Rotational Motion and Flexibility of Ca^{2+},Mg^{2+}-Dependent Adenosine 5'-Triphosphatase in Sarcoplasmic Reticulum Membranes†

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ABSTRACT: Ca^{2+},Mg^{2+}-dependent adenosine 5'-triphosphatase (ATPase) in sarcoplasmic reticulum vesicles is labeled with the triplet probe, 5-iodoacetamidoeosin. Rotational mobility of the ATPase is investigated by measuring flash-induced transient dichroism of the eosin probe. The absorption anisotropy measured 20 μs after the exciting flash is found to be small at 37 °C but increases considerably with decreasing temperature and upon fixation with glutaraldehyde. A purified Ca^{2+},Mg^{2+}-dependent ATPase preparation partially depleted of membrane lipids exhibits similar properties. The low value of the anisotropy at 37 °C is due to the existence of a fast motion which in part is assigned to independent segmental motion of the protein. This internal flexibility of the ATPase may have considerable significance for the functional properties of the enzyme. At times longer than 20 μs, the anisotropy decays with a time constant which varies from ~90 μs at 0 °C to ~40 μs at 37 °C. This decay is assigned to rotation of the ATPase about an axis normal to the plane of the membrane. There is some evidence for self-aggregation of the protein at lower temperatures.

Rotational diffusion of membrane proteins may be investigated by either optical spectroscopy or saturation transfer EPR[1] [for recent reviews, see Thomas (1978), Hyde (1978), and Cherry (1979)]. In the optical method, rotation is measured by observing flash-induced transient dichroism of a triplet probe (Cherry & Schneider, 1976; Lavalette et al., 1977; Vaz et al., 1979). The long lifetime of the triplet state permits relaxation times in the microsecond–millisecond time range to be measured. Most measurements have been made with derivatives of the triplet probe, eosin. It has recently been shown that polarized phosphorescence and delayed fluorescence from these and similar probes may also be used to study rotational mobility of membrane proteins (Austin et al., 1979; Garland & Moore, 1979; Moore et al., 1979; Greinert et al., 1979).

The most detailed studies using triplet probes have so far been made with band 3, the anion transport system of the human erythrocyte membrane (Cherry et al., 1976; Nigg & Cherry, 1979a,b, 1980; Nigg et al., 1980; Austin et al., 1979). These studies demonstrate that rotational diffusion measurements are particularly valuable for investigating self-association and other protein–protein interactions in the membrane.

Here we have extended the application of triplet probes to the investigation of rotational motion of the Ca^{2+}-ATPase in sarcoplasmic reticulum (SR) membranes. The function of the membrane is to regulate calcium fluxes during muscle contraction and relaxation [for reviews, see Maclennan & Holland (1975) and Tada et al. (1978)]. Ca^{2+}-ATPase, the calcium pump, constitutes 60–90% of the total membrane protein (Meissner, 1975). Rotational mobility of this protein has previously been studied by two groups using saturation transfer EPR techniques (Kirino et al., 1978; Thomas & Hidalgo, 1978). These studies both detected the existence of rotational motion in the microsecond time range, although rather different quantitative results were obtained. Preliminary results of the present studies have also previously been presented (Bürkli & Cherry, 1978; Cherry, 1979).

Materials and Methods

Buffers. The following buffers were used in preparative procedures: 50 mM potassium phosphate, pH 8.0, containing 0.3 M sucrose and 1 M KCl (buffer I); 0.1 M $NaHCO_3$, pH 8.0, containing 0.5 M NaCl (buffer II); 10 mM Tris, pH 7.2, containing 0.3 M sucrose and 0.1 M KCl (buffer III); 10 mM Tris, pH 7.2, containing 0.3 M sucrose (buffer IV).

Preparation of SR and Purified Ca^{2+}-ATPase Vesicles. SR was prepared from rabbit white leg muscle as described by Warren et al. (1974). The final step of the preparation consisted of dialysis for 12 h at 4 °C against buffer I. The vesicles were then frozen in liquid N_2 and stored at −80 °C.

Ca^{2+}-ATPase was purified from SR according to Warren et al. (1974), except that DTT was omitted throughout, and stored as above. The preparation consists of vesicles with a phospholipid/ATPase mole ratio of 25–30:1.

Labeling with Eosin. 5-Iodoacetamidoeosin (IA-eosin) was dissolved in buffer II at a concentration of 0.5–5 μg/μL. SR was preincubated for 5 min at 4 °C in buffer I containing 5 mM MgATP and 0.1 mM $CaCl_2$, and then the probe solution was added in a ratio of 10–30 μg IA-eosin/mg of protein. The reaction proceeded for 60–120 min in the dark at room temperature. All subsequent steps were carried out in the dark or under dim red light to avoid eosin-sensitized photooxidative damage. At the end of the reaction time, the preparation was loaded onto a Sephadex G-25 column (1 mL of Sephadex/20 μg of IA-eosin equilibrated with buffer I) to separate unreacted probe. The labeled SR was collected from the void volume and contained 10–60 μg of eosin/mL. Labeled samples were either used directly or frozen in liquid N_2 and stored at −80 °C. For flash photolysis measurements, samples were diluted with buffer I to a concentration of 8–20 μg of eosin/mL, either with or without 50% sucrose. Prior to flash photolysis measurements, oxygen was displaced from the samples by a stream of argon as described previously (Cherry, 1978). Some samples were fixed before measurement by adding 0.3% glutaraldehyde and incubating for either 30 min in buffer or 12 h in 50% sucrose at room temperature.

In some experiments, labeled SR was dialyzed against buffer III and centrifuged at 12000*g* for 30 min at 4 °C, resulting

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[1] Abbreviations used: EPR, electron paramagnetic resonance; SR, sarcoplasmic reticulum; IA-eosin, 5-iodoacetamidoeosin; $NaDodSO_4$, sodium dodecyl sulfate; Ca^{2+}-ATPase, Ca^{2+},Mg^{2+}-dependent adenosine 5'-triphosphatase; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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in partial pelleting. After removing the supernatant, the pellet was resuspended in buffer III by mild stirring. Flash photolysis measurements and negative stain electron microscopy were performed on each of the three samples (pellet, supernatant, and original total sample).

