Chapter 2

Dynamic Structure of Membranes and Subcellular Components Revealed by Optical Anisotropy Decay Methods

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1. INTRODUCTION

Molecules in living organisms, as well as those in dead matter, undergo continual, irregular motions. It is thermal fluctuations, or the Brownian motions.

The optical anisotropy decay method is a means of visualizing the Brownian motion of molecules. The method, applied to biology, reveals how the molecules composing living things move about in complex organized structures. Looking at the molecular motion is not simply a biophysicist’s fun. The thermal motions provide many important clues toward the understanding of life at the molecular level.

Through the molecular motion one sees, first of all, structure: the structure of the molecule itself and/or of the surroundings. Currently, the major tools in structural biology at the molecular level are x-ray crystallography and electron microscopy, which are unsurpassed in the information content. Analysis of thermal motion, as explained below, complements these tech-
niques in that it provides information about the structure in an environment where the biological molecule works. The optical anisotropy decay method, in particular, has a high sensitivity and focuses on a selected molecular species in a complex biological system. The method is therefore a powerful means of studying the structure of a key molecule incorporated in a higher-order structure.

Second, the structural information reported by the thermal motion is necessarily a dynamic one: flexibility or mobility is directly expressed in the thermal motion. Changes in conformation can also be followed by time-resolved measurements.

Third, it should not be overlooked that biological reactions rely heavily on thermal motions. Encounter between an enzyme and substrate is an obvious example, which may appear trivial. In membranes or cells, however, the motions are often hindered and impeded, possibly under biological controls. Catalysis is by itself a thermal reaction. Even in an energy-consuming reaction, most steps are thermally activated ones. In the photochemical reaction of rhodopsin and bacteriorhodopsin, for example, only the first step is activated by light; subsequent steps do not proceed at low temperature.

In what follows, we first discuss briefly the principle of the optical anisotropy decay method and then describe several applications chosen from our recent work. Our aim is to explain, in simple words, what can be learned from an optical anisotropy decay. We avoid the discussion of technical details and problems, for which the readers are referred to the references cited and those therein. This chapter is not meant to be a review. Although each topic contains a few references as an access to a fuller explanation, those by no means constitute a complete set. Many important contributions are left out.

2. OPTICAL ANISOTROPY DECAY

2.1. Principle of Optical Anisotropy Decay Method

The optical anisotropy decay method is based on the work of Perrin (1936) and Weber (1953), who showed that optical anisotropy such as the polarization of fluorescence reflects the rotational mobility of the dye molecules. Later, introduction of the decay method has enabled time-resolved measurement of the rotational motion (e.g., see Yguerabide, 1972; Rigler and Ehrenberg, 1973; Wahl, 1975; Kinosita et al., 1984b).

First, we take the example of fluorescence depolarization, through which one sees the rotational motion of fluorescent molecules. Imagine that the
circles in Figure 1 represent fluorescent molecules. Each molecule has a unique axis fixed to it, represented by the bar; light polarized parallel to the axis is absorbed by the molecule while perpendicularly polarized light does not interact with the molecule. In an ordinary sample contained in a cuvette, molecules adopt random orientations by virtue of the thermal motion (Figure 1A). Now illuminate the sample, at time 0, with pulsed light that is vertically polarized. Then, only those molecules that happen to be oriented more or less vertically at this instant can absorb the light and get excited (thick molecules in Figure 1B). The excited molecule will sooner or later emit a photon as fluorescence and become deexcited. The light emitted by each molecule is completely polarized, again along the unique axis (the bar in the figure). At time 0, therefore, the fluorescence from the whole sample is strongly polarized along the vertical direction: the intensity of fluorescence observed through a vertically oriented polarizer is much higher than the intensity through a horizontal polarizer.

As time passes the orientations of the excited molecules become random due to the thermal motion (heavy circles in Figure 1C). Fluorescence at this stage is depolarized. The degree of polarization is expressed in terms of a quantity called anisotropy, which is a function of the ratio between the vertically and horizontally polarized intensity components. The anisotropy is maximal at the instant of the pulsed illumination and becomes zero for completely random orientations. Thus, the rotational Brownian motion is visualized as the decay of anisotropy of the optical signal (fluorescence). A fast decay reflects a fast motion, and a slow decay a slow motion. If the anisotropy does not decrease below a certain value, it implies that the rotational motion is restricted to a certain angular range.

In Figure 1, from B to C, the number of excited molecules decreases. When an excited molecule emits a photon, the molecule is deexcited; it never emits a second photon unless reexcited by absorbing light. The average lifetime of the excited state, or the fluorescence lifetime since this is the time during which one can observe fluorescence, is at most 100 nsec for ordinary

![Figure 1](image-url)  
**FIGURE 1.** Principle of the optical anisotropy decay method. Molecules shown immediately before (A), immediately after (B), and some time after (C) illumination with a vertically polarized light pulse. Heavy circles represent molecules in the excited state.
fluorescent molecules (1 nsec = 10\(^{-9}\) sec). Therefore, the motion that can be studied by the fluorescence anisotropy decay is a fast motion occurring in the nanosecond range. Fortunately, the thermal rotation of proteins and nucleic acids in aqueous environment occurs in this time range. The motion of these biological macromolecules is visualized by fixing a suitable fluorescent probe (dye) to the target molecule.

Slower motions, such as the rotation of proteins in a membrane, which takes place in the microsecond range, can be visualized with a dye that emits phosphorescence or delayed fluorescence (Jovin et al., 1981). The lifetime of these emissions reaches milliseconds at room temperature in the absence of oxygen. Anisotropy of the emission reflects the rotational Brownian motion of the dye (hence of the host molecule that rigidly holds the dye molecule) as in the fluorescence anisotropy decay. Such dyes are called triplet probes (since it is the excited triplet state that is given the long lifetime).

Another method is the absorption anisotropy decay. A dye molecule that has been excited by absorbing light does not absorb light (of the same wavelength as the first one) again until it returns to the ground state. Thus, if one illuminates the sample in Figure 1A with a polarized light pulse of high intensity, the molecules drawn heavy in Figure 1B and 1C become "transparent," resulting in an absorbance loss. The change in the absorbance of the sample is detected by passing a weak light beam through the sample and monitoring the transmitted intensity. The absorbance change is anisotropic: the transmittance is higher for a vertically polarized beam than for a horizontally polarized beam. This absorption anisotropy also decays with time (from Figure 1B to 1C) as the dye molecules rotate. The triplet probe above was first introduced by Cherry and co-workers as the absorption anisotropy probe (Cherry, 1979). The long lifetime of the excited (triplet) state of the probe has enabled extensive studies of slow rotational motion, mainly of membrane proteins.

Of the variety of methods, the fluorescence anisotropy decay is suited for measuring rotations in the nanosecond or faster time range. The absorption method, though less sensitive, covers a wide time range from nanosecond to second. High sensitivity in the micro- to millisecond range is obtained by the phosphorescence or delayed fluorescence methods, or by a fluorescence depletion method which is a high-sensitivity version of the absorption method (Johnson and Garland, 1981). The absorption method in particular requires only an absorbance change upon light absorption; light emission from the excited state is not necessary. Thus, any sample that undergoes a photochemical reaction can be studied by the absorption anisotropy decay method. For example, rhodopsin in retina is bleached by light. Using this reaction, Cone (1972) detected the rotation of this protein in the disc membrane, the first successful observation of the rotation of a membrane protein.
2.2. Experimental Techniques

Here we illustrate only the basic principle. For details, see, for example, the following: for fluorescence, Yguerabide (1972), Wahl (1975), Badea and Brand (1979), Kinosita (1983), Lakowicz (1983), and Meech et al. (1984); for triplet signals, Cherry (1979), Jovin et al. (1981), Chan and Austin (1984), and Thomas (1986).

