Segmental motion and rotational diffusion of the Ca^{2+} -translocating adenosine triphosphatase of sarcoplasmic reticulum, measured by time-resolved phosphorescence depolarization

Alastair SPEIRS,* Christopher H. MOORE,† David H. BOXER* and Peter B. GARLAND*

*Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, U.K., and †Department of Chemistry and Biochemistry, Massey University, Palmerston North, New Zealand

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We studied the rotational mobility of the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -activated ATPase in skeletal-muscle sarcoplasmic-reticulum vesicles, using time-resolved measurements of the depolarization of laser-flash-excited phosphorescence of the extrinsic triplet probe erythrosin. Our results are in general agreement with those of others [Bürkli & Cherry (1981) *Biochemistry* 20, 138–145] obtained by linear dichroism methods. In addition, we directly observed fast depolarization in the 1–5 μs time range that can be attributed to limited motion of part of the protein (segmental motion). Temperature-dependent changes in phosphorescence anisotropy indicated the onset of a conformational change in structure of the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -activated ATPase at 11–13°C. We also describe the synthesis of 5-iodoacetamidoerythrosin.

We have previously described the synthesis and photochemistry of erythrosin isothiocyanate and its use as an extrinsic probe for studies of the rotational diffusion of membrane proteins by time-resolved measurements of phosphorescence depolarization (Moore & Garland, 1979; Garland & Moore, 1979; Moore *et al.*, 1979). In view of the novelty of this technique, we have applied it to a membrane-bound protein (the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase of sarcoplasmic reticulum, or ' Ca^{2+} -ATPase') that has previously been studied by alternative methods of measuring rotational diffusion, namely linear dichroism (Hoffman *et al.*, 1979; Bürkli & Cherry, 1981) or saturation transfer e.p.r. spectroscopy (Thomas & Hidalgo, 1978; Kirino *et al.*, 1978; Thomas *et al.*, 1982). Apart from seeking to validate the phosphorescence depolarization method in an apparently well-described system, we were also interested in determining whether the improved sensitivity and time resolution of our method would reveal as yet undescribed dynamic features of the Ca^{2+} -ATPase.

We now describe time-resolved measurements of the decay of phosphorescence anisotropy of erythrosin isothiocyanate-labelled Ca^{2+} -ATPase. We confirm in general the rotational relaxation times described by Bürkli & Cherry (1981) in the 40–300 μs time region. In addition, we have directly measured the phosphorescence anisotropy over the first few microseconds after the laser flash, and found fast decays with a relaxation time of 2–4 μs at

5°C. We attribute these fast decays to segmental motion of the ATPase. A marked slowing of the fast decay that occurred as the temperature increased through 11–13°C was attributed to a temperature-dependent conformational change of the ATPase structure.

Experimental

Synthesis of erythrosin derivatives

Erythrosin 6-isothiocyanate was synthesized from 6-aminofluorescein as described by Moore & Garland (1979). 5-Aminoerythrosin was prepared from 5-aminofluorescein by a method similar to that used by Moore & Garland (1979), and used for the synthesis of 5-iodoacetamidoerythrosin by the following method. A stock solution of iodoacetic anhydride in dioxan was prepared by adding dicyclohexylcarbodi-imide (1 mmol) to iodoacetic acid (2 mmol) in 5 ml of dioxan and stirring for 45 min at 10°C. The insoluble dicyclohexylurea was filtered off, and the solution of iodoacetic anhydride was used without further purification. Then 100 mg of 5-aminoerythrosin was dissolved in aq. 70% (v/v) dioxan (2 ml), and 200 μmol of stock iodoacetic anhydride (2 ml stock solution) was added. After the mixture had been stirred for 1 h at 4°C, the iodoacetamidoerythrosin was precipitated by the addition of cold dilute (10 mM) H_3PO_4 , collected by

centrifugation in a bench-top centrifuge, washed with water and freeze-dried. The dried material was dissolved in ethyl acetate, and an equal volume of diethyl ether was added, which caused a small precipitate to form. This precipitate was removed by centrifugation, and the iodoacetamidoerythrosin was precipitated from the organic solvent as the *N*-ethylmorpholine salt. The derivative was assayed by reaction with sodium thioglycollate in 0.1 M-sodium phosphate buffer, pH 7.0. An estimation of the extent of the reaction after separation of the products by paper electrophoresis at pH 6.5 and extraction of the spots with ethanol showed that at least 75% of the derivative was capable of reaction with thiol groups.