Prereaction of SH groups in SR was achieved by incubating SR for 5 min in buffer III containing 5 mM MgATP and 0.1 mM $CaCl_2$ and then adding NEM (dissolved in buffer III) at a ratio of 1 μg of NEM/mg of protein. After 1 h at 4 °C, the reaction was stopped by adding 1 μg of L-cysteine/mg of protein. The sample was diluted 50-fold with the same buffer and centrifuged at 100000*g* for 1 h. The NEM-labeled SR and unlabeled SR were reacted with eosin-5-maleimide (Molecular Probes). The labeling procedure followed that described for IA-eosin except that buffer III and 120 μg of eosin maleimide/mg of protein were used. Reaction for 1 h resulted in binding of ~1 eosin/ATPase molecule.

Prereaction of the probe was achieved by reacting IA-eosin with equimolar or 10 times higher concentration of β-mercaptoethanol for 1 h at room temperature in buffer I. The prereacted probe was used for labeling SR as described above.

SR lipids were extracted as described by Folch et al. (1957), redispersed in buffer III, and sonicated to a clear suspension with a bath sonifier. The lipid dispersion was labeled with IA-eosin as described above by using buffer III and replacing 1 mg of SR protein by 0.57 mg of extracted phospholipids.

Analytical Methods. The amount of bound eosin was determined spectrophotometrically as previously described (Cherry, 1978) after first solubilizing vesicles in either 0.2% sodium deoxycholate or 5% $NaDodSO_4$. Protein was determined by a modified Lowry procedure (Bensadoun & Weinstein, 1976). Phospholipid phosphorous was measured according to Chen et al. (1956). SR lipids were extracted according to Folch et al. (1957). The eosin content of the upper and lower phases was measured after redispersion in buffer IV containing 5% $NaDodSO_4$.

For $NaDodSO_4$–polyacrylamide gel electrophoresis, samples were dialyzed against buffer IV and then solubilized in the same buffer containing 8% $NaDodSO_4$, 8% β-mercaptoethanol, and 8 M urea at room temperature. A total of 100–300 μg per sample was run on 5-mm slab gels [12% acrylamide, bis(acrylamide) 2.7% of acrylamide monomer] as described by Laemmli (1970). The tracking dye was omitted since its presence masks free eosin running near the gel front. Eosin fluorescence was photographed through an orange filter, and the gels were subsequently stained with Coomassie blue.

ATPase activity was measured by using the coupled enzyme test described by Warren et al. (1974). Both Ca^{2+}-dependent and Ca^{2+}-independent activities were measured; the latter was ~10% of the Ca^{2+}-dependent activity for SR and <0.1% for the purified ATPase. Ca^{2+} accumulation was measured spectrophotometrically according to Scarpa (1979) in a buffer containing 5 mM oxalate, 0.2 mM arsenazo III, 5 mM MgATP, 0.2 mM $CaCl_2$, 50 mM KCl, and 100 mM triethanolamine hydrochloride, pH 7.2.

The morphology of the vesicles was examined by electron microscopy of freeze-fractured and phosphotungstate negatively stained specimens. Vesicle size distributions were determined by the latter method. The appearance of both labeled and unlabeled SR vesicles in freeze-fracture electron micrographs was similar to that previously reported (Wang et al., 1979; Stewart & Maclennan, 1974). The viscosity of vesicle suspensions was measured with an Ostwald viscometer.

The effect of photooxidation was measured by illuminating an air-saturated, continuously stirred suspension of eosin-labeled SR vesicles with a 150-W slide projector. The light intensity at the sample was 170 ± 50 mW/cm^2. After illumination for a given time interval, ATPase activity, Ca^{2+} accumulation, and eosin concentration were measured as described above. In some samples, air was displaced by a stream of argon prior to illumination, and the sample was maintained under an argon pressure.

Flash Photolysis Measurements. The flash photolysis apparatus used in these experiments is described in detail elsewhere (Cherry, 1978). Briefly, protein-bound eosin probes were excited at 540 nm by a linearly polarized laser pulse of duration 1–2 μs. Transient absorbance changes at time t after the flash arising from ground-state depletion were simultaneously measured at 520 nm for light polarized parallel [$A_\parallel(t)$] and perpendicular [$A_\perp(t)$] with respect to the polarization of the exciting flash. Data were analyzed by calculating the absorption anisotropy $r(t)$ defined by

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

$r(t)$ is independent of the signal lifetime and depends only on rotational motion providing the absorption transient exhibits a single exponential decay.

In each measurement, 32–64 signals were averaged with a Datalab DL 102A signal averager. A further improvement in signal to noise was obtained by averaging several sets of measurements; data analysis was accomplished by a Hewlett-Packard HP 9825A desktop computer. The computer was also used to fit the experimental $r(t)$ by exponential decays using an iterative nonlinear least-squares program.

Fluorescence Anisotropy Measurements. Steady-state fluorescence polarization was measured with a Schoeffel RRS 1000 recording fluorometer. The excitation beam was vertically polarized by a Glan-Thompson polarizer, and the emitted light was analyzed with Polacoat sheet polarizers. Excitation was at 500 nm and emission was measured at 570 nm after passing through a 550-nm cutoff filter. The steady-state emission anisotropy (r_s) was calculated by using an equation analogous to eq 1. Standard corrections were made for instrumental depolarization (Azumi & McGlynn, 1962).

Results

Binding of IA-Eosin to SR. Reaction of IA-eosin with SR as described under Materials and Methods typically resulted in binding of 8–16 μg of eosin/mg of membrane protein, corresponding to about 1–2 eosin molecules/ATPase. It might be anticipated that most of the bound probe would be associated with the ATPase, since this component constitutes 60–90% of the total membrane protein (Meissner, 1975). This was confirmed by $NaDodSO_4$ gel electrophoresis (Figure 1) which shows a strong eosin band coincident with the ATPase, together with bands at higher molecular weights corresponding to ATPase oligomers. A much weaker band is found near the front of the gel; this is assigned to free eosin on the basis of control gels containing only the unbound probe. Other faint eosin bands are associated with other minor components. Little eosin is seen in the expected position of a minor glycoprotein which has previously been claimed to have a highly reactive SH group (Hidalgo & Thomas, 1977).