The optical diagram of our apparatus for phosphorescence and absorption anisotropy decay measurements is shown in Figure 2A. In the case of phosphorescence, we illuminate the sample with a pulsed light from a dye laser through a vertically oriented polarizer. The phosphorescence emission at right angles to the excitation beam is passed through the polarizers A\textsubscript{1} and A\textsubscript{2}, deflected by the mirrors M\textsubscript{1} and M\textsubscript{2}, and detected by the photomultiplier tubes PM\textsubscript{1} and PM\textsubscript{2}. By setting A\textsubscript{1} vertical and A\textsubscript{2} horizontal the two polarized components of the phosphorescence can be measured simultaneously. The time courses of the two signals are recorded on a signal averager. The quality of the signals is improved by averaging over many excitation pulses. Finally, the phosphorescence anisotropy decay is calculated from the two signals.

In absorption measurements the polarizers A\textsubscript{1} and A\textsubscript{2} and the mirrors M\textsubscript{1} and M\textsubscript{2} are removed. The sample is exposed to a weak monitor beam of constant intensity from the halogen lamp. A desired wavelength component of the monitor beam is selected by the monochromator. The vertically and horizontally polarized components of the beam are separated by the beam-splitting polarizer and detected by the photomultiplier tubes PM\textsubscript{3} and PM\textsubscript{4}. Changes in the beam intensity accompanying the laser illumination are amplified and fed to the signal averager.

Time resolution of our apparatus, determined by the electronics shown in Figure 2B, is 1 \(\mu\)sec. With a triplet probe such as eosin, which has a lifetime of a few milliseconds, rotational motions in the entire microsecond range can be examined. Techniques for achieving a higher resolution are discussed by Chan and Austin (1984). Measurements under a microscope have also been reported (Johnson and Garland, 1981).

Optical setup for fluorescence measurement is essentially the same as that for phosphorescence measurement. Time resolution of less than 1 nsec is obtained by detecting individual photons of fluorescence: the time lapse between the pulsed excitation and the arrival of a single photon at the photomultiplier tube is measured precisely by a special circuit. As a reference the apparatus that we have been using is shown in Figure 3.

To observe the rotational motion of a biological macromolecule, one needs a probe (dye) on the molecule. Ideally, the probe should be attached rigidly and specifically, via a chemical bond and/or adsorption, to a single
FIGURE 2. Optical (A) and electrical (B) diagrams of the apparatus for phosphorescence and absorption anisotropy decay measurement. In (A), D, iris diaphragm; S, shutter; F, optical filter; M, mirror; A, polarization analyzer; PM, photomultiplier tube. In (B), $I_v$ and $I_h$, vertically and horizontally polarized components of emitted (phosphorescence) or transmitted (absorption) light; PMTs, a pair of photomultiplier tubes corresponding to PM₁ and PM₂ (phosphorescence) or PM₃ and PM₄ (absorption) in (A). In phosphorescence measurement, the photomultiplier tubes set at a high sensitivity are gated off during the laser illumination. The phosphorescence anisotropy (as well as fluorescence anisotropy) is defined as $(I_v - I_h)/(I_v + 2I_h)$. The absorption anisotropy is defined similarly except that the intensity $I$ is replaced by the absorbance change $\Delta A$. Diagram (A) is from Kinoshita et al. (1984a).
FIGURE 3. Optical (A) and electrical (B) diagrams of the apparatus for fluorescence anisotropy decay measurement. In (A), M, mirror; S, shutter; MC, monochromator; P, polarizer; A, polarization analyzer; SH, sample holder; PM, photomultiplier tube; ID, iris diaphragm. The nanosecond light pulser at the top provides a light pulse of duration 0.8 nsec. The light is monochromatized by MC_{EX}, polarized by P_{B}, and focused on the sample in chamber E. The temperature-controlled sample holder can accommodate sample cuvettes of various sizes, including dewars, and is equipped with a magnetic stirrer. Emitted fluorescence is decomposed into the polarized components by A_{E1} and A_{E2} and detected by PM_{D1} and PM_{F1}, or optionally by PM_{M0} and PM_{M1} through MC_{EM1} and MC_{EM2}. Additional mirrors in chamber E allow front surface illumination. By placing a light-scattering solution in chambers A and C, the time profile of the excitation light pulse can be monitored through the path B→A→F or B→C→D. The xenon lamp at the top is for steady-state measurements. In this case the mirror M_{B} deflects part of the excitation light to a reference sample (quantum counter) in chamber A. Fluorescence from the reference is monitored by PM_{AC} and is used for the correction of the variation of excitation light intensity. Most of the optical components are driven electrically under computer control. In (B), PMR and PML, either of the pair PM_{F1} and PM_{M1} or PM_{M0} and PM_{M0} in (A); TAC, time-to-amplitude converter; PHA, pulse height analyzer; numbers with an asterisk indicate modules made by Ortec. Fluorescence from the sample is detected as single photons by PMR and PML. The electrical signal corresponding to the detection of a photon is fed to the stop input of TAC. The start input synchronous with the excitation light pulse is generated in the nanosecond light pulser. The TAC converts the time lapse between the start and stop signals into
FIGURE 3. A voltage signal, which in turn is digitized and accumulated in the PHA. After many excitations, the data in PHA are the number of occurrences versus the time lapse, that is, the fluorescence intensity versus the time after excitation. The dual electrical paths to the stop input allow the simultaneous measurement of the vertical and horizontal polarization components. The PHA memory selector judges which of the two PMs has detected the photon and issues a proper instruction to PHA. When signals from the two PMs overlap, the event is discarded. At intervals the roles of the two paths, vertical and horizontal, are interchanged by rotating the analyzers \( A_{E1} \) and \( A_{E2} \) so as to cancel the slight mismatch between the two. The optics control watches all the optical components. If any of them deviates from the assigned position (not-OK signal), the measurement is immediately blocked and a mending cycle is initiated by the computer.

site in the target molecule. Finding an appropriate probe and the method of specific attachment is the key to successful measurement of anisotropy decay. When available, an intrinsic probe, such as the retinal chromophore in rhodopsin, often proves to be satisfactory.

2.3. Information Contained in an Anisotropy Decay

The motion that one sees via the anisotropy decay is the thermal motion of molecules. In principle, "biological" motions such as the conformational change of a protein in a biological reaction should also be visualized. In fact, however, these motions are usually buried under the continual thermal motion. How then does the anisotropy decay method contribute to biological science?

The data in the anisotropy measurement are given in terms of anisotropy versus time (Figure 4). Usually, the ordinate is in the log scale. The decrease of the anisotropy with time implies the change from the state B to C in Figure 1.
Figure 4. Typical anisotropy decay curves. Solid curve, free rotation of globular particles; dashed curve, angle-restricted rotation in or on a structure.

