two correlation times (15), differing by a factor of 4, to a limiting value r_∞. In addition, wobbling motions as those described above are also possible, though less likely; the distinction between both models is only possible if very accurate anisotropy decay curves can be obtained. The reader interested in a more detailed analysis may consult refs. 6-11,14-16.

3. FLUORESCENT AND PHOSPHORESCENT POLARIZATION PROBES (ref. 17)

Intrinsic probes

Many proteins incorporate aminoacids with aromatic side-chains (tryptophan, tyrosine, phenylalanine), and a sizable fraction of which contain additional chromophores (enzyme cofactors, chlorophylls, etc.) that may be used as intrinsic luminescent probes in the study of their dynamic processes. However, as the topological and photophysical requirements of a polarization probe are very demanding, only the polarized fluorescence of Trp has found extensive use with that purpose. In proteins, the rigid indole side-chain of Trp shows a fluorescent emission (4) with modest yields (<0.2) and short lifetime (0.1-6 ns), almost invariably multiexponential. Since the photophysics of Trp is extremely complex (4) the analysis of the fluorescence excitation polarization spectra is not trivial. Apparently, the ^1L_a and ^1L_b states, well separated in indole (18), invert and overlap extensively in Trp. Nevertheless, with excitation at 295-300 nm the parallel component is initially twice as intense as the perpendicular counterpart, and r(0) is in the 0.2-0.25 range. Rotational blurring of this difference has been used to extract detailed information of ps-ns internal motions in proteins containing one or two Trp residues and, more recently, in protein mutants constructed with an unique Trp residue directed to critical locations (19). The rigid-body rotational hydrodynamics of several of these proteins could be also investigated from the fluorescence depolarization of Trp by extending the observation time to 5-8 Trp fluorescence lifetimes. That gives an idea of the sensitivity of present day instrumentation and also of the constrains imposed by the emission kinetics. The phosphorescence of Trp in proteins may last from ms to seconds and is polarized perpendicular to the indol plane, $r(0) = -0.14$. This emission has been used to detect slow rotational motions and rigid segments in proteins (20). On the other hand, in multi-Trp biomolecules the lack of precise spectral assignment prevents the application of the intrinsic luminiscence anisotropy of this residue.

In natural lipid bilayers there are a few lipids that are luminescent. Unfortunately these are not so widespread as Trp in proteins and, in addition, they occur usually in very low concentration. This prevents the use of the intrinsic polarized emission in bilayer studies, unless it is artificially enriched in the emissive components.

Extrinsic probes

Using appropiate dyes as extrinsic protein probes allows, in principle, greater flexibility in choosing a particular photophysical process. Thus, fluorescent probes may be used to explore the full ns time range, and phosphorescent derivatives would reveal μs and ms dynamic phenomena. Note that the dye must possess, in addition to the chromophore, a reactive group capable of forming a covalent link with the protein. A large number of fluorescent dyes have been proposed as protein tags; nonetheless important gaps remain. The situation can be grasped more easily if we first recall the requisite photophysical properties for probing rotational dynamics. The quantum yield of emission and the intrinsic polarization should be high; the absorption and emission spectra should not overlap those of the protein; the location of the transition moment directions in the molecular frame should be known so that macroscopic observables can be related to molecular properties. These criteria are easily met by e.g. the in-plane π-π^* transitions of rigid, multi-ring aromatics; unfortunately, the water solubility of these compounds is too low. To overcome this difficulty, molecules with two or three rings, containing perhaps additional heteroatoms and polar groups, would be necessary. The additional structural complexity renders unpredictable the intrinsic polarization of the emission and patient screening of prospective fluorophores becomes inescapable. Satisfactory probes, mostly two-ring heterocycles with high r(0) values and emission lifetimes in the 0.5-30 ns range, are abundant (17). For observation windows encompassing few tenths of a μs, the choice is limited to pyrene derivatives (which-due to their hydrophobicity, low polarization and weak absorption-are otherwise unattractive). Suitable fluorophores in this time interval would be characterized by symmetry-forbidden dipolar transitions; to avoid undesirably high probe

concentrations, photoabsorption to a higher (allowed) singlet state would be required; to maximize r(0), the two electronic transition moments will have to be (almost) parallel. Simultaneous fulfilment of these conditions is a tall order indeed.