Measurements of polarized phosphorescence

The apparatus used the 'T'-format geometry described previously, wherein two photomultipliers on opposite sides of the sample were equipped with polarizer filters oriented parallel ($\parallel$) or perpendicular ($\perp$) to the plane of polarization of the laser flash (Moore *et al.*, 1979). However, several improvements have been made. Firstly, the laser-flash duration was decreased from 2–3 μ s to about 0.5 μ s by use of a dye laser with a coaxial flash lamp (model DL 1200v; Phase-R Corp., New Durham, NH, U.S.A.). Secondly, signal averaging was introduced by use of a Nicolet model 1170 signal averager, giving 12-bit resolution and a minimal address time of 1 μ s. For each experiment 32–64 traces were averaged. Thirdly, the anisotropy parameter r (see below) was obtained with the computational facilities incorporated in the averager. For this purpose it was convenient to use a fast analogue amplifier circuit that accepted the two photomultiplier signals, $I(\parallel)$ and $I(\perp)$, and presented voltages proportional to $[I(\parallel) - I(\perp)]$ and $[I(\parallel) + 2I(\perp)]$ to the signal averager. Fourthly, an additional filter (Schott KV 550, 3 mm thick) was placed between the sample and each of the red-pass filters (Schott RG 695, 3 mm thick) protecting the photomultiplier tubes. The dye laser was operated with 0.15 mM-Coumarin 6 in methanol and at a repetition rate of about 1–2 Hz. An extra-cavity Glan-Taylor prism polarized the laser output. A blue-green filter (Schott glass BG 18, 3 mm thick) removed any red components arising from the flash-lamp. The output energy received at the sample was 1–2 mJ per shot at 540 nm. In some experiments where faster resolution was required, fast pre-amplifiers (rise-time 50 ns) were introduced close to the photomultipliers and the ($\parallel$) and ($\perp$) signals were recorded in a 20 MHz transient recorder (Datalab model DL 922) as unaveraged single-flash experiments. In these unaveraged experiments the laser energy was increased to 5–10 mJ at 540 nm.

Analysis of data

The anisotropy parameter r is defined as (Jablonski, 1961):

$$r = \frac{I(\parallel) - I(\perp)}{I(\parallel) + 2I(\perp)} \quad (1)$$

where $I(\parallel)$ and $I(\perp)$ are the phosphorescence intensities measured through polarizing filters aligned either parallel ($\parallel$) or perpendicular ($\perp$) to the plane of polarization of the exciting beam. When the plane of polarization of the exciting beam was rotated through 90°, to lie parallel to the plane occupied by the sample and photomultipliers, anisotropy should be absent (Albrecht, 1970). This position was used to balance the gain of the photomultiplier to give $I(\parallel) = I(\perp)$. Rotation of the polarization plane was performed by rotation of the Glan-Taylor prism. For measurements collected in the signal averager (address time 1 μ s), the time-dependent values of r were plotted directly as the ratio of the two analogue-computed signals $[I(\parallel) - I(\perp)]$ and $[I(\parallel) + 2I(\perp)]$. For the fast single-shot measurements made with the transient recorder (address time 100 ns), r values were calculated from manual measurement of the recorder outputs of $I(\parallel)$ and $I(\perp)$.

Preparation and erythrosin labelling of sarcoplasmic reticulum

Sarcoplasmic reticulum was purified from rabbit skeletal muscle essentially as described by Nakamura *et al.* (1976), with the addition of a further purification step on a discontinuous sucrose density gradient. The Ca^{2+} -dependent ATPase activity was assayed by measuring with a coupled enzyme system the Ca^{2+} -dependent release of ADP from ATP (Warren *et al.*, 1974). At 30°C the specific activity for Ca^{2+} -dependent ATP hydrolysis was 1.5–2.0 μ mol/min per mg of membrane protein. Polyacrylamide-gel electrophoresis of detergent-solubilized sarcoplasmic reticulum in the presence of sodium dodecyl sulphate showed that the major polypeptide had M_r 10⁵, corresponding as expected to the ATPase. The purified sarcoplasmic-reticulum preparations were stored at a protein concentration of approx. 10 mg/ml in 100 mM-KCl/20 mM-Mes (4-morpholine-ethanesulphonic acid) buffer, pH 7.2, and kept in liquid N_2 until required. To avoid cycles of freezing and thawing, the stored samples were of small volume (0.2 ml) in plastic tubes.

