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LIBRATIONAL MOTIONS OF MEMBRANE-EMBEDDED Ca^{2+}-ATPase OF SARCOPLASMIC RETICULUM LABELED WITH FLUORESCEIN ISOTHIOCYANATE

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Continuing our investigation of the relationships between internal motions and functional properties of soluble and membrane-bound proteins we have explored the lifetimes and correlation times associated with the fluorescence emission of fluorescein-labeled Ca^{2+}-dependent ATPase of sarcoplasmic reticulum. The emission was characterized by two lifetime components near 1.8 and 4.1 ns, probably due to exposure of the probe to environments of different polarities. The time-dependent anisotropy showed the presence of two correlation times near 0.8 and 6.6 ns. The shorter correlation time was due to motions of the probe around its point of attachment on the surface of the protein. The longer correlation time indicated the presence of internal motions of the protein. Both lifetimes and correlation times were insensitive to temperature between 2 and 10 °C. They were also insensitive to addition and removal of 100 μM free Ca^{2+}

1. Introduction

Allosteric properties of proteins are consistent with internal motions which should produce in the system the appearance of correlation times shorter than those expected for the rotational diffusion of the entire molecule as a rigid unit. In this laboratory we were able to detect short correlation times, in the nanosecond region, in hemoglobin and in band 3 of human erythrocytes. In both cases these internal motions were dependent on the presence or absence of ligands specific for the proteins, suggesting their involvement in the allosteric transitions of the systems [1–3]. Continuing this line of investigation, we chose to explore the behavior of sarcoplasmic reticulum (SR) ATPase.

This enzyme is one of the most extensively studied ion-transport systems. The protein has been recognized as an allosteric system whose conformational changes regulate cooperative binding of ligands [4,5]. The structural mechanism of transport is still the object of hypothesis and speculations, and the only accepted notion is that transport does not occur because the protein rotates through the membrane so as to shuttle physically the ion from one side to the other of the vesicles. This immediately implies that internal motions of the protein must direct the transport, coupling it to the hydrolysis of ATP.

The rotational diffusion of SR ATPase labeled with eosin isothiocyanate has been investigated in natural and reconstituted systems in the laboratories of Cherry [6] and Hoffman [7]. Their experimental approach could monitor motions occurring in the microsecond range, therefore only rotations of the entire protein and of the vesicles could be followed. Consistent with our predictions, Cherry and Burkli [6] noticed that the initial dichroism of

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their samples was low, as if motions were present in the system at times much shorter than 1 μs.

We have investigated the time-resolved anisotropy decay of the fluorescence emission of SR vesicles labeled with fluorescein isothiocyanate (FITC), which Pick et al. [8,9] have shown to be a probe specific for ATPase which binds at or very near the ATP-binding site. Our results indicate the presence in the protein of correlation times in the nanosecond region consistent with the existence in the molecule of internal domains capable of independent motions. Addition of Ca^{2+} did not modify the time-resolved emission characteristics of the labeled vesicles.

2. Materials and methods

SR vesicles were prepared from rabbit skeletal muscles following the procedure of Eletr and Inesi [10]. They were labeled with FITC as described by Pick and Karlish [8]. To 1.9 ml of vesicles containing 14 mg protein in 0.05 M Tris-HCl, 0.05 M glycine, 100 μM EGTA, and 0.2 M sucrose at pH 9.0, 0.1 ml were added of a 0.35 mg/ml solution of FITC in dimethyl formamide. The samples were left in the dark at room temperature with gentle stirring for 30 min. To eliminate the excess FITC the vesicles were filtered through a 1 × 10 cm Sephadex G-50 column equilibrated with the same buffer at pH 7.5. When the labeled vesicles appeared in the eluate the first 2 ml were collected and refiltered in the same way through a second column. Again the first 2 ml of eluate containing labeled vesicles were selected and used for the subsequent measurements. Using an SLM 3000 spectrofluorometer, the samples showed the same emission spectra as described by Pick and Karlish [8], addition of 100 μM free Ca^{2+} decreased the maximum of emission intensity by 6%, and SDS electrophoresis indicated that the label was confined to one band corresponding to the Ca^{2+}-dependent ATPase.