The solid line in Figure 4 is the curve expected for a globular protein undergoing thermal rotation in water. The (log of) anisotropy falls linearly with time toward zero, that is, toward the completely random orientations. The slope is proportional to the rate of rotational motion, as is evident from Figure 1. The rate, or the rotational diffusion coefficient $D$, is inversely proportional to the cube of the diameter $L$ of the rotating molecule:

$$ D = \frac{kT}{\pi \eta L^3} \quad (1) $$

where $k$ is the Boltzmann constant, $T$ the absolute temperature, and $\eta$ the viscosity of the surrounding medium (in this case water). Since $T$ and $\eta$ are experimental quantities, one can estimate the size of the molecule $L$ from the rate of anisotropy decay. Conversely, by putting a probe molecule of known size in cell membrane, for example, the viscosity of the membrane is estimated. Thus, the optical anisotropy decay reveals, first of all, the size of the rotating object or the viscosity of the medium.

The important point is the cubic dependence of the rotational rate on the size. Complex formation between two protein molecules, for example, will be detected as a marked slowing down of the rotational motion. Conformational change of a molecule will also be detected if it is accompanied by a change in the diameter $L$. [When the shape of the molecule is not spherical, $L$ in Eq. (1) stands, very roughly, for the largest diameter.]

A curve like the dashed one in Figure 4 is often observed for a probe dye in a supramolecular structure such as a membrane or muscle. The anisotropy levels off at a finite value instead of falling toward zero. The data imply that the orientations of the molecules do not become random: the angular range of the rotational motion is restricted. Such is expected when the molecule carrying the probe dye is connected with a “spring” to a larger structure, or when the molecule is trapped in a gap between “walls.” The harder the spring or the narrower the gap, the higher the final plateau level of the anisotropy. Thus, from the final anisotropy value one sees how the
molecule is incorporated in the supramolecular structure. This is the second role of the anisotropy decay method.

Even when the angular range of rotational motion is restricted, the initial slope of the anisotropy decay is proportional to the rotational rate $D$ in Eq. (1). The initial slope is determined solely by the size of the rotating molecule and the viscosity of the surrounding medium; the spring constant or the gap width does not affect the initial slope.

In summary, the initial slope of the anisotropy decay reflects the rate of rotational motion and the final plateau level the angular range. The rate tells one the size of the molecule (or the medium viscosity) and the range the organization of the supramolecular architecture. The thermal motion elucidates structure. [For more rigorous discussion, see, e.g., Kinoshita et al. (1984b) and references therein.]

2.4. Optical Anisotropy Decay as a Tool in Bioscience

Below we list the major characteristics of this tool.

1. Visualization of thermal motion. The information the thermal motion conveys has already been discussed above. Examples will be given in the next section.

2. Visualization of rotational motion. The two types of thermal motion, translational (fluctuation in position) and rotational (fluctuation in orientation), can be studied by a variety of physical methods. Some sense both types of motion. The anisotropy decay reports only on rotation and hence is free from separation problems. The rate of rotation is by far more sensitive to the size of the molecule than the rate of translational motion: the latter is inversely proportional to the first power of the diameter whereas the rotational rate is inversely proportional to the third power.

3. Visualization only of the target molecules. Of the variety of molecules in a sample, only those with a probe dye give rise to the anisotropy decay. This tool is therefore particularly suited for the analysis of complex supramolecular architectures. Different parts of the structure may be stained with different probes, each giving specific information.

4. High sensitivity. For the measurement of emission anisotropy decay, 1 ml of a sample with a dye concentration of the order of 10–100 nM is sufficient. Single cell measurement under a microscope is also possible. Absorption anisotropy requires a dye concentration about 100 times as high. Yet the sensitivity is higher than many other methods.

5. Broad time range. Calculation based on Eq. (1) shows that the time by which a protein molecule of diameter 6 nm changes its orientation by 45° in water is, on the average, 25 nsec. For a dye molecule of diameter 1 nm
this number is 0.1 nsec. In cell membranes for which the effective viscosity is about 100 times as high as that of water (see below), the rotational motions are 100 times as slow. All these motions are within the range of the anisotropy decay method.

6. Time-resolved measurement. The time course of the rotational process is followed by the anisotropy decay method. Thus, distinction between the rate and range is straightforward. This is not the case with time-averaged techniques. For example, with the magnetic resonance spectroscopy one may see that the rotational “mobility” of a molecule is low. However, whether the low mobility implies slow motion, restriction in the angular range, or both is not immediately obvious. In contrast, coexistence of mobile and immobile components is easily detected with magnetic resonance, whereas this case will give rise to an anisotropy decay similar to the dashed curve in Figure 4. The distinction between the two-component case and the restricted rotation is not straightforward in the anisotropy decay. The two methods complement each other, optical on the time axis and magnetic on the component axis.

3. EXAMPLES OF APPLICATION

3.1. Dynamic Structure of Membranes Probed by Diphenylhexatriene

3.1.1. Diphenylhexatriene as a Probe of Lipid Dynamics

The fluorescent dye 1,6-diphenyl-1,3,5-hexatriene, introduced by Shinitzky and Barenholz (1974), has been widely used as a probe of lipid dynamics in biological and model membranes. As is seen in Figure 5, it is an approximately rod-shaped molecule with a thickness similar to that of a fatty acyl chain of a lipid molecule. The dye taken up by a membrane presumably enters in-between lipid molecules and replaces an acyl chain. The motion of the dye molecule will reflect the lipid chain dynamics.

Fluorescence anisotropy decay of diphenylhexatriene in various membranes was always biphasic as the dashed curve in Figure 4. The rod-shaped molecule “wobbled” in a restricted angular range rather than undergoing free rotation. This is expected in the lipid bilayer, since the lipid chains have a tendency to lie parallel to each other. Whether the tendency is strong or weak is judged from the final plateau level of the anisotropy decay. A high level points to a high degree of orientational order. From the initial part of the anisotropy decay, in contrast, the rate of wobbling is estimated. The higher the rate, the lower the effective viscosity of the membrane interior.

The topic of lipid dynamics, particularly that probed by diphenylhexa-
triene, is also dealt with by other authors of this volume. The following are excerpts from the studies made in our laboratory (Ikegami et al., 1982; Kinosita et al., 1984b).

3.1.2. Lipid Bilayers

Liposomal membranes made of pure phospholipid undergo an order to disorder (crystalline to liquid crystalline) phase transition at a temperature characteristic of the lipid species. The changes in the lipid chain dynamics accompanying the transition, as probed by diphenylhexatriene, are shown in Figure 6 for a saturated phospholipid with a transition temperature of 41°C (solid circles). At low temperatures the wobbling motion of the probe molecule was slow and restricted to a narrow angular range. Above the transition
FIGURE 6. Temperature dependence of the rate and range of wobbling motion of diphenylhexatriene in pure phospholipid membranes. $D_w$, the rate of wobbling (wobbling diffusion coefficient); $\theta_c$, the range of wobbling (half-angle of the cone); ●, dipalmitoylphosphatidylcholine; ○, 1-palmitoyl-2-linoleoylphosphatidylcholine; △, 1-palmitoyl-2-oleoylphosphatidylcholine; ▲, 1-palmitoyl-2-arachidonoylphosphatidylcholine; ×, dioleoylphosphatidylcholine. Adapted from Stubbs et al. (1981).

temperature, in contrast, the wobbling rate was an order of magnitude higher. The effective viscosity of the membrane interior was 0.15 poise at 50°C (only 30 times the viscosity of water at this temperature), compared to 2.7 poise at 10°C (200 times the viscosity of water). The wobbling range at high temperatures was quite wide, which is consistent with the laterally expanded state of the bilayer above the phase transition (Trauble and Haynes, 1971). An interesting finding was that the increase in the wobbling rate at the transition preceded that in the wobbling range (arrows in Figure 6). Fluctuation in the ordered array of lipid chains at low temperature is activated to a high level before the array melts into the disordered liquid-crystalline state.