Labelling of sarcoplasmic reticulum with either erythrosin isothiocyanate or iodoacetamidoerythrosin was performed as follows. A sample of sarcoplasmic reticulum (0.2 ml, 2 mg of protein) was removed from storage in liquid N_2 , thawed and diluted to 1 ml by adding 0.8 ml of 250 mM-sucrose/100 mM-KCl/20 mM-Mes buffer, pH 8.0 (referred to

below as pH8 buffer). The membrane vesicles were sedimented by centrifugation for 10 min at maximum speed in an Eppendorf microcentrifuge, the supernatant was discarded and the pellet was resuspended in 0.4 ml of pH8 buffer. Next, erythrosin isothiocyanate or iodoacetamidoerythrosin was added as 5 μl of a 0.3–0.35 mM solution in 0.1 M-solution phosphate buffer, pH8.0, and the mixture was left for 1 h at room temperature in the dark. The mixture was then added to the top of a small column (10 cm $\times$ 1 cm diam.) of Sephadex G-25 equilibrated with pH8 buffer, and washed through with 15 ml of the same buffer. Erythrosin derivatives unattached to the membrane adsorbed strongly to the column.

Characterization of erythrosin-labelled sarcoplasmic reticulum

Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The erythrosin content of labelled sarcoplasmic reticulum was calculated from the absorption spectrum of a sample clarified by the presence of sodium dodecyl sulphate (2%, w/v) at 50°C, taking $\epsilon_{\text{mm}} = 9.9 \times 10^4 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 545 nm (Garland & Moore, 1979). The distribution of fluorescence in polyacrylamide gels used for electrophoresis (see above) was determined by illuminating the gel with the expanded beam (514.5 nm) of an argon-ion laser (100–200 mW) and observing fluorescence visually through safety goggles that blocked the laser emission but transmitted the orange fluorescence of erythrosin. The use of conventional u.v. illumination, from either low-pressure or medium-pressure mercury arc sources, was less sensitive and excited far greater background fluorescence from the gel.

Preparation of anaerobic samples of labelled sarcoplasmic reticulum

The labelled membrane obtained after removal of free erythrosin with Sephadex G-25 had in general a protein concentration of about 1 mg/ml and an erythrosin concentration of 0.3–0.7 μM . Labelled sample (1 ml) was mixed with an equal volume of 94% (w/v) glycerol, placed in a 1 cm-diam. stoppered tube fitted with a stopcock, and gassed with argon admitted by a fine polythene tube passed through the bore of the stopcock. After 10–15 min of gassing the polythene tube was removed and the stopcock was closed. During this and all other procedures the labelled sample was shielded from ambient lighting. The closed sample tube was immersed in water in a small Dewar flask in the laser-flash apparatus. The water temperature was adjusted with ice or heating, and measured with a thermistor and meter. For temperatures below 0°C a mixture of ice and NaCl solution was used in the Dewar flask.

Results

Labelling of Ca^{2+} -ATPase with erythrosin isothiocyanate or iodoacetamidoerythrosin

The ATPase accounts for most of the protein of sarcoplasmic reticulum, and it was to be expected that most of the bound erythrosin after exposure to the isothiocyanate or iodoacetamido derivatives would be attached to the ATPase. This was indeed the case. Less than 5% of the bound erythrosin could be extracted from labelled sarcoplasmic reticulum with chloroform/methanol (2:1, v/v). These findings are similar to those reported by Bürkli & Cherry (1981) with iodoacetamido-eosin. It should be noted that erythrosin (tetraiodofluorescein) is considerably less fluorescent than is eosin (tetrabromofluorescein), their quantum yields for prompt fluorescence being 0.02 and 0.19 respectively (Bowers & Porter, 1967). Accordingly erythrosin detection on gels by fluorescence is less sensitive than for eosin, but not disablingly so.