For blank subtractions, labeled vesicles were treated in the same way as the labeled ones, only FITC was not present in the added dimethyl formamide.

The lifetimes and correlation times associated with the emission of the label were measured with a nanosecond PRA pulse fluorometer. Blanks were prepared by matching the absorbance of suspensions of labeled and unlabeled vesicles at 480 nm. Vertically polarized light at 480 nm was used in excitation and the emission was monitored at 535 nm. All measurements were performed using front face techniques.

Lifetimes and correlation times were estimated following described procedures, based on nonlinear least-squares deconvoluting algorithms [3]. The fits of experimental and simulated data gave average residuals of ±2–4% of the experimental values. The reduced χ^2 were 1.2 ± 0.5.

Protein concentration was measured by the method of Lowry et al. [11], using standards of serum albumin.

The amount of free Ca^{2+} in the EGTA/Ca^{2+} buffer system was estimated with the procedure of Fabiato and Fabiato [12], using the equilibrium constants of Schwartzenbach et al. [13].

The semiangle θ of the apex of the cone of rotation of the probe was estimated from the initial (A_0) and final (A_∞) anisotropy of the samples from

$$\frac{A_\infty}{A_0} = \frac{1}{4}(3\cos^2\theta - 1)^2 \qquad (1)$$

as described for the hindered rotations of membrane-embedded band 3 of human erythrocytes [5], where A_0 is the initial detectable anisotropy of the system and A_∞ the final anisotropy.

Results

Table 1 shows that the time-dependent emission of the labeled vesicles could be resolved into

Table 1

Lifetimes of FITC-labeled SR vesicles

Measurements were performed in the presence and absence of 100 μM free Ca^{2+} at 2, 6 and 10 °C. No significant difference was found at the various temperatures and the data here reported are averages of all measurements.

$[Ca^{2+}]$ (μM)	α_1	τ_1 (ns)	α_2	τ_2 (ns)
0	0.36 ± 0.07	1.85 ± 0.27	0.64 ± 0.15	4.11 ± 0.23
100	0.33 ± 0.11	1.74 ± 0.42	0.67 ± 0.16	4.05 ± 0.16

Table 2

Correlation times of FITC-labeled SR vesicles

Measurements were performed in the presence and absence of 100 μM free Ca^{2+}, at 2, 6 and 10°C. No significant difference was found at the various temperatures, and the data here reported are averages of all measurements. f_i is the fractional depolarization corresponding to the correlation time σ_i. A_0 and A_∞ are the initial and final detectable anisotropies, respectively.

$[Ca^{2+}]$ (μM)	f_1	σ_1 (ns)	f_2	σ_2 (ns)	A_0	A_∞
0	0.52 ± 0.05	0.75 ± 0.28	0.48 ± 0.03	6.60 ± 0.35	0.32 ± 0.01	0.07 ± 0.01
100	0.53 ± 0.10	0.83 ± 0.34	0.47 ± 0.03	6.69 ± 0.57	0.32 ± 0.01	0.07 ± 0.02

at least two components with similar amplitudes. They were insensitive within our errors, to addition or removal of 100 μM free Ca^{2+}. They were also insensitive to temperature between 2 and 10°C. Table 2 shows the presence of at least two correlation times associated with the emission of the label, one below 1 ns, the other near 6.6 ns. Also in this case they were independent of Ca^{2+} and temperature.

Table 2 also shows that the final anisotropy failed to reach a zero value, as expected from hindered rotations. From the value of the initial and final anisotropies, assuming that the rotation was only around a single axis perpendicular to the plane of the membrane, the semiapex angle of the cone of rotation was estimated from eq. 1 to be 36.6°.