Symbols other than the solid circles in Figure 6 are for unsaturated
phospholipids, for which the transition temperatures are all below 0°C. The degree of unsaturation was quite different among the four lipids: one double bond in chain 2 (△), two double bonds in chain 2 (○), four double bonds in chain 2 (▲), and one double bond each in chains 1 and 2 (✗). Yet the wobbling parameters were rather similar to each other. The first double bond introduced in a phospholipid molecule renders the bilayer in the disordered state at physiological temperatures. The second and subsequent double bonds make only a minor contribution to the dynamic structure of the membrane. The importance of the first double bond in a molecule was also confirmed in mixtures of unsaturated and saturated phospholipids. The primary factor that determines the lipid chain dynamics is the percentage of unsaturated phospholipid molecules in the membrane, not the percentage of unsaturated fatty acyl chains or the average number of double bonds per molecule.

Cholesterol, one of the major components of biological membranes, exerted pronounced effects on lipid chain dynamics. In the disordered state above the phase transition, addition of cholesterol greatly reduced the wobbling range. The range approached the value in the ordered state as the cholesterol content was increased from 0 to 40 mol %. The wobbling rate, in contrast, remained close to that of the disordered state, although a slight decrease was observed. An opposite effect was found in the ordered state. There the already narrow wobbling rate did not respond to the addition of cholesterol. Instead, the wobbling rate increased and approached the value in the disordered state. Thus, cholesterol, a molecule with a rigid steroid nucleus, tends to realize a state with a narrow wobbling range (characteristic of the ordered state) and a high wobbling rate (disordered state), irrespective of the starting state. The state at high cholesterol content is reminiscent of the state of pure phospholipid at the onset of phase transition.

3.1.3. Lipid–Protein Interaction

The effects of membrane proteins on lipid chain dynamics appear to be qualitatively similar to the effects of cholesterol. In the presence of cytochrome oxidase, a typical membrane protein, the wobbling range of diphenylhexatriene was narrow and the wobbling rate was high, whether the membrane was above or below the transition temperature. Both proteins and cholesterol may be regarded as rigid particles. Insertion of the rigid particles in the fluid bilayer above the transition will naturally reduce the wobbling range. In the bilayer in the ordered state, the lipid chains are closely packed, allowing only cooperative motions. At the interface between lipid chains and the particle surface, the cooperativity may well be disrupted, leading to a high-frequency (but narrow) wobbling.

At the time we were studying the lipid–protein interaction, the dynamics
of the boundary lipid, those lipid molecules that are in contact with membrane proteins, was still controversial. Earlier ESR studies had suggested that the boundary lipid was immobile, distinct from the highly mobile bilayer lipid away from protein molecules. NMR studies, in contrast, did not detect any immobile component. NMR further indicated that the average angular range of the chain motion slightly increased upon insertion of membrane proteins into lipid bilayers.

Our fluorescence anisotropy results are shown in Figure 7B. The wobbling motion of diphenylhexatriene was severely restricted in angular range in the boundary region (curve b), compared to the motion in the bilayer region (curve a). The restriction was consistent with the ESR results above. The anisotropy decay, however, also revealed a small-amplitude wobbling with a rate of the same order of magnitude as that in the bilayer region (curve b).

Lipid chains are constrained on the irregular protein surface by the pressure from neighboring chains and/or by possible interactions between the lipid head group and the membrane protein. The chains pressed against the irregular surface do not have a large freedom of reorientation. Yet they undergo a rapid (nanosecond) thermal wobbling. Occasionally, a chain will acquire a sufficient thermal energy to overcome the constraint and depart from the protein surface. If the chains go into and out of the boundary region once in microseconds, the boundary and bilayer chains are not resolved by NMR, which averages out all motions faster than a millisecond. For NMR all chains appear to be highly mobile. On the nanosecond time scale for which ESR

![Figure 7](image.png)

**FIGURE 7.** The effect of membrane protein on the lipid chain dynamics. (A) Schematic diagram with cones representing the angular range of diphenylhexatriene wobbling. (B) Fluorescence anisotropy decays of diphenylhexatriene in pure phospholipid (dimyristoylphosphatidylcholine) membrane (a), and in the same phospholipid membrane containing cytochrome oxidase at such a concentration that most lipid chains are in contact with the protein surface (b). Adapted from Kinosita et al. (1981).
and fluorescence are sensitive, in contrast, the motion in the bilayer part is relatively free, whereas the motion of the boundary chains is severely restricted. On the irregular protein surface, the axis of the restricted wobbling may well differ from site to site, reflecting the surface structure. This situation is illustrated in Figure 7A where the axis and angular range of wobbling are represented by a cone. The bilayer cone a and the boundary cones b and c are for nanosecond motions. For NMR, there is only one wide vertical cone, which is a superposition of all nanosecond cones. Thus, the overall angular range on the NMR time scale is greater in the presence of the membrane protein than in its absence. The controversy is settled.

3.1.4. Biological Membranes

Investigation of biological membranes from several sources has suggested that the lipid chain dynamics is explained, basically, by a simple formula: a biological membrane ~ unsaturated phospholipid + protein + cholesterol. Most phospholipid molecules in biological membranes contain at least one double bond. The chain dynamics is therefore expected to be of the type characteristic of unsaturated phospholipids. In fact, the temperature dependencies of both the rate and range of the wobbling motion of diphenylhexatriene in biological membranes were quite similar to those for unsaturated lipids in Figure 6. The major difference between the pure unsaturated phospholipids and biological membranes was the smaller angular range in the latter. The difference is accounted for by the effect of protein and cholesterol. As discussed above, these molecules act as range reducers in phospholipids above the phase transition. The effect appeared to be additive.

Of course, the above formula is only a first-order approximation referring to average motional properties in a membrane. Microheterogeneity may well exist both in the plane of the membrane and in the vertical direction, as suggested, for example, by the distinction between the boundary and bilayer lipids. Various membrane processes will respond to either the average or local molecular dynamics depending on the spatial and temporal scales of the reaction. The reader is referred to other chapters in this volume.

3.1.5. Steady-State Measurement

Direct measurement of an optical anisotropy decay requires a pulsed light source and a fast detection system. Indirect information, however, is obtained by the steady-state measurement of the anisotropy of the optical signal under constant illumination. Steady-state measurement of fluorescence anisotropy is quite popular, since it is easy to perform and since the high precision in the steady-state measurement allows the detection of small changes in the motional properties.
The steady-state fluorescence anisotropy is a weighted average of the time-dependent anisotropy decay that would be obtained after a pulsed excitation. Essentially, the decay over the time period equal to the fluorescence lifetime of the probe is averaged. If the decay to the final plateau level is much faster than the lifetime, then the steady-state anisotropy reflects the plateau level or the angular range of the rotational motion. If, on the other hand, the decay is slow compared to the lifetime, then the steady-state value reflects the initial slope of the decay or the rate of motion.