In the μs range, optical polarization techniques are based largely on the room-temperature phosphorescence of extrinsic probes in fluid solution (5-6). Closely related methods, which combine the long lifetime of the triplet state with fluorescence detection, as E-type delayed emission (21) and polarized fluorescence depletion (6), may provide much higher sensitivity, affording μs-dynamic experiments to be carried out on individual cells (22).

The xanthene dyes eosin and erythrosin, with reactive groups for conjugation to proteins, are the current favourites for time-resolved phosphorescence polarization and fluorescence depletion work. At room-temperature the emission is in the 620-720 nm range, with lifetimes around 1 ms and r(0) values close to 0.2. The spectral parameters of these dyes, both free and protein-bound, have been discussed in great detail (see eg. ref.6). In general, the photophysics and photochemistry of both probes in aqueous solution are fairly complex. For the application of the polarized phosphorescence to biodynamic research there certainly is a dire need of better extrinsic triplet probes.

Extrinsic polarization probes for the study of the lipid bilayer are frequently rod-shaped dyes incorporated non-covalently into the lipid matrix. Alternatively, the dye may be covalently attached at one of the acyl chains of a lipid molecule. In either case, the fluorophore will adopt an orientational distribution which mimics that of membrane components. This information is contained in r_∞, the limiting anisotropy of the probe. In addition, the rate of relaxation of the two polarized intensities is indicative of the friction that hinders the angular motions of the probe (in its electronically excited state). Accurate quantification of those two pieces of information in a heterogeneous system, like a bio-membrane, is not simple. Nevertheless, the answer to questions of biological relevance may only require relative measurements of r_∞ and the wobbling rate, as a function of some specific parameter characterizing the membrane state or composition. The presence of a hydrophobic group helps to define the transverse region of the lipid bilayer being sampled by the depolarizing motions of the emitting molecule. As with the protein probes, the precise knowledge of the transition moment orientation within the molecular coordinates and the range of the emission lifetime are two of the most important spectral properties of a dye molecule which is to serve as a membrane probe.

4. MOLECULAR DYNAMICS IN BLOOD PLATELETS

Platelets are $\sim$1 μm width cells which play a central role in blood haemostasis. One of the key steps in this process is the binding of a soluble protein, fibrinogen (FIB), to its receptor in the platelet surface, the integral membrane protein GPIIb/IIIa (23). Since *any* information concerning the structure and dynamics of the two reactants, FIB and GPIIb/IIIa, would facilitate the formulation of the reaction scheme, we decided to determine first the range of the internal motions of the FIB molecule under physiological conditions, using the methods summarized above. Flexibility of large segments was ruled out by monitoring the decay of the polarized fluorescence of FIB randomly labelled with 1-methyl-pyrene (12). The result obtained by repeating these experiments with a new pyrene bearing probe (24) with superior properties is reproduced in Fig.2. The decay of r(t) reveals only very rapid motions (1-20 ns), indicating small-fragment dynamics. The much slower sub-μs relaxation (ca. 900 ns) is caused by the free rotational tumbling of FIB. Based on this observation of a stiff protein core, the global hydrodynamics of FIB could be studied in a second investigation (25), from the decay, in a few μs, of the dichroism of triplet-labelled FIB.