In a control experiment, IA-eosin was prereacted with β-mercaptoethanol before incubation with SR. This experiment gave the result that the amount of eosin associated with SR after separation on a Sephadex G-25 column was 30–50% of that obtained without prereaction. However, $NaDodSO_4$ gels of this preparation exhibited little eosin associated with the

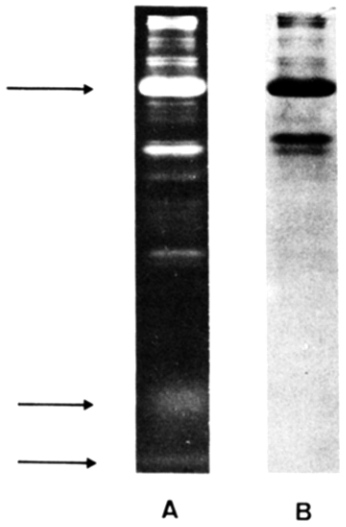

FIGURE 1: Fluorescence distribution in polyacrylamide gels of IA-eosin labeled SR. The labeling ratio was 14 μg of eosin/mg of membrane protein. Arrows from top to bottom indicate positions of Ca^{2+}-ATPase monomer, free eosin, and gel front, respectively. (A) Eosin fluorescence in unstained gel. Note that the photograph is overexposed to reveal minor bands; the eosin fluorescence in these minor bands relative to the Ca^{2+}-ATPase monomer is therefore exaggerated. (B) Coomassise blue staining of same gel.

ATPase. It was therefore concluded that the prereacted probes has bound noncovalently to SR.

The above experiment suggested a possible complication in the binding of IA-eosin. Conceivably, the probe could bind noncovalently to SR with the covalent attachment occurring during the solubilization in $NaDodSO_4$. If this were true, the exact location of the probe would be in doubt. To check this possibility, we incubated IA-eosin for 1 h at room temperature with $NaDodSO_4$-solubilized SR and ran gels as before. It was found that very little eosin was associated with the ATPase, demonstrating that the covalent reaction does not occur during solubilization.

Several experiments were performed to check possible association of the probe with lipids. After extraction of eosin-labeled SR with chloroform–methanol, ~2% eosin was detectable in the organic phase. A small amount of eosin (~10%) appeared in the aqueous phase. In another experiment, SR lipids were extracted, redispersed in buffer solution, sonicated, and labeled with IA-eosin. After gel chromatography, it was found that the amount of probe associated with lipid vesicles was only ~10% of the amount which binds to SR under identical conditions.

From the above experiments, we conclude that at least 80% of bound eosin is associated with the ATPase. The results of flash photolysis measurements may therefore be related to the rotational motion of this protein.[2]

Effects of Eosin Labeling on Activity of ATPase. Ca^{2+}-dependent ATPase activity and Ca^{2+} accumulation were measured before and after eosin labeling of the enzyme. Before labeling, the ATPase activity was typically 2–3.5 IU, in agreement with other determinations (Warren et al., 1974). No loss of activity occurred upon eosin labeling; in fact, an activation (~40%) was observed. In the case of Ca^{2+} transport, some loss was observed after eosin labeling. Typically the ability of the vesicles to accumulate calcium was

[2] Qualitatively similar results to those reported here were obtained for rotation of purified Ca^{2+}-ATPase reconstituted into SR lipid vesicles (provided by Professor S. Fleischer, Vanderbilt University). Thus, the effect of the small amount of eosin associated with other membrane proteins is not significant.

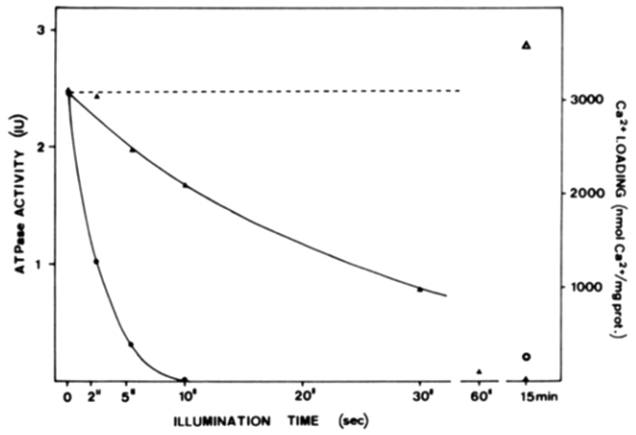

FIGURE 2: Eosin-sensitized photoinactivation. IA-eosin labeled SR in buffer III (20 μg of eosin/mL) was illuminated as described under Materials and Methods. (▲, △) Total ATPase activity (left side scale); (●, ○) Ca^{2+}-accumulation (right side scale). (Closed symbols) Air-equilibrated samples; (open symbols) at 15 min, samples under argon. The dashed line connects the initial ATPase activity with the value measured at 15 min under argon.

reduced 30–40% by the labeling procedure. Since standing at room temperature also results in loss of Ca^{2+} accumulation, this effect was probably only in part due to the presence of the eosin label.

Eosin-Sensitized Photoinactivation. It has previously been shown that free eosin sensitizes inactivation of the ATPase in SR by photooxidation (Kondo & Kasai, 1974). Figure 2 shows that bound eosin has a similar effect. Under the illumination conditions described under Materials and Methods, all the Ca^{2+}-dependent ATPase activity is lost within ~1 min. The ability of the vesicles to accumulate calcium was lost at an even more rapid rate. Illumination of eosin-labeled SR also produced two further reactions, namely cross-linking of all membrane proteins into high molecular weight aggregates (detected by $NaDodSO_4$ gel electrophoresis) and bleaching of the eosin chromophore. These latter two reactions, however, occurred at a considerably slower rate than the loss of ATPase activity.

Experiments were performed to test whether the above photochemical reactions occur when air in the sample is displaced by argon. It was found that argon afforded almost complete protection against loss of ATPase activity and protein cross-linking, while loss of Ca^{2+} accumulation and eosin bleaching were little protected.

Some further measurements were made on samples which had been used for flash photolysis experiments. It should be noted that such samples always have air displaced by argon in order to maximize the eosin triplet lifetime. As expected from the experiments described above, no cross-linking and little loss of ATPase activity (<10%) occurred under the conditions of the flash photolysis experiments. However, Ca^{2+} accumulation was mostly lost and eosin bleaching occurred to a variable extent, depending on the duration of the set of experiments performed with a given sample. In previous studies of Ca^{2+}-ATPase rotational motion using probes, Ca^{2+} uptake was either lost during labeling (Kirino et al., 1978) or apparently not measured (Thomas & Hidalgo, 1978; Hoffman et al., 1979). Although the loss of Ca^{2+} accumulation is clearly undesirable, it should be noted that activity can be changed by a subtle modification involving, for example, a single amino acid, which in itself would not affect rotational motion. The preservation of ATPase activity suggests that relatively gross conformational changes which might affect rotational motion are unlikely to occur.