For the diphenylhexatriene in lipid environment, the anisotropy decay in most cases is substantially faster than the fluorescence lifetime. The steady-state anisotropy of diphenylhexatriene fluorescence therefore mainly reflects the wobbling range of the probe in the membrane. A high anisotropy points to a narrow range and a low anisotropy a wide range. Such interpretation, however, is not always warranted. Direct confirmation by time-resolved measurement is desirable. For more quantitative discussion, see Kinosita et al. (1984b).

3.2. Protein Rotations in Membrane and on Membrane Surface

3.2.1. In-Plane Rotation of Membrane Proteins

A transmembrane protein, like cytochrome oxidase, that vertically spans the bilayer membrane rotates mainly in the plane of membrane. Flip-flop across the bilayer hardly takes place. Since those portions of the protein molecule that protrude into the aqueous phase are hydrophilic, pulling them into the hydrophobic core of the membrane requires a lot of energy. In-plane rotation, in contrast, requires little energy since it preserves the hydrophobic contact between the lipid chains and the body of the protein.

Since the size of a protein molecule is more than an order of magnitude larger than the size of diphenylhexatriene, protein rotation in membrane occurs in the microsecond time range. Hence, its detection requires an optical probe with a long lifetime. The triplet probes with a chemical handle for conjugation have been introduced for this purpose. In addition, many membrane proteins contain a chromophore that serves as an intrinsic probe.

Figure 8 shows an example in which the rotational motion of a protein bacteriorhodopsin was examined. This protein, a typical transmembrane protein of molecular weight about 26,000, has retinal as a rigidly bound internal chromophore. Light absorption by the chromophore initiates a cycle of reaction, during which the purple color of the chromoprotein disappears. The large absorbance change is an excellent signal for the anisotropy decay measurement.
Dynamic Structure by Anisotropy Decay

FIGURE 8. Absorption anisotropy decay arising from the in-plane rotation of bacteriorhodopsin in a phospholipid (dimeristoylphosphatidylcholine) membrane (K. Kinosita, Jr., and A. Ikegami, unpublished data). From the final plateau level of the anisotropy decay at the high temperature where the protein rotates in the monomeric form, the angle between the membrane normal and the axis of the chromophore, retinal, can be estimated (Heyn et al., 1977).

In the experiment in Figure 8, bacteriorhodopsin was put in a phospholipid membrane. At 25°C, where the lipid was in the disordered state, the anisotropy decayed to a low constant value in several microseconds. The in-plane rotation of monomeric bacteriorhodopsin in the bilayer lipid, in which the effective viscosity is of the order of 1 poise, is expected to occur in this time range. The finite plateau level is explained by the restriction in the rotational mode, that is, by the suppression of the flip-flop rotation. As the temperature was lowered beyond the transition temperature of this lipid of 23°C, the protein motion greatly slowed down. At 10°C the protein was almost immobile. There is evidence that bacteriorhodopsin tends to aggregate and form patches in lipid membranes below the phase transition. The extreme slowing down of the rotational motion must therefore in large part be due to the increase in the size of the rotating unit.

That the anisotropy decay is sensitive to the association state of membrane proteins was put forward by Cherry and co-workers and has been widely evidenced. In biological membranes the final level of anisotropy decay is often high, which is taken to mean the presence of an immobile population. One reason is that the membrane is crowded with many proteins to the extent that nonspecific protein microaggregates form. In fact a high final level, for the anisotropy of cytochrome oxidase, in mitochondrial membrane was shown to decrease upon dilution of the membrane with exogenous lipid (Kawato et al., 1982). In plasma membranes the immobilization also results from the interaction with cytoskeleton (Nigg and Cherry, 1980). Specific interaction between membrane proteins, the formation of one-to-one complex, has also
been revealed (Gut et al., 1983). The anisotropy decay method has proved to be an excellent tool for studies of protein–protein interactions in biological membranes.

3.2.2. Wobbling Motion of Antibody on the Membrane Surface

Complex formation on the membrane surface, between a membrane component (e.g., a receptor or transporter protein) and an external ligand or substrate, is the initial event in many biological processes. Elucidation of the dynamic structure of the complex is a key to the understanding of the molecular process. Individual components can be studied by the anisotropy decay method. In addition, the method may reveal the presence, degree, or site of flexibility in the complex. Whether the complex (or part of it) is buried in the membrane or is held in the aqueous phase can also be inferred from the rotational rate.

As an example we studied the interaction between an antibody and hapten on the membrane surface. To observe the rotational motion, an antibody against a fluorescent hapten was prepared. The small hapten molecule (▲ in Figure 9A) was bound rigidly by the Y-shaped antibody (Figure 9A-a) as well as by its isolated arm portion (\( F_{\text{arm}} \) fragment, Figure 9A-b). Fluorescence anisotropy decays for these complexes in solution are shown in curves a and b in Figure 9B. The anisotropy fell toward zero, indicating free rotation. The rotational motion of the whole antibody (curve a) was slower than that of the

![Diagram](image)

**FIGURE 9.** Rotational motion of antibody in solution and on membrane surface. (A) Schematic diagram. ▲, \( N \)-iodoacetyl-\( N' \)-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) as a fluorescent hapten; Y-shaped body in a, antibody (immunoglobulin G) raised against the hapten; ellipsoid in b, \( F_{\text{arm}} \) fragment of the antibody; in c and d the hapten was conjugated to phosphatidylethanolamine and mixed with dipalmitoyl- or dimyristoylphosphatidylcholine to form bilayer membranes. (B) Fluorescence anisotropy decays of the hapten in the configurations shown in (A). Adapted from Osada et al. (1984).
arm portion (curve b), reflecting the difference in the molecular size. The size difference alone, however, would predict a much slower decay for the whole antibody. The relatively fast decay as observed indicates, as is known, that the antibody molecule is flexible: reorientation of the fluorescent dye (hapten) was accelerated by internal rotational motions (e.g., the arm against the rest).

To model a cell surface antigen, the fluorescent hapten was planted on the surface of a lipid bilayer membrane (Figure 9A-c,d). Both the antibody and isolated arm rigidly held the hapten, which was covalently linked to the head group of a lipid molecule. The linkage produced a large effect on the late portion of the anisotropy decays (Figure 9B-c,d): the anisotropy remained at a high level, indicating a restriction of the rotation angle. The initial decay rate, however, was not different from the rate of free rotation in solution. The friction against the rotational motion [the viscosity $\eta$ in Eq. (1)] still comes from water. The picture that has emerged is shown in Figure 9A-c,d. The antibody does not penetrate into the membrane interior. It moves about in the aqueous phase and is "reflected" by the membrane surface. As expected from the size difference, the motion of the whole antibody is more restricted in angular range than the motion of the isolated arm. The initial decay rates in Figure 9B also suggest that the arm (or the whole antibody) on the membrane surface still rotated around its own center. If the hapten had been firmly attached on the membrane surface and served as a pivot, the decay would have been much slower (due to the larger friction for the rotation around the pivot than for the rotation around the molecular center). The hapten on the membrane must have an appreciable freedom of translational motion, which presumably comes from the flexible linkage between the hapten dye and the lipid head.

In the above experiment the antibody was in large excess of the hapten. The dynamics thus refers to the antibody with one arm binding the hapten and the other arm free. When hapten on the membrane is in excess, the nanosecond rotational motion of the antibody in the one-to-one complex will help the free arm bind a second hapten. The dynamics of the two-to-one complex on the membrane is yet to be analyzed.