Measurements of phosphorescence depolarization

Fig. 1 shows recorder tracings for the total phosphorescence signal, free from rotational effects [i.e. $I(\parallel) + 2I(\perp)$], of erythrosin isothiocyanate-labelled sarcoplasmic reticulum. The recordings are for sample temperatures from -5°C to 40°C . The phosphorescence recordings, plotted semi-logarith-

Fig. 1. Phosphorescence decay curves from sarcoplasmic-reticulum vesicles labelled with erythrosin isothiocyanate

Both parallel $I(\parallel)$ and perpendicular $I(\perp)$ signals were recorded, and plotted as $\log[I(\parallel) + 2I(\perp)]$ against time after the laser flash. The temperature of the sample ranged from -5°C to 40°C , as shown in the Figure. Each curve has been off-set to avoid overlaps.

mically, were non-linear (Fig. 1). The half-times averaged over the first 90% of decay increased with temperature from 160 μ s at -7°C to 30 μ s at 40°C . The anisotropy parameter recordings (Fig. 2) were relatively noise-free for at least 400 μ s at -7°C , but only 100 μ s at 40°C . In all cases the value for the anisotropy parameter r was positive at zero time and subsequently decayed. At -7°C and -5°C r decayed monotonically, starting at zero time at $r = 0.15$. As the temperature was further increased, the decay of anisotropy became biphasic, with a faster decay over the first few microseconds followed by a slower decay. The zero-time anisotropy also fell as the temperature increased, probably because part of the fast phase became too fast to resolve when it occurred during the flash (0.3 μ s for the flash width at half maximal height) and in the first microsecond after the flash.

If the ATPase could rotate only anisotropically, with no rotation around axes parallel to the membrane plane, then it would be expected that r would decay to a time-independent value, except in the unlikely circumstance that the transition dipole moments for erythrosin absorption and emission (phosphorescence) were not only parallel but also at an angle of approx. 55° to the axis normal to the membrane around which rotation of the ATPase presumably occurs (see the Discussion section). At higher temperatures (20– 40°C) the value of r did decay within the measurable duration of phosphorescence to a limiting value of 0.05–0.06. At lower temperatures the slower decay of r did not permit attainment of a limiting value before phosphorescence decayed to unmeasurable values.

Although there was a steady and apparently continuous progression in the shortening of phosphorescence lifetimes as temperature was increased (Fig. 1), this was not so with the anisotropy-decay curves (Fig. 2). At around 11– 13°C , varying between samples, the value of r at zero time was markedly higher than that at lower temperature, but then decayed more rapidly (Fig. 2).

In preparations of sarcoplasmic reticulum that had been thawed and left at 0– 4°C overnight before labelling, or similarly left after labelling, anisotropy-decay curves showed lower initial values (e.g. 0.1), absence of the fast decay in the first few microseconds, absence of the unusual transition around 11– 13°C and a time-independent limiting anisotropy of 0.07–0.08. Samples that had been cross-linked by overnight exposure at 0– 4°C to 0.5% (v/v) glutaraldehyde gave no decay of anisotropy from the zero-time value of about 0.13.

Results with iodoacetamidoerythrosin

Although sarcoplasmic reticulum labelled with iodoacetamidoerythrosin yielded satisfactory phosphorescence signals, the initial anisotropy value was

Fig. 2. Anisotropy-decay curves of phosphorescence from sarcoplasmic-reticulum vesicles labelled with erythrosin isothiocyanate

The anisotropy parameter r is defined in eqn. (2). The temperatures of the samples are shown in the Figure.

low (0.0625) and if anything increased slightly with time (Fig. 3). It seems unlikely that the erythrosin was attached to part of the ATPase that allowed a very fast ($<1\mu$ s) but limited motion as the only motion, because the analogous triplet probe iodoacetamidoerythrosin gave (with linear dichroism measurements) anisotropy-decay curves that in many features were similar to those described above for erythrosin isothiocyanate (Bürkli & Cherry, 1981). A more probable reason for time-independent anisotropy with iodoacetamido-erythrosin labelling is that the angles between the absorption dipole moments, the emission dipole moment and the axis of rotation were such that the anisotropy parameter was independent of rotation.