All experiments were performed in 0.05 M Tris-HCl, 0.05 M glycine, 100 μM EGTA, 0.2 M sucrose at pH 7.5. Three different preparations were used in the various experiments. Average values are reported in the tables.

4. Discussion

FITC-labeled SR vesicles showed an emission characterized by the presence of at least two lifetime components. The lifetime near 4.1 ns was that expected from fluorescein in water. The shorter lifetime component was consistent with exposure of the label to a hydrophobic environment. This could be explained either by the presence of a second binding site for FITC on the SR ATPase, or by different positions of the label on the same site on the surface of the protein. The data reported by Pick and Karlish [8] and Pick and Bassilian [9] seem to exclude the presence of a second binding site for FITC on the enzyme. It is likely that the lifetime heterogeneity was produced by different positions of the probe, moving around its point of attachment to the protein and going through environments of different polarity in times longer than its lifetime. The presence in the system of a lipid/water interface is consistent with this proposition.

It is also conceivable that charge-transfer complexes were formed by the interaction of fluorescein in the excited state with tryptophanyl or tyrosyl residues, similar to the findings of Cathou and Bunting [14] for the quenching of fluorescein in solutions of these amino acids. FITC is known to react with Lys 515 of SR ATPase [15], and the nearest tryptophanyl residue in the sequences is at position 552. In the absence of detailed information with regard to the tertiary structure of the protein, the quenching hypothesis appears improbable.

As reported by Pick and Karlish [8] and confirmed by us, at room temperature addition of free Ca^{2+} decreased the fluorescence intensity of FITC-labeled SR vesicles by 5–8%. Table 1 does not indicate a decrease of the lifetimes in the presence of Ca^{2+}, which would go beyond their standard deviations.

It is possible that a small decrease of intensity distributed between two lifetimes would not be detectable above the noise of our measurements. It is also possible that part of the observed steady-state intensity decrease was due not to quenching of the probes, but to modifications of the optical properties of the samples.

With regard to correlation times, the short components, below 1 ns, could be easily referred to

motions of the probe around its point of attachment to the protein. The longer correlation times, near 6.6 ns, indicated the presence of internal motions in the protein molecule. In fact, the minimum correlation time expected for a spherical protein of the size of ATPase (110 kDa) is in excess of 100 ns in water at 5°C. The viscosity of the lipids surrounding the protein in the membrane should make this relaxation even longer.

Conformational changes in SR ATPase upon addition of Ca^{2+} have been proven in a number of ways [4,5,16]. Thus, it was disappointing that the correlation times of the system were not sensitive to the presence or absence of free Ca^{2+}. It is conceivable that the insertion of FITC near the binding site of ATP could freeze the system in a particular conformation, and it is known that low temperatures stabilize the E_1 form of the enzyme [17]. It is also possible that the conformational changes produced by Ca^{2+} binding did not interfere with the internal motions of the protein, as detected by the FITC probe.

The limiting anisotropy of the system appeared to be different from zero, as expected from hindered rotations [18]. This confirms again that membrane proteins cannot tumble inside the membrane on the nanosecond time scale, and that their rotation is probably confined around a single axis perpendicular to the plane of the membrane. The semiangle of the apex of the cone of rotation was 36.6°, which suggests a high degree of freedom of these internal motions. Similarly, a high degree of freedom was also found in the librational motions of membrane-embedded band 3 of human erythrocytes [5].

Both lifetimes and correlation times were insensitive to temperature between 2 and 10°C. Unfortunately we could not explore higher temperatures, because the several hours needed for data collection would have resulted in some denaturation of the samples. The temperature independence indicates that the fluorophore was somewhat shielded from the solvent and suggests that the detectable motions were confined to an internal region of the protein, not exposed to the bulk viscosity of the solvent.

It should be stressed that a similar temperature independence of lifetimes and correlation times of extrinsic fluorophores has been reported for band 3 of human erythrocytes [5]. It is possible that this is a general characteristic of membrane-embedded systems, in which the internal dynamics of proteins is controlled by the lipid components.

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