3.3. Internal Motion of DNA

3.3.1. Long DNA in Solution

Double-stranded DNA in solution normally takes the form of right-handed double helix (B-form DNA). A fluorescent dye ethidium, with a planar structure, fits into the narrow spacing between neighboring nucleotide base pairs,
which form the ladderlike backbone of the helix. Fluorescence anisotropy of the intercalated ethidium reports the thermal motion of the DNA helix.

The anisotropy decay for a filamentous structure, like the DNA helix, is best understood by referring to the model in Figure 10: small cylinders connected by springs. Suppose that the reporter dye is fixed on one of the cylinders. Initially, the anisotropy decays due to the rotation of this cylinder. The rotation is rapid since the cylinder is small, but the motion is soon arrested by the springs holding the cylinder. The anisotropy decreases only slightly. Then comes the concerted and therefore much slower motion of three cylinders, the dye-carrying one together with the neighboring two, leading to a further decrease of anisotropy. This is followed by a concerted motion of five, then seven, and so on, cylinders. In this way, the rate of anisotropy decay decreases rapidly with time. The expected decay is in the form shown in Figure 11 (dashed line a). [For a rigorous theory, see Allison and Schurr (1979).] The noisy solid line is experimental, obtained for B-form DNA in solution. Of the two kinds of motion, bending (reorientation of the filament axis) and twisting (rotation around the filament axis), the bending motion is much slower since friction from water is larger. What one sees in the anisotropy decay is therefore mainly the twisting motion. Curve a in Figure 11 represents the twisting motion of DNA.

FIGURE 10. A model of an elastic filament.
Dynamic Structure by Anisotropy Decay

FIGURE 11. Fluorescence anisotropy decays of ethidium intercalated in DNA. a, Calf thymus DNA in solution (Ashikawa et al., 1983); b, DNA in the head of immature sperm (late spermatid) from boar (Ashikawa et al., 1987); c, DNA in chicken erythrocyte nucleus (Ashikawa et al., 1985); d, DNA in the head of mature sperm (cauda spermatozoon) (Ashikawa et al., 1987). Zigzag solid lines are experimental. Smooth dashed lines are theoretical curves for free twisting (a) or for twisting of a short stretch of filament with both ends fixed (b–d).

The overall decay rate is a function of the spring constant. The decay is slower for stronger springs, since the decrease of anisotropy in each step above is then smaller. From the anisotropy decay, one can therefore estimate the rigidity of the filamentous structure. The torsional (twisting) rigidity of B-form DNA, estimated from the data, is about one-thousandth the rigidity of an iron filament of the same thickness. Expressed differently, the neighboring base pairs in the DNA helix thermally rotate over each other by as much as $\pm 5^\circ$ on the average. The DNA helix is flexible.

Yet another way of expressing the rigidity is in terms of the persistence length. It is defined as such that the mutual direction, either bending-wise or twisting-wise, between two short segments in the filament becomes random when the two are separated by more than the persistence length along the filament. This length for twisting is about 50 nm for B-form DNA. The value agrees with the persistence length for bending estimated by other means. The DNA helix happens to be equally flexible bending-wise and twisting-wise.

A particular sequence of DNA forms a left-handed helix (Z-form DNA) in high salt solutions. The anisotropy for Z-form DNA decayed much faster than that for B-form DNA. The estimated torsional rigidity was several times smaller than that for B-form DNA (Ashikawa et al., 1984a).

3.3.2. Chromatin

In the nucleus of eukaryotic cells DNA exists as chromatin, a "beads-on-a-string" structure. The bead, nucleosome bead, is a complex of histone proteins around which the DNA is wound. The DNA extends to the next
bead with a short connecting stretch called linker DNA. The entire string is packed in a helical fashion, forming a "solenoid."

The dynamics of DNA on the surface of the histone complex was investigated by intercalating the dye ethidium in DNA in isolated nucleosome beads. Analysis of the fluorescence anisotropy decay indicated that the rigidity of the wound DNA was not very different from that of free DNA and that the DNA had a considerable motional freedom in the nucleosome bead. Since the histone core and the wound DNA appear to be in close contact only on a few points (Kornberg and Klug, 1981), angle-restricted twisting of intermediary stretches is not surprising. The motional freedom indicated in the fluorescence, however, was more than that: some flexibility even in the contact regions was suggested.

When ethidium was added to the whole chromatin, the dye was intercalated primarily in the linker portion. The early portion of the anisotropy decay was rather similar to that for free DNA, indicating a similar rigidity, as may be expected. The late portion, in contrast, was quite high, reflecting the constraint of motion imposed by the beads on both ends of the linker DNA. A very slow decay was seen in the late portion, presumably arising from a concerted motion of the linker and neighboring beads. The slow decay was sensitive to the higher-order structure of the chromatin: the decay became faster in a low-salt solution where the solenoidal chromatin melted into an extended beads-on-a-string form. Measurement on intact nuclei produced a decay curve (Figure 11, curve c) indistinguishable from that for the isolated chromatin in the solenoidal form.

The higher-order structure of chicken erythrocyte chromatin was more stable than that of calf thymus chromatin. The anisotropy decay in the late portion was slower in chicken erythrocyte chromatin than in calf thymus chromatin, both for the solenoidal and extended forms. Furthermore, solenoidal form of chicken chromatin was more resistant to low-salt conditions. In chicken erythrocyte the DNA is dormant: it is no longer duplicated or transcribed. The immobilization, or stabilization, of the higher-order structure in chicken chromatin may be related to the loss of activity.

A class of histone, histone H1, is important in maintaining the higher-order structure. In chicken erythrocyte, histone H5 plays the role. When chicken erythrocyte chromatin was depleted of the protein, the anisotropy decay in the late portion became notably faster.

3.3.3. DNA in Phage Head

Double-stranded DNA in bacteriophages such as λ or T4 is closely packed in the phage head. Structural studies have indicated that the DNA helices are
aligned parallel with each other with an interhelix distance almost equal to the diameter of a hydrated DNA helix.

The consequence of the packaging on the torsional dynamics was investigated on suspensions of phages stained with ethidium (Ashikawa et al., 1984b). In the bacteriophage λ, an anisotropy decay close to curve c in Figure 11 was observed, indicating a restriction in the twist angle. Presumably, motion of the DNA strand was hindered at many points by neighboring strands. Stretches in between were relatively free, although the ends were fixed, giving rise to the observed small-amplitude decay of anisotropy. The motion of the stretches, however, was not as fast as would be expected in water: the initial portion of the anisotropy decay in the phage head was somewhat slower than that in the erythrocyte nucleus in curve c. Friction by neighboring strands may account for the slower motion.

If the suppression of DNA motion really resulted from the dense packing, it would be relieved by decreasing the density. This was in fact the case: in a λ mutant whose DNA content was deficient by 18%, the anisotropy decay was significantly enhanced particularly in the late portion. Also, when soluble DNA was collapsed artificially with ethanol to almost the same density as in λ wild, an anisotropy decay very similar to that in the phage was observed.