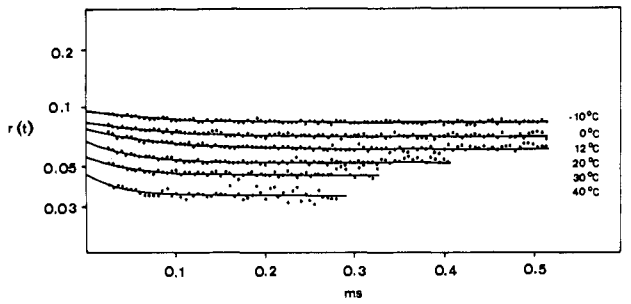

FIGURE 3: Decay of absorption anisotropy of IA-eosin labeled SR at different temperatures. Fresh aliquots from the same sample were used at each temperature. Each set of points is the average of three to five experiments, in each of which 32–64 signals were averaged. The solid lines were obtained from fitting the data by eq 2. Samples were in buffer I containing 50% sucrose.

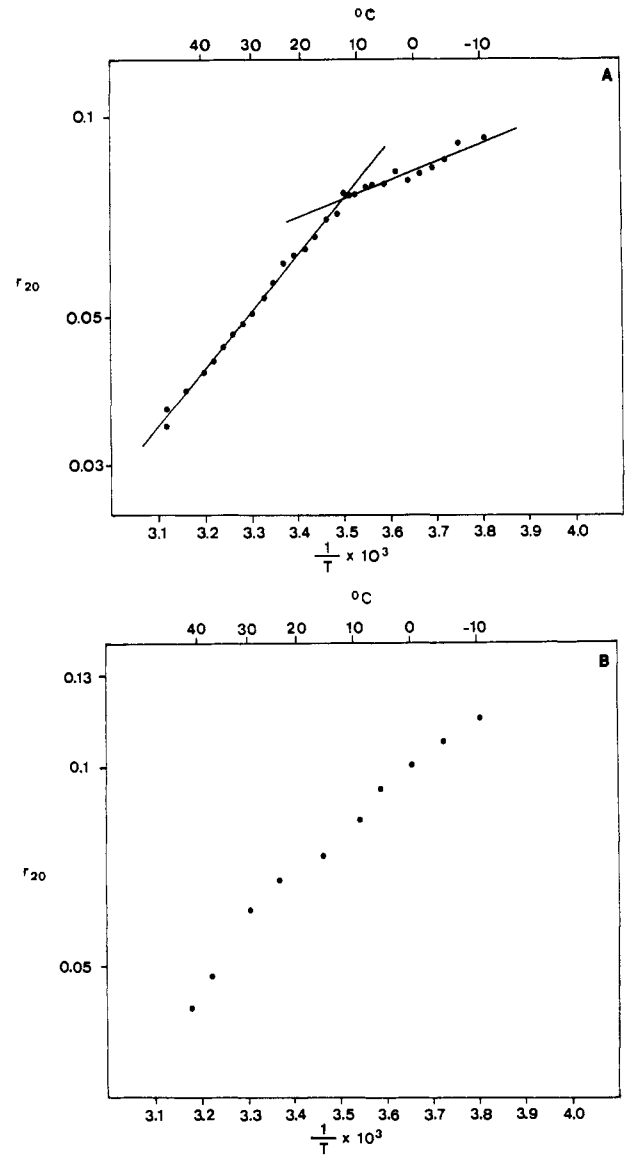

FIGURE 4: Temperature dependence of r_{20}. (A) IA-eosin labeled SR. (B) IA-eosin labeled purified Ca^{2+}-ATPase vesicles. Values of r_{20} were determined as described in the text from plots similar to those shown in Figure 3. In (A) the lines through the points above and below 13 °C were obtained by linear regression. It was not possible to meaningfully follow a similar procedure in the case of (B). All samples were in buffer I containing 50% sucrose.

Flash Photolysis Measurements. The decay of the absorption anisotropy obtained with eosin labeled SR at different temperatures is shown in Figure 3. The data points begin

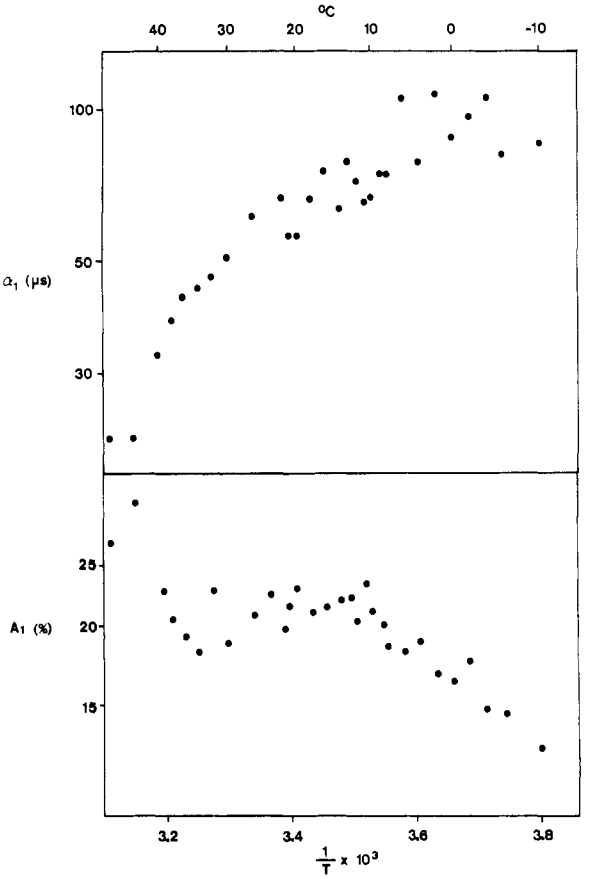

FIGURE 5: Temperature dependence of constants A_1 and α_1. The constants were obtained from fitting data similar to those shown in Figure 3 by eq 2. All measurements were for IA-eosin labeled SR in buffer I containing 50% sucrose.

at $t = 20$ μs, since it was difficult to be sure that artifacts were absent at shorter times. The data were fitted by eq 2.

$$r(t) = A_1 \exp(-t/\alpha_1) + A_3 \quad (2)$$

By use of the parameters obtained from the best fit, the value of the anisotropy at $t = 20$ μs (r_{20}) was then determined. Figure 4 shows the temperature dependence of r_{20} for SR and for purified ATPase. In both cases, it may be seen that r_{20} decreases substantially with increasing temperature.