Results with faster time resolution

Anisotropy-decay curves for phosphorescence from erythrosin isothiocyanate-labelled sarcoplasmic reticulum measured with a 50 ns address time are shown in Fig. 4. Because the transient recorder used for these measurements time-shares its input between two memory halves, the true time-resolution for the $I(\parallel)$ and $I(\perp)$ signals was 0.1 μ s. This compares with 1 μ s per address and a resolution of 2 μ s for the experiments of Figs. 1–3. Gated photomultipliers were not used for either the fast (0.1 μ s) or the slow (2 μ s) measurements. The far-red tail of the emission spectrum of prompt fluorescence of erythrosin is not excessively bright compared with the ensuing phosphorescence, and does not cause instrumental interference provided that fast-settling amplifiers are used. It is clear from the recordings in Fig. 4 that anisotropy decays rapidly in the first 5 μ s after excitation of phosphorescence. Samples previously cross-linked by

Fig. 3. Phosphorescence and anisotropy-decay curves for sarcoplasmic-reticulum vesicles labelled with iodoacetamido-erythrosin

The sample was at 4°C . The phosphorescence-decay curves are for the difference $[I(\parallel) - I(\perp)]$ between the parallel $I(\parallel)$ and perpendicular $I(\perp)$ signals, and for the sum $[I(\parallel) + 2I(\perp)]$. The $[I(\parallel) - I(\perp)]$ recording is at $4\times$ greater sensitivity than the $[I(\parallel) + 2I(\perp)]$ recording.

overnight exposure at $0-4^{\circ}\text{C}$ to 0.5% glutaraldehyde gave no decay of anisotropy from the zero-time value of approx. 0.125.

Discussion

The value of the anisotropy parameter r at time t after plane-polarized laser-flash excitation of phosphorescence from membrane molecules undergoing Brownian rotation about an axis normal to the plane of the membrane in a non-oriented collection of such membranes is given by (Rigler & Ehrenberg, 1973):

$$r(t) = A_1 e^{-t/\sigma} + A_2 e^{-4t/\sigma} + A_3 \quad (2)$$

where σ is the rotational relaxation time (the reciprocal of the rotational diffusion coefficient D) and

$$A_1 = 1.2 \sin \theta_a \cdot \sin \theta_e \cdot \cos \theta_a \cdot \cos \theta_e \cdot \cos \phi \quad (3)$$

$$A_2 = 0.3 \sin^2 \theta_a \cdot \sin^2 \theta_e \cdot (\cos^2 \phi - \sin^2 \phi) \quad (4)$$

$$A_3 = 0.1 (3 \cos^2 \theta_a - 1) \cdot (3 \cos^2 \theta_e - 1) \quad (5)$$

Fig. 4. Anisotropy-decay curves of phosphorescence from sarcoplasmic-reticulum vesicles labelled with erythrosin isothiocyanate, measured at faster time resolution

Only a single sweep was recorded, lasting $100 \mu\text{s}$ at a time resolution (after the end of the laser flash) of $0.1 \mu\text{s}$. The temperature for each experiment, from above downwards, was 5, 10, 22 and 32°C .

where θ_a is the angle between the absorption dipole moment and the axis of rotation, θ_e is the corresponding angle for the emission dipole moment, and ϕ is the angle between the respective projections of the absorption and emission dipole moments on to the plane of the membrane.

If the absorption and emission dipole moments are parallel, then $\phi = 0$, the values of A_1 and A_2 are positive, and any time-dependent change in r will consist of a decay towards the lower limiting value set by the time-independent term A_3 . The values of A_1 , A_2 and A_3 all depend on θ , the angle between the absorption (or emission) dipole moment and the axis of rotation. It is instructive to note that the decay of anisotropy due to rotation has two exponential terms, $e^{-t/\sigma}$ and $e^{-4t/\sigma}$, and their relative contributions depend on A_1 and A_2 and therefore θ . It is not usually possible to measure θ unless, as with bacteriorhodopsin, there are favourable circumstances (Cherry & Godfrey, 1981). Nor may θ have a single unique value when an extrinsic probe is used, because labelling of more than one site is possible. Owing to these uncertainties about θ , it is usual to obtain only an apparent rotational

relaxation time that may be anywhere between σ and $\sigma/4$.