In the bacteriophage T4, the ethidium anisotropy decay was much more extensive than in λ wild, although the DNA in T4 was also fully packed. The reason turned out to be the difference in the chemical structure of DNA: in T4 DNA, cytosine residues are glucosylated. A T4 mutant having normal cytosine residues gave an anisotropy decay similar to that in λ wild. When isolated DNA was examined in solution, the rigidity of the glucosylated DNA from T4 wild was similar to or even slightly higher than that of the normal DNA from λ or the T4 mutant. Thus, the enhanced mobility of the DNA in T4 wild phage should be ascribed to an alteration in the DNA–DNA interaction, or the packaging mode, induced by the glucosylation.

### 3.3.4. DNA in Sperm Head

Dramatic changes in chromatin structure take place in the differentiation stage of sperm production. A diffuse chromatin characteristic of a genetically active cell is transformed into a highly condensed inactive state. In this course the histone proteins interacting with DNA are replaced with protamine.

The change in DNA mobility is also dramatic. In the nuclei of immature sperm, the ethidium anisotropy was seen to decay extensively as shown in curve b in Figure 11. The sperm were at a late stage of differentiation and their nucleus had already acquired a nonround shape characteristic of the
mature sperm. In the nuclei, however, the exchange of histone with protamine was still in progress. The DNA mobility in these differentiating sperm was much higher than that in the chicken erythrocyte (curve c). In the mature sperm, in contrast, the decay was almost absent (curve d). Presumably, all stretches of DNA were complexed with protamine, forming a tightly condensed chromatin.

Under a microscope the nuclei of the immature and mature sperm were indistinguishable. The size, shape, and ethidium stain pattern were exactly alike. The difference in the mobility cannot be attributed to different packing densities.

From the comparison of various chromatins, including the case of sperm above, we have an impression that functionally dormant DNA tends to be thermally motionless; or, functional activity requires thermal mobility. Whether this is a general rule or not remains to be seen.

3.4. **Internal Motion of Actin Filament**

3.4.1. **Naked F-Actin**

Actin is a globular protein with a molecular weight of about 42,000. At physiological ionic strength it polymerizes into a double-helical filament called F-actin. Actin filaments, together with myosin filaments, form the contractile apparatus of muscle. These proteins are also implicated in various forms of motility in nonmuscle cells.

The twisting motion of DNA takes place in the nanosecond time range (Figure 11). The motion of F-actin is expected in the microsecond range, since the radius of F-actin, 5 nm, is about four times as large as that of DNA. (The filament rigidity, as well as the friction from water, is an increasing function of the radius.) To study the filament dynamics, therefore, F-actin was labeled with the triplet dye eosin, which gave an excited-state lifetime of a few milliseconds at room temperature.

The labeled F-actin in solution gave an anisotropy decay in microseconds, which was similar to curve a in Figure 11 in that the decay rate decreased continuously with time. Analysis revealed that F-actin is torsionally very flexible. The rigidity of F-actin against twist was found to be as much as an order of magnitude smaller than that against bend estimated by other means. (The torsional persistence length was about 500 nm whereas the bending persistence length is several micrometers.) The anisotropic rigidity of F-actin contrasts with the case of DNA, for which twisting and bending rigidities are similar to each other. Electron microscopy has also indicated a high torsional flexibility of F-actin (Egelman et al., 1982).

In the double-helical structure of F-actin, two neighboring actin mono-
mers on one strand are separated by 5.5 nm along the filament axis. During thermal motion these monomers are twisted over each other by $\pm 6^\circ$ on the average. At a radius of 3 nm from the filament axis, the twist induces a displacement of 0.3 nm. If actin monomers in the filament are rigid, bonds between neighboring monomers will be broken. (Note that instantaneous displacement in the thermal motion will occasionally exceed the average value above.) Either the actin monomers are by themselves very flexible, and/or the interaction between neighboring monomers allows many twisted arrangements separated by low-energy barriers. The fluctuation angle between neighboring base pairs of DNA is also as large as $\pm 5^\circ$. In terms of displacement, however, the fluctuation in DNA is modest.

3.4.2. Regulated F-Actin

In skeletal muscle, regulatory proteins tropomyosin and troponin decorate F-actin, forming the thin filament. These proteins modify the myosin–actin interaction in response to calcium ions.

The anisotropy decay for the regulated F-actin, or the thin filament, was several-fold slower than the decay for naked F-actin. Since the regulatory proteins do not increase the filament radius greatly, the regulated F-actin must be torsionally stiffer than naked F-actin. The increase in rigidity, however, is rather small. Regulated F-actin is also more flexible against twist than against bend. The form of anisotropy decay also suggested that the length of torsional unit, the cylinder in Figure 10, is larger in the regulated F-actin. In naked F-actin, the unit is small and probably corresponds to an actin monomer. In regulated F-actin, in contrast, a long stretch containing many monomers rotates as a unit. The long tropomyosin molecules, which lie parallel to the filament, may be responsible for this effect. The results are shown diagrammatically in Figure 12. Calcium ions, to which the thin filament responds, did not affect the anisotropy decay in both the presence and absence of the regulatory proteins.

3.4.3. Interaction with Myosin

Myosin is a Y-shaped molecule with two globular heads and a long rod portion. In muscle, the rods are bundled parallel to each other to form myosin filament or thick filament. The head portions protrude from the filament backbone into water and interact with the thin filament. The head alone, cut out by an enzyme, can interact with actin.

Myosin–actin interaction was studied with the eosin-labeled F-actin and the isolated myosin heads. When the heads were added to regulated F-actin in the absence of ATP (the condition that favors the formation of actin–myosin complex), the anisotropy slowed down in proportion to the amount
FIGURE 12. A highly schematic diagram representing the torsional dynamics of actin filament. The filament made of short cylinders represents naked F-actin, while that of long cylinders the regulated F-actin, a complex of F-actin with the regulatory proteins tropomyosin (TM) and troponin (TN). The ellipsoids represent the head of myosin (S1, or myosin subfragment 1). Based on Yoshimura et al. (1984) and Mihashi et al. (1988) [a preliminary report appears in Kinosita et al. (1985)].

added. Calculation showed that the slowdown is explained by the increase in the effective radius of the filament due to the bound heads. Regulated F-actin decorated with myosin heads is still torsionally flexible.

The effect of myosin heads was modulated by calcium. Removing calcium ions from the solution, either before or after the addition of the heads, partially reversed the reduction in the decay rate. Apparently, the affinity of regulated actin for myosin is reduced in the absence of free calcium ions (Figure 12).

When ATP was added to the mixture of regulated F-actin and myosin heads, the original anisotropy decay for regulated F-actin alone was restored whether calcium was present or not. ATP is a dissociating agent for actin–myosin complex. In the absence of calcium ions muscle relaxes (myosin heads are detached from the thin filament). The restoration of the original anisotropy decay above is consistent with the relaxation. When both ATP and calcium ions are present, muscle contracts. The anisotropy decays under relaxing and contracting conditions were indistinguishable, suggesting apparently the same torsional rigidity. Whether the thin filament in contracting muscle really remains as flexible as that in relaxed muscle, however, has to be answered by the measurement on the whole muscle, since the protein concentration in the measurement in vitro was too low to simulate the situation in muscle.

A quite different result was obtained when myosin heads were added to naked F-actin. The anisotropy decay became flat (no decay) at a head/actin molar ratio as small as 0.05. The result may be explained by the formation
of actin bundles in which the myosin head cross-links actin filaments (Ando and Scales, 1985). In regulated F-actin, the regulatory proteins presumably blocked the head binding site(s) other than the genuine one; cross-linking was thus inhibited.