It should be pointed out that the absolute values of the anisotropy can vary with the intensity of the exciting flash due to excitation of a nonnegligible fraction of molecules. In separate experiments it was established that this effect could be neglected provided that the degree of excitation (as measured by the total absorption change $S = A_{\parallel} + 2A_{\perp}$) did not vary by more than ±5%. This condition was fulfilled by the data shown in Figure 4; hence, the temperature dependence of r_{20} is a property of the sample.

A further trivial explanation of the temperature dependence of r_{20} is that it results from changes in depolarization due to light scattering. In separate experiments, it was determined that this effect is small and, if present, would lead to an opposite temperature dependence to that actually observed.

The values of the constants α_1 and A_1 obtained from curve fitting are shown in Figure 5. The wide spread of the values is due to the small amplitudes of the decays and the difficulty of accurately determining the residual anisotropy A_3. As a result, a good fit to the experimental points can be obtained within a rather wide range of the adjustable parameters. It may be seen, however, that α_1 is temperature dependent, varying from ~40 μs at 37 °C to ~90 μs at 0 °C. The coefficient A_1 becomes smaller as the temperature is reduced

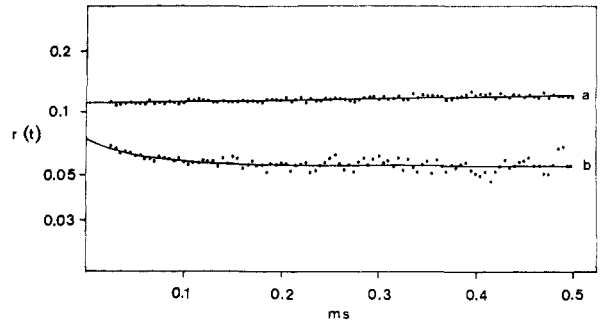

FIGURE 6: Effect of glutaraldehyde on $r(t)$ curve of IA-eosin labeled SR at 22 °C. (Lower curve) Control sample; (upper curve) sample fixed for 12 h with 0.3% glutaraldehyde. Samples were in buffer I containing 50% sucrose.

below ~15 °C. The parameters obtained from the curve fitting were not noticeably different when 10 mM Ca^{2+} was added to the medium.

Most of the studies reported here were made with the probe IA-eosin. A few measurements were also made with a different derivative, eosin-5-maleimide, which is also expected to principally react with SH groups. Essentially similar results were obtained with the two different probes. Since it has previously been reported that maleimide reagents preferentially label a highly mobile component of SR (Hidalgo & Thomas, 1977), we carried out a further experiment in which membranes were labeled with NEM prior to the reaction with eosin maleimide. This prelabeling had no effect on the observed results.

Figure 6 shows the effect of glutaraldehyde fixation on the decay of $r(t)$. It may be seen that at 22 °C, fixation produces a considerable increase in the anisotropy. The anisotropy no longer decreases with time but becomes virtually constant.

Effects of Vesicle Size. The sizes of the SR vesicles used in the present study were estimated by negative-stain electron microscopy. The diameters were found to be in the range 400–3000 Å with a mean value (weighted according to surface area) of 1200 Å. A computer simulation based on the Stokes–Einstein equation showed that the contribution to $r(t)$ from vesicle tumbling is negligible over the time range 0–20 μs but could have an effect over the range 0–1 ms.

Experimentally, possible effects of vesicle tumbling on the measured $r(t)$ were tested by separating SR vesicles into two fractions by low-speed centrifugation. Electron micrographs showed that the pellet contained more large vesicles than the supernatant. $r(t)$ curves for the pellet, supernatant, and original total sample were then measured both in buffer and in buffer plus 50% sucrose. It was found that only the supernatant showed a significant change in the shape of $r(t)$ upon addition of 50% sucrose. Moreover, the $r(t)$ curves for all three samples in 50% sucrose were essentially the same. From these results, we conclude that the contribution of vesicle tumbling to $r(t)$ is negligible in 50% sucrose and that the presence of 50% sucrose does not significantly change the protein motion in the membrane.

Fluorescence Polarization. Table I shows steady-state fluorescence anisotropy values for IA-eosin labeled SR under different conditions. A relatively high value of the anisotropy is obtained for vesicles in buffer at room temperature. The value is little changed by fixation with glutaraldehyde or by lowering the temperature from 20 to 0 °C.

Table I: Fluorescence Anisotropy Values[a]

sample	temp (°C)	r_s
IA-eosin labeled SR	20	0.26
IA-eosin labeled SR	0	0.26
IA-eosin labeled SR, glutaraldehyde fixed	20	0.27
eosin immobilized in poly(methyl methacrylate)	20	0.33
1 μM eosin in glycerol	−15	0.33

[a] SR samples were measured in buffer at an eosin concentration of 1.2 μM and an eosin/ATPase mole ratio of 0.7. Experimental error in r_s was <5%.

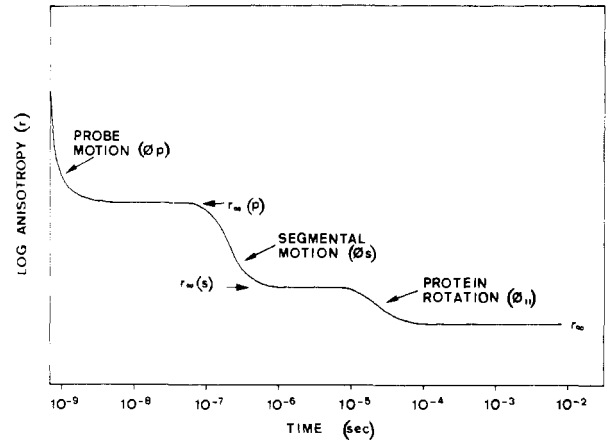

FIGURE 7: Schematic representation of expected decay of anisotropy when independent probe motion, segmental motion, and protein rotation are simultaneously present with well-separated relaxation times. Note that the time axis is logarithmic.

Discussion

"Fast" Rotational Motion of Ca^{2+}-ATPase. The anisotropy measured 20 μs after the exciting flash at 37 °C is typically ~0.045. This is extremely low when compared to the maximum anisotropy of 0.4 expected for eosin ground-state depletion signals. Although the anisotropy is normally reduced by instrumental factors (principally by excitation of a non-negligible fraction of molecules), this effect could not possibly account for the low value of r_{20} observed in the present experiments. We therefore conclude that a rapid decay of the dichroism must occur in times shorter than 20 μs.