In studies of membrane-protein rotation with either linear dichroism measured by ground-state depletion (Cherry & Schneider, 1976) or fluorescence depletion (Johnson & Garland, 1981, 1982), the same absorption dipole moment is used both for establishing anisotropy (by photoselection) and for its measurement [i.e. in eqns. (3), (4) and (5), $\theta_a = \theta_e$]. For phosphorescence there is no certainty that the absorption and emission dipole moments will be parallel: indeed, for many molecules they are not (Pilipovich, 1961). Furthermore, θ_a , θ_e and ϕ appear to be sensitive to their environment (Jovin *et al.*, 1981; Bartholdi *et al.*, 1981), compounding the difficulties raised by not knowing their values. To illustrate the severity of this problem, we show in Fig. 5 some computer-simulated curves for the time-dependence of r for uniaxial rotation of a phosphorescent molecule, by using a constant value for σ (the rotational relaxation time or D^{-1}) and selected values for θ_a , θ_e and ϕ . Although the anisotropy may decay or grow after the excitation flash, the relaxation time for this process is still governed by σ and $\sigma/4$, just as for the case when $\theta_a = \theta_e = \theta$.

Rotation of a membrane protein is not the only cause of time-dependent changes of phosphorescence anisotropy. Other possible causes are (i) free but limited motion of the phosphorescent probe at its site of attachment, (ii) motion of the segment or domain of protein carrying the probe (segmental motion), and (iii) rocking of the whole protein in the lipid bilayer (Kawato & Kinosita, 1981). The lower

limit for the rotational relaxation time of an integral membrane protein is probably about 20 μ s, corresponding to that measured by Cone (1972) for rhodopsin in retinal rod outer-segment discs. There is no upper limit. Free but limited motion of probe at the attachment site is expected to occur on a nanosecond time scale (Cherry *et al.*, 1980). Segmental motion can occur in as little as 33 ns, as shown for flexion of the Fab segment of immunoglobulin G (Yguerabide *et al.*, 1970): there is no reason to expect an upper time limit. Wobbling of the whole protein in the bilayer (as distinct from segmental flexion of a hydrophilic domain protruding from the membrane) could, like rotation, be considered as arising from phospholipid displacement by a 'hopping' mechanism (Trauble & Sackmann, 1973). If this were so, wobbling and rotation would occupy similar time domains.

With these considerations in mind, we turn now to the results obtained with the Ca^{2+} -ATPase. The relatively high positive values for the zero-time anisotropies and the subsequent decay to considerably lower but still positive values, very similar to those obtained by linear dichroism (Bürkli & Cherry, 1981), indicate that in this experimental situation the absorption and emission dipole moments for erythrosin excitation and phosphorescence are approximately parallel. This is also the case for erythrosin label when attached to the following proteins: a variety of water-soluble proteins (Moore *et al.*, 1979), band 3 (the anion translocator) of erythrocyte 'ghosts' (Moore & Garland, 1979), erythrocyte glycophorin reconstituted in dimyristoyl phosphatidylcholine vesicles (Jovin *et al.*, 1981), and rhodopsin in rod outer-segment discs (Chapman & Restall, 1981). By contrast, a low initial value for anisotropy followed by a time-dependent increase has been observed with alkali-extracted electric-organ membranes from *Torpedo marmorata*, where the acetylcholine receptor was labelled with erythrosin-labelled α -bungarotoxin (Lo *et al.*, 1980; Bartholdi *et al.*, 1981); similar behaviour was observed for erythrosin-labelled epidermal growth-factor bound to epidermoid carcinoma cells (Zidovetzki *et al.*, 1981).