3.5. Dynamic Structure of Myosin Filament

3.5.1. Myosin Filament in Solution

Contraction of muscle results from the sliding of actin and myosin filaments over each other. The molecular basis of this sliding motion is not fully understood yet. The prevailing model has been one in which the myosin head protruding from the myosin filament attaches to the actin filament and rotates in such a way as to pull the two filaments against each other. Recent experiments, for example, one by Yanagida et al. (1985), cast a serious doubt on the rotating–head model. Resolution calls for more experimental facts.

Myosin head, on which actin-binding and ATP-hydrolyzing sites reside, is undoubtedly a key part of the contractile apparatus. The thermal motion of the head was revealed in the anisotropy decay of the triplet probe eosin tightly conjugated to the head (Figure 13).

In Figure 13A is shown the anisotropy decay for a solution of individual myosin heads obtained by cutting away the rod portion enzymatically. The heads rotated freely. The decay curve indicated that the head is rigid and is approximated by a prolate ellipsoid of revolution with a size between 16 nm × 4.7 nm and 17 nm × 4.5 nm.

When myosin filaments in solution were examined, the curve shown in Figure 13B was obtained. Since the myosin filament is thicker than the actin filament, the microsecond decay in the figure is too fast to be accounted for by the motion of the filament backbone. The decay must have arisen from

![Graph A](image1)

**FIGURE 13.** The absorption anisotropy decays of eosin bound to the head of myosin. (A) Solution of isolated heads (myosin subfragment 1). (B) Suspension of myosin filaments made in vitro of purified myosin. Adapted from Kinosita et al. (1984a).
the motion of the protruding heads relative to the filament backbone. The motion of the heads connected to the backbone is naturally restricted in angular range, as is suggested by the decay curve. The initial decay rate in Figure 13B is lower than the rate in Figure 13A by a factor of about 3.5. (Note the difference in time scales.) The reduction in the rate is accounted for by postulating a swivel at the head–rod junction. The rotation about the swivel is slower than the rotation about the center of the head just by the observed factor above. This one-swivel model, however, predicts an anisotropy curve consisting of a submicrosecond decay followed immediately by a constant phase. The observed decay in Figure 13B exhibits an additional component with a decay time of a few microseconds before the final plateau level is reached. The slow component must reflect the rotational motion of an object larger than the head, that is, a combined rotation of the head and something attached to it. A natural candidate is a part of the rod portion next to the head–rod junction. In this way a model depicted in Figure 14 has been reached.

The model places a second swivel joint in the rod portion at 14 nm from the head–rod junction. The experiment does not exclude an alternative possibility that a portion of the rod is bent uniformly rather than at the unique site. Then the estimated length of 14 nm should be understood as the effective length of the distributed flexibility. In any case the bend of the rod near the head–rod junction is a new finding. Previous studies have shown the presence of possibly flexible sites that are highly susceptible to enzymatic attacks. These sites, however, are remote from the head–rod junction and are distinct from the flexibility site inferred from the anisotropy decay.

3.5.2. Myofibril

A muscle fiber is a bundle of myofibrils. The myofibril is a thin thread that contains the contractile apparatus. Whether the above model obtained in solution is applicable to myosin in the contractile apparatus was investigated.

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**FIGURE 14.** A model of head dynamics on a myosin filament. Only one of the two heads of myosin is shown. Adapted from Kinosita *et al.* (1984a).
in a suspension of myofibrils in which myosin heads were selectively labeled with the eosin probe (Ishiwata et al., 1987).

The anisotropy decay in relaxed myofibrils, in the presence of ATP and in the absence of calcium ions, was quite similar to the one in Figure 13B. The scheme in Figure 14 applies, except for slight differences in the parameter values, when myosin is detached from actin. The major consequence of this finding is that the myosin heads detached from actin fluctuate translationally, by virtue of the two-swigel structure, over a distance of a few tens of nanometers in a matter of a few microseconds. The gap between myosin and actin filaments is about 20 nm. Thus, the heads make continual searches for a binding site(s) on the actin filament from various directions and with different orientations. The search frequency is enormous. A myosin head interacting with actin hydrolyzes ATP only 10 times a second.

In the absence of ATP where the heads were expected to attach to the actin filament, the anisotropy did not decay in the time range of 1 msec. Attached heads were immobile. Apparently, the result is not consistent with the finding that the regulated actin filament remains torsionally flexible in the presence of attached myosin heads. Actin was mobile while the attached myosin was not. The discrepancy is explained by the fact that an actin filament in muscle is surrounded by several myosin filaments from which many heads protrude. When all heads attach, the actin filament cannot be twisted even though it is flexible as the solution study has indicated.

The head dynamics during contraction has to be investigated on a muscle fiber with fixed ends, since myofibrils contract quickly and irreversibly when ATP is added in the presence of calcium ions. We have not completed the analysis. Preliminary data suggest, however, that the behavior of heads when detached from actin is qualitatively similar to that in the relaxed myofibrils above. How an attached head pulls, or is pulled by, the actin filament is not known yet.

4. CONCLUDING REMARKS

Dynamic structures of various biological molecules and supramolecular systems have been elucidated by the optical anisotropy decay method. The "molecular machines" working in living organisms appear highly flexible. The whole machine as well as its parts undergoes continual fluctuations. Without the fluctuation, the biomolecular machine probably will not work.

The fluctuation is important in two ways: one is to drive the molecular machines properly, while the other is to reveal the architecture of the machine via, for example, the anisotropy decay.
The first applications of the time-resolved optical anisotropy decay method to biological systems appeared in the 1960s. With the development of techniques both experimental and theoretical, the area of applications has expanded from simple determinations of rotational rates to analyses of complex supramolecular structures. Currently, efforts are being made toward the application of the method to oriented samples, as is also discussed in several other chapters in this volume.

Measurement on an oriented sample adds a new class of information: the direction of rotation with respect to the structure. In Figure 14, for example, two cones are depicted that represent the rotation ranges determined from the anisotropy decay. The directions of the cone axes, however, are drawn arbitrarily since the measurement on suspension did not give the information. Measurements on an oriented muscle fiber, in which the myosin filaments are aligned parallel, will yield the required information.

The added information implies an increased number of parameters. Experimental determination as well as theoretical analysis is naturally more complicated. We believe that the development of simplifying procedures, appropriate to each system studied, is the key to successful analyses. Progress is being made in many laboratories including our own.

ACKNOWLEDGMENTS. The optical system in Figure 3A was built by Mr. Y. Sawada, Mr. T. Kishimoto, and Mr. T. Nagamura, who were then at Union Giken Co., Ltd. The system in Figure 2A was made by Sigma Koki Co., Ltd. Many of the parts were also made at the Technology Division of the Institute of Physical and Chemical Research. Mr. T. Hakamada and his colleagues at Hamamatsu Photonics Co., Ltd. gave us valuable suggestions on the use of photomultiplier tubes. The work described here would have been impossible without these supports.

The work is a product of fruitful collaborations with many colleagues. In particular, we thank Dr. I. Ashikawa, Dr. S. Ishiwata, Dr. S. Kawato, Dr. T. Kouyama, Dr. K. Mihashi, and Dr. M. Nakanishi for critically reading the manuscript.

This work was supported by Special Coordination Funds for Promoting Science and Technology and a research grant for Solar Energy-Photosynthesis given by the Agency of Science and Technology of Japan, and by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

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