This rapid decay could in principle be due to any combination of the following three types of motion: (1) independent motion by the eosin probe; (2) independent segmental motion of part of the protein containing the probe; (3) rotation of the whole protein in the membrane.

In order to facilitate the discussion, we illustrate in Figure 7 an idealized graph of how the anisotropy would be expected to decay when all three types of motion are present with well-separated relaxation times. In this graph, it is assumed that excitation is by an instantaneous pulse of light so that the initial anisotropy (r_0) is determined only by instrumental factors which reduce its value from the theoretical maximum. It is further assumed that independent probe motion and segmental motion are both restricted in angular amplitude, so that for each type of motion the anisotropy decays with relaxation times ϕ_p and ϕ_s to constant values given by $r_\infty(p)$ and $r_\infty(s)$, where the symbols p and s refer to probe motion and segmental motion, respectively. A theoretical treatment applicable to these motions has been presented by Kinosita et al. (1977). Rotation of the whole protein in the membrane is assumed to occur only around the membrane normal and is characterized by the relaxation time $\phi_\parallel$ and the residual anisotropy r_∞ (Cherry, 1978). The time at which the present transient dichroism measurements begin is 20 μs. Decays of the anisotropy which occur at shorter times will not be seen directly but will affect the measured values of r_{20}. The possible contributions of each of the three types of motion to the rapid decay of anisotropy are discussed in turn below.

(*a*) *Independent Motion of the Eosin Probe.* Studies with eosin-labeled water-soluble proteins demonstrate that independent probe motion does in general occur (Cherry & Schneider, 1976). This motion is expected to be very fast because of the dependence of Brownian motion on molecular size. It was in fact shown that the relaxation time for independent motion of eosin bound to ovalbumin is only 2–3 times slower than that of free eosin. Independent probe motion generally results in only a partial loss of anisotropy because the angular amplitude is restricted by the surrounding protein. Nanosecond-pulse fluorometry measurements have in fact demonstrated that fluorescent SH reagents bound to Ca^{2+}-ATPase undergo a rapid restricted wobbling motion within a few nanoseconds (R. Kataoka, private communication).

It is unlikely that independent probe motion is the sole reason for the low values of r_{20} in the case of the Ca^{2+}-ATPase for the following reasons. (1) Independent probe motion would have to be almost isotropic to account for the low anisotropies observed. In all other proteins which have been investigated, anisotropy values in excess of 0.1 have been measured in transient dichroism experiments. (2) The value of r_{20} is strongly increased by fixation with glutaraldehyde. Cross-linking would be expected to inhibit protein rotation and probably segmental motion but would be unlikely to drastically affect independent probe motion. (3) Independent probe motion occurs in a time comparable to the eosin fluorescence lifetime [1.7 ns (Fleming et al., 1976)]. Hence the steady-state fluorescence anisotropy (r_s) should be sensitive to such motion. In agreement with this prediction, r_s of IA-eosin labeled SR is less than that of immobilized eosin (see Table I). However, in contrast to r_{20}, r_s does not vary significantly with temperature and is unaffected by glutaraldehyde fixation. Since the value of r_s arises from a combination of the fluorescence lifetime, the rate of motion, and the angular amplitude (Heyn, 1979), it is conceivable that changes in individual parameters could occur which coincidentally might cancel out to leave r_s unchanged. If we reject this interpretation as improbable, then the data indicate that independent motion is not significantly affected by fixation and change of temperature. Hence, an additional fast motion must be postulated to explain the behavior of r_{20}.

(*b*) *Segmental Motion.* It is conceivable that the Ca^{2+}-ATPase is a flexible structure in which part of the molecule undergoes independent segmental motion. Such motion would be expected to be slower than independent probe motion but could be fast relative to rotation of the whole protein in the membrane. Approximately half of the Ca^{2+}-ATPase appears to protrude from the membrane into the aqueous phase (Saito et al., 1978; Shamoo & Goldstein, 1977). Possibly all or part of this hydrophilic moiety undergoes segmental motion whose rate is determined by the local viscosity at the membrane surface. The viscosity of this surface layer is not known but it is likely to be considerably lower than that of the membrane.

Fixation by glutaraldehyde might be expected to inhibit segmental motion either by inter- or intramolecular cross-linking. Thus, the observation that r_{20} increases substantially upon fixation is consistent with the existence of segmental motion in unfixed samples.

A further experimental finding is that r_{20} increases with decreasing temperature. From Figure 7 it may be seen that r_{20} will vary with $r(s)$, which in turn is determined by the amplitude of segmental motion. Thus, the temperature dependence of r_{20} could be explained on the basis of a decrease in the amplitude of segmental motion with decreasing temperature. A temperature-dependent angular amplitude of independent probe motion would have a similar effect but appears unlikely on the basis of the fluorescence anisotropy data discussed above. Other interpretations are possible; for example, self-aggregation of the Ca^{2+}-ATPase could occur at lower temperature, leading to inhibition of segmental motion by protein–protein contact.

(*c*) *Protein Rotation in the Membrane.* The remaining possibility is that rotation of the whole protein in the membrane reduces the anisotropy to low values within a time of 20 μs. The fastest value of $\phi_\parallel$ which has been measured for a membrane protein is 20 μs for rhodopsin (Cone, 1972); other proteins have slower relaxation times (Cherry, 1979). The Ca^{2+}-ATPase is a larger molecule than rhodopsin, and it is unlikely that the viscosity of SR membranes is markedly lower than that of rod outer segment disk membranes. Hence, 20 μs appears to be a lower limit for the relaxation time of the Ca^{2+}-ATPase; it is possible that such a relaxation time would be just below the resolution of the present experiments. If the rotation of the ATPase were to be as fast as this, it would be highly likely that the protein is in a monomeric state.

The temperature dependence of the $r(t)$ curves shown in Figure 3 cannot straightforwardly be explained in terms of rotation of the whole protein. It might be expected that reducing the temperature would slow the rotation. Thus, even if the decay of anisotropy is too fast to measure at 37 °C, it would be expected that at some lower temperature the whole decay would fall within the time range of the measurement. This is not observed experimentally. To explain the results, it would be necessary to evoke a temperature-dependent equilibrium between rapidly rotating and virtually immobilized (probably aggregated) ATPase molecules, as was previously proposed for band 3 proteins in the human erythrocyte membrane (Nigg & Cherry, 1979b).