The anisotropy decay curves that we obtained from erythrosin-labelled sarcoplasmic reticulum were clearly biphasic (Fig. 2), there being a fast decay in the first few microseconds followed by a slower phase. The relaxation times of the slower phase varied from approx. 40 μ s at 34°C to several hundred μ s at 0°C. These values are in broad agreement with those obtained by Hoffman *et al.* (1979) and Bürkli & Cherry (1981) by using linear dichroism and eosin isothiocyanate or eosin maleimide to label the ATPase.

The faster decay of anisotropy observed in the first few microseconds has not been directly ob-

Fig. 5. Time-dependence of the anisotropy parameter. Plots of $r(t)$ versus time were computed according to eqn. (2). The values (in degrees) of θ_a , θ_e and ϕ for individual plots were: A, 15, 15, 0; B, 25, 15, 180; C, 25, 25, 0; D, 15, 45, 90; E, 54.7, 54.7, 0; F, 90, 45, 90; G, 15, 75, 0. The abscissa represents time, in arbitrary units of D^{-1} , where D is the rotational diffusion coefficient.

served previously. The linear-dichroism instruments used by Hoffman *et al.* (1979) and Bürkli & Cherry (1981) suffered from electrical or optical artifacts that interfered with the first 10–20 μs after the laser flash. However, a fast decay occurring during this dead time could be inferred from the data of Hoffman *et al.* (1979). Bürkli & Cherry (1981) also noted the presence of a fast but unresolved early decay, and attributed it to segmental motion occurring during the instrumental dead time. We observed that at 0°C the fast component decayed over several microseconds, but as the temperature increased the decay accelerated such that it was largely complete by the time of the first observation point 1 μs after the flash (Fig. 4). Bürkli & Cherry (1981) have advanced cogent reasons for regarding the early decay of anisotropy (i.e. before 10 μs) as being due, at least for the ATPase, to segmental protein motion rather than rotation of the whole molecule. The greater sensitivity and time resolution achieved by the phosphorescence-depolarization method has enabled us to provide direct confirmation of the segmental motions.

A further benefit of improved time resolution is illustrated by the unusual behaviour of the anisotropy-decay curves as the temperature was increased in the region from 10°C to 15°C. The zero-time anisotropy, rather than falling with increased temperature (Fig. 2), increased rather abruptly at 11–13°C to a value of 0.17 or even greater, well in excess of the value observed at lower temperatures. We consider that this effect of temperature is unlikely to be due to increased parallelism of the absorption and emission dipole moments of the erythrosin label. The very similar r values that were measured from 20 μs onwards by either linear dichroism (Bürkli & Cherry, 1981) or phosphorescence (Figs. 2 and 4) indicate that these dipole moments were already rather parallel. A more likely explanation is that part of the protein had undergone a conformational change that slowed the segmental motion sufficiently to bring much more of it into the observation time range of the measurements. The slower phase of anisotropy decay from 20 μs onwards did not show altered behaviour, and we cannot conclude that the conformational change at 11–13°C had altered the diameter of the transmembranous part of the ATPase, or had caused oligomer formation. Other evidence for a temperature-induced conformation change of the ATPase has come from Arrhenius plots of ATPase activity in delipidated sarcoplasmic-reticulum vesicles (Dean & Tanford, 1978) and from laser Raman spectroscopy (Lippert *et al.*, 1981). The Arrhenius plots of ATPase activity have a break at 18–20°C (Madden & Quinn, 1979; Inesi *et al.*, 1973; Lee *et al.*, 1974; Davis *et al.*, 1976), some 5°C higher than the temperature at which we

observed a transition in the anisotropy-decay curves. Laser Raman spectroscopy (Lippert *et al.*, 1981) showed a transition in the ATPase structure commencing at 13°C.

In conclusion, we have successfully used erythrosin isothiocyanate as a probe for measurements of rotation of sarcoplasmic-reticulum Ca^{2+} -ATPase. The transition dipole moments for absorption and emission were approximately parallel, a circumstance that was fortunate but not predictable. Estimates of the uniaxial rotation of the ATPase were in good agreement with those obtained by others by using linear-dichroism methods. Superior time resolution enabled us to make direct observations of segmental protein motion in the 1–5 μs time range, and to detect a temperature-induced conformational change that at 13°C markedly slowed the segmental motion.

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