Restricted motions of protein components may be exemplified by the ns mobility of the oligosaccharide side chains of FIB (Fig.3), labelled in the distal N-acetylneuraminic acid residues with the

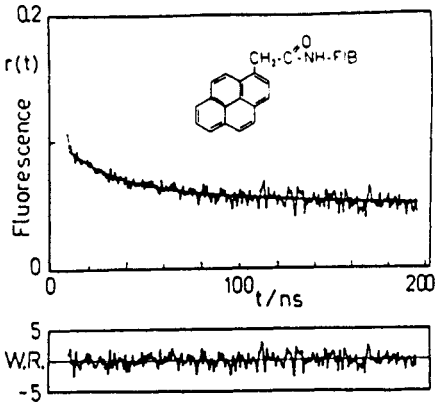

Fig.2

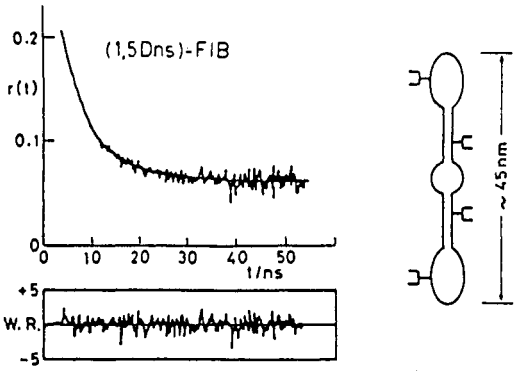

Fig.3 (from ref. 10)

fluorescent tag 5-dimethylamino-naphtalene-1-sulfonyl hydrazine (26). The average lifetime (13 ns) and high initial anisotropy (0.31) were well suited to the considerable task of disentangling two correlation times (0.7 and 7 ns). In addition, two kinds of depolarizing motion, segmental mobility and overall free rotation, may be observed in the anisotropy decay of an antibody molecule (IgG-P37) raised against the GPIIIa subunit of the receptor (27) and labelled with the new pyrene probe (24). The analysis of r(t) shows (Fig. 4) three correlation times: a fast component (< 300 ps) due to the local reorientation of the label, an intermediate value (18 ns) arising from segmental flexibility, and a slow component (176 ns) produced by overall IgG rotation.

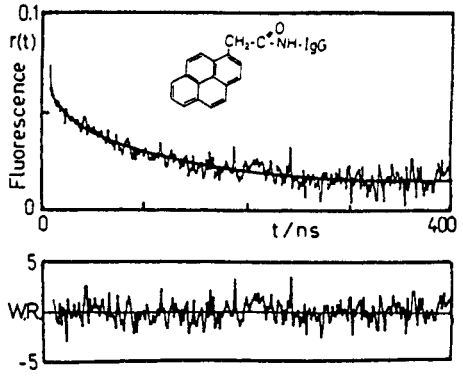

Fig 4

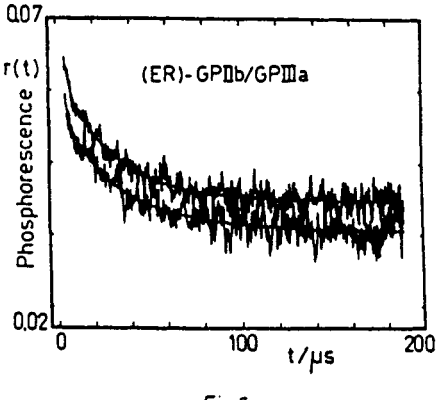

Fig.5

The μs dynamics of the platelet membrane receptor GPIIb/IIIa was investigated, as might be expected, by means of the polarized phosphorescence of an erythrosin label bonded to the receptor through a series of immunoreagents (28). This technique was useful in determining the dependence of the dynamics of the macromolecule on several chemical and physical parameters. The phosphorescence anisotroy decays presented in Fig.5 show, for example, that (in this range) the receptor dynamics in the intact living cell (upper curve) is virtually the same as in a suspension of membrane fragments (lower curve). Small but significant changes in the r_∞ value may be indicative of restrictions on the amplitude of rotational motions imposed by intracellular interactions. Finally, the properties of the platelet lipid bilayer where the receptor is embedded could also be investigated by the polarized fluorescence of several lipophilic probes (29).

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