The strongest argument against rapid rotation of the whole protein in the time range 0–20 μs is obtained from measurements with purified Ca^{2+}-ATPase which has a phospholipid/ATPase mole ratio of 25–30:1 compared with 90:1 in SR (Warren et al., 1974). These lipid-depleted samples show a similar temperature dependence and a similar low value of r_{20} at 37 °C as SR vesicles (Figure 4). It would be surprising if the same rapid rotation of the whole protein were to be maintained under conditions where there is little or no bulk lipid bilayer. Model system studies in fact confirm that protein rotation slows considerably at low lipid/protein ratios (Cherry et al., 1977).

(*d*) *Conclusions.* On the basis of the above discussion, we conclude that both independent probe motion and segmental motion probably occur and in combination are responsible for the low value of r_{20} observed at 37 °C. Independent probe motion and instrumental factors reduce the anisotropy to a value in the range 0.1–0.15 as judged from results with glutaraldehyde-fixed samples. Segmental motion further reduces the anisotropy to ~0.045 at 37 °C. Since eosin fluorescence is partially depolarized, the relaxation time for probe motion is probably on the order of 10^{-9} s. The relaxation time for segmental motion must be slow compared with the eosin fluorescence lifetime (since r_s is relatively high) but fast compared with the time resolution of the transient dichroism measurements, placing it in the range 10^{-8}–10^{-5} s.

"Slow" Rotational Motion of Ca^{2+}-ATPase. As argued above, the decay of anisotropy in times faster than 20 μs is probably not due to rotation of the whole protein in the membrane. However, it is likely to be this rotation which is responsible for the decay of anisotropy at times longer than 20 μs. Due to its large hydrophilic moiety, the Ca^{2+}-ATPase

is expected to maintain a fixed orientation with respect to the sidedness of the membrane (Martonosi & Fortier, 1974; Dutton et al., 1976). When rotation of the protein occurs only about the membrane normal, the decay of anisotropy is given by

$$r(t) = A_1 \exp(-t/\phi_\parallel) + A_2 \exp(-4t/\phi_\parallel) + A_3 \quad (3)$$

where A_1, A_2, and A_3 are constants (Cherry, 1978). The relaxation time $\phi_\parallel = 1/D_\parallel$, where $D_\parallel$ is the diffusion coefficient for rotation about the membrane normal. Since the experimental data are not sufficiently accurate to resolve the expected double-exponential decay, the data were fitted by the simpler eq 2. The values of α_1 obtained by this procedure are therefore not exact relaxation times. Also, they may represent average values if different populations of Ca^{2+}-ATPase with different rotational mobilities are present in the membrane. An additional complication is that the decay of the absorption transient ($S = A_\parallel + 2A_\perp$) is not monoexponential. In this case the decay of $r(t)$ cannot safely be assumed to be determined only by rotational motion independent of the signal lifetime (Rigler & Ehrenberg, 1973). Thus, α_1 should be regarded as an order of magnitude estimate of the relaxation time.

The time constant α_1 varies from ~90 μs at 0 °C to ~40 μs at 37 °C. A temperature dependence is also seen in the coefficient A_1 which is more pronounced in the lower temperature range. This could indicate self-aggregation of the Ca^{2+}-ATPase at the lower temperatures, resulting in an increasing contribution to A_3 from a relatively immobile population.

Comparison with Other Data. Previously, two studies of the rotation of the Ca^{2+}-ATPase in SR using saturation transfer EPR have been reported. Thomas & Hidalgo (1978) found that the correlation time τ_2 varies from 60 to 20 μs over the range 4–20 °C, while Kirino et al. (1978) obtained values of 800–200 μs over the same temperature range. Both groups agree that there is a change in temperature dependence at about 15–20 °C, with τ_2 becoming relatively temperature insensitive in the region of 20–37 °C. It should be noted that the above values are calculated on the basis of calibration spectra for isotropic rotation, for which $\tau_2 = 1/6D_R$ (D_R is the rotational diffusion coefficient for isotropic rotation). They cannot therefore be compared directly with the data from transient dichroism measurements which take into account the expected anisotropic rotation of the protein. More importantly in the present case, the flash photolysis data indicate that the motion of the ATPase is complex, with segmental motion superimposed on the rotation of the protein in the membrane. Line shape analysis of EPR spectra is unlikely to resolve these different motions, so that the reported correlation times may represent an average of different motions present in the sample. (Note that the further complication of independent probe motion appears not to occur with the maleimide spin-labels used in the EPR experiments.)

During the completion of this project, Hoffmann et al. (1979) independently reported transient dichroism studies with eosin isothiocyanate labeled SR. It is interesting to note the initial anisotropy in their experiments (see their Figure 3) also appears to be temperature dependent, although they interpret this finding as due to the trivial effect of variation in laser intensity. Over the time range of 10–300 μs their $r(t)$ curves exhibit more pronounced decays than ours and appear to be approximately monoexponential. The plateau region which they observe in Arrhenius plots of the decay time is not apparent in our data (see Figure 5, upper plot).

From NMR spectra of bound nucleotides, Manuk & Sykes (1977) previously deduced the existence of a relatively fast motion ($\tau_2 < 0.3$ μs) in the Ca^{2+}-ATPase which they interpreted as rotation of the protein in the membrane. Such a fast motion seems improbable for the whole protein but would be compatible with the segmental motion indicated by the present investigations.

Very recently, P. Garland and D. Boxer (private communication) have labeled SR with erythrosin isothiocyanate and studied rotational motion of the Ca^{2+}-ATPase by using time-resolved phosphorescence depolarization. At times longer than 20 μs, their data are generally in good agreement with those presented here. Furthermore, they observe a fast decay of the anisotropy which occurs within a few microseconds. The contribution of the fast decay decreases with decreasing temperature. Thus, these measurements provide direct confirmation of the fast motion inferred from the present experiments.

Rotational Motion and Function. A change in slope occurring between 15 and 25 °C in the Arrhenius plot of the Ca^{2+}-ATPase activity in SR has frequently been reported (Inesi et al., 1973; Lee et al., 1974; Hidalgo et al., 1976; Moore et al., 1978; Dean & Tanford, 1978; Anzai et al., 1978; Madden & Quinn, 1979; Hoffmann et al., 1979). In some cases, the break has been assigned to an effect of the lipids (Inesi et al., 1973; Lee et al., 1974; Hidalgo et al., 1976). However, the occurrence of a similar break in lipid-depleted ATPase and in membranes reconstituted with different lipids indicates that a conformational change of the protein is more likely to be responsible (Dean & Tanford, 1978; Anzai et al., 1978).

In recent studies, it has been proposed that a change in the temperature dependence of rotational mobility of the ATPase accompanies the change in temperature dependence of the activity occurring between 15 and 25 °C (Thomas & Hidalgo, 1978; Hoffmann et al., 1979). It is, however, not immediately obvious why rotation of the whole protein about the membrane normal should be related to activity. A major conclusion of the present study is that segmental motion occurs in the Ca^{2+}-ATPase. The Arrhenius plot of the parameter r_{20}, which is sensitive to this motion, shows a change of slope at 13 °C. We do not place undue emphasis on this finding, since the factors which determine r_{20} are complex. It is possible that r_{20} and parameters observed in other rotational studies reflect a subtle combination of contributions from segmental motion and rotation of the whole protein. Until these different contributions can be separated, it is difficult to ascertain their functional significance. However, there can be little doubt that internal motions of the enzyme are in some way involved in the activity. The Ca^{2+}-ATPase appears to be the first membrane protein for which evidence of segmental motion has been obtained. It is possible that further studies of this motion will make an important contribution toward understanding the functional properties of this protein.

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References

Anzai, K., Kirino, Y., & Shimizu, H. (1978) *J. Biochem. (Tokyo) 84*, 815–821.

Austin, R. H., Chan, S. S., & Jovin, T. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5650–5654.
Azumi, T., & McGlynn, S. P. (1962) *J. Chem. Phys. 37*, 2413–2420.
Bensadoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241–250.
Bürkli, A., & Cherry, R. J. (1978) *Experientia 34*, 907.
Chen, P. S., Toribara, T. V., & Huber, W. (1956) *Anal. Chem. 28*, 1756–1758.
Cherry, R. J. (1978) *Methods Enzymol. 54*, 47–61.
Cherry, R. J. (1979) *Biochim. Biophys. Acta 559*, 289–327.
Cherry, R. J., & Schneider, G. (1976) *Biochemistry 15*, 3657–3661.
Cherry, R. J., Bürkli, A., Busslinger, M., Schneider, G., & Parish, G. R. (1976) *Nature (London) 263*, 389–393.
Cherry, R. J., Müller, U., & Schneider, G. (1977) *FEBS Lett. 80*, 465–469.
Cone, R. A. (1972) *Nature (London), New Biol. 236*, 39–43.
Dean, W. L., & Tanford, C. (1978) *Biochemistry 17*, 1683–1690.
Dutton, A., Rees, E. D., & Singer, S. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1532–1536.
Fleming, G. R., Morris, J. M., & Robinson, G. W. (1976) *Chem. Phys. 17*, 91–100.
Folch, J., Lees, M., & Stanley, G. H. S. (1957) *J. Biol. Chem. 226*, 497–509.
Garland, P. B., & Moore, C. H. (1979) *Biochem. J. 183*, 561–572.
Greinert, R., Stärk, H., & Weller, A. (1979) *J. Biochem. Biophys. Methods 1*, 77–83.
Heyn, M. P. (1979) *FEBS Lett. 108*, 359–364.
Hidalgo, C., & Thomas, D. D. (1977) *Biochem. Biophys. Res. Commun. 78*, 1175–1182.
Hidalgo, C., Ikemoto, N., & Gergely, J. (1976) *J. Biol. Chem. 251*, 4224–4232.
Hoffmann, W., Sarzala, M. G., & Chapman, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3860–3864.
Hyde, J. S. (1978) *Methods Enzymol. 49*, 480–511.
Inesi, G., Millman, M., & Eletr, S. (1973) *J. Mol. Biol. 81*, 483–504.
Kinosita, K., Jr., Kawato, S., & Ikegami, A. (1977) *Biophys. J. 20*, 289–305.
Kirino, Y., Ohkuma, T., & Shimizu, H. (1978) *J. Biochem. (Tokyo) 84*, 111–115.
Kondo, M., & Kasai, M. (1974) *Photochem. Photobiol. 19*, 35–41.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lavalette, D., Amand, B., & Pochon, F. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1407–1411.
Lee, A. G., Birdsall, N. J. M., Metcalfe, J. C., Toon, P. A., & Warren, G. B. (1974) *Biochemistry 13*, 3699–3705.
Maclennan, D. H., & Holland, P. C. (1975) *Annu. Rev. Biophys. Bioeng. 4*, 377–404.
Madden, T. D., & Quinn, P. J. (1979) *FEBS Lett. 107*, 110–112.
Manuck, B. A., & Sykes, B. D. (1977) *Can. J. Biochem. 55*, 587–596.
Martonosi, A., & Fortier, F. (1974) *Biochem. Biophys. Res. Commun. 60*, 382–389.
Meissner, G. (1975) *Biochim. Biophys. Acta 389*, 51–68.
Moore, B. M., Lentz, B. R., & Meissner, G. (1978) *Biochemistry 17*, 5248–5255.
Moore, C., Boxer, D., & Garland, P. (1979) *FEBS Lett. 108*, 161–166.
Nigg, E. A., & Cherry, R. J. (1979a) *Nature (London) 277*, 493–494.
Nigg, E. A., & Cherry, R. J. (1979b) *Biochemistry 18*, 3457–3465.
Nigg, E. A., & Cherry, R. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4702–4706.
Nigg, E. A., Bron, C., Girardet, M., & Cherry, R. J. (1980) *Biochemistry 19*, 1887–1893.
Rigler, R., & Ehrenberg, M. (1973) *Q. Rev. Biophys. 6*, 139–199.
Saito, A., Wang, C.-T., & Fleischer, S. (1978) *J. Cell Biol. 79*, 601–616.
Scarpa, A. (1979) *Methods Enzymol. 56*, 301–338.
Shamoo, A. E., & Goldstein, D. A. (1977) *Biochim. Biophys. Acta 472*, 13–53.
Stewart, P. S., & Maclennan, D. H. (1974) *J. Biol. Chem. 249*, 985–993.
Tada, M., Yamamoto, T., & Tonomura, Y. (1978) *Physiol. Rev. 58*, 1–79.
Thomas, D. D. (1978) *Biophys. J. 24*, 439–462.
Thomas, D. D., & Hidalgo, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5488–5492.
Wang, C.-T., Saito, A., & Fleischer, S. (1979) *J. Biol. Chem. 254*, 9209–9219.
Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 622–626.
Vaz, W. C., Austin, R. H., & Vogel, H. (1979) *Biophys. J. 26*, 415–426.