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SOLVENT PERTURBATION EVIDENCE FOR A TWO-STATE SYSTEM REGULATED BY CALCIUM IN SARCOPLASMIC RETICULUM ATPase

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Perturbation of sarcoplasmic reticulum ATPase with the nonionic detergent $C_{12}E_8$ is modulated by the amount of free Ca^{2+} present in the solvent prior to the addition of detergent. CD measurements show that the enzyme exists in solution in two different conformations that react differently with the detergent. They probably represent the free enzyme, and its complex with Ca^{2+}. On this assumption, titrations with increasing amounts of Ca^{2+} produced data superimposable on curves obtained measuring Ca^{2+} bound to sarcoplasmic reticulum vesicles.

1. Introduction

It is an accepted proposition that the functional characteristics of Ca^{2+}-dependent ATPase of skeletal muscle SR results from conformational changes elicited by the binding of ligands [1,2]. Direct evidence on the effects of Ca^{2+} on the enzyme conformation has been recently obtained by Dupont and Leight [3] using fluorescence spectroscopy.

Solvent perturbation techniques can be used for exploring conformational states in proteins. In SR vesicles a potential useful perturbant is $C_{12}E_8$ which solubilizes the vesicles, therefore improving the quality of data for optical measurements. Also, this detergent is known to produce CD spectra in the far-ultraviolet region, which are different in the presence and absence of Ca^{2+} [4], suggesting that it is able to distinguish between the free enzyme and its complex with Ca^{2+}.

The results presented here indicate that the kinetics of interaction of SR vesicles with $C_{12}E_8$ is biphasic, due to the presence of at least two components reacting with the detergent at different rates. If the slow reacting species is taken as the fraction of enzyme saturated with Ca^{2+}, titrations are produced which are very similar to those obtained measuring the amount of Ca^{2+} bound to SR vesicles [5].

Abbreviations: SR, sarcoplasmic reticulum; SR ATPase, (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum; CD, circular dichroism; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $C_{12}E_8$, dodecyloctaethylene glycol monoether.

2. Materials and Methods

SR vesicles were prepared according to the method of Eletr and Inesi [6]. ATPase was purified as described by Meissner et al. [7]. They were suspended in 0.01 M Mops or Hepes buffers and 0.2 M sucrose. Various concentrations of free Ca^{2+} were obtained using Ca^{2+}/EGTA buffers whose composition was determined by the dissociation

equilibrium constants of Schwartzenbach et al. [8], as calculated by the procedure of Fabiato and Fabiato [9]. The total concentration of EGTA was kept at 100 μM.

Protein concentration was measured spectrophotometrically at 280 nm in 1% SDS using $\epsilon = 1.0$ for the vesicles and $\epsilon = 1.2$ for the purified ATPase.

Spectrophotometric measurements were conducted with a Cary 14 spectrophotometer.

Optical activity was measured with a Jasco-20 spectropolarimeter. All experiments were performed at room temperature.

3. Results

Fig. 1 shows the CD spectra obtained with vesicles equilibrated with 10^{-7} and 10^{-5} M free Ca^{2+}, respectively, and solubilized with 3 mg/ml $C_{12}E_8$. The spectra were analyzed with a curve-fitting program, standard in our laboratory [10], for

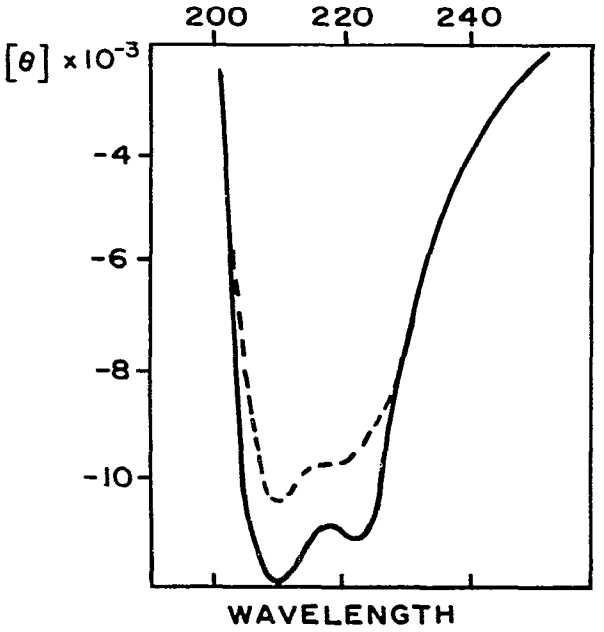

Fig. 1. Far-ultraviolet CD spectra of SR vesicles solubilized with 3 mg/ml $C_{12}E_8$ in the presence of 10^{-7} M Ca^{2+} (- - - - -) and of 10^{-5} M Ca^{2+} (———), in 100 mM Mops, 200 mM sucrose, 100 mM EGTA, at pH 7.0.

Table 1

Relative amounts of secondary structures computed from the parameter of Greenfield and Fasman [11] for SR vesicles saturated or not saturated with Ca

Conditions: 10 mM Mops (pH 7.0), 200 mM sucrose, 100 μM EGTA, 3 mg/ml $C_{12}E_8$.

Amount of free Ca^{2+} (M)	% α-helix	% β-conformation	% nonordered structure
10^{-5}	38.2	9.48	52.4
10^{-7}	29.8	20.7	49.5

estimating the relative proportions of α-helix, β-conformation and nonordered structures of the system. The results are listed in table 1. They suggest that Ca^{2+} modulates the secondary structure of the system, producing a 10% interconversion of α-helix and β-conformation, without modifying the total amount of nonordered structure. Similar values were obtained at pH 8.0 and 6.3. Also, similar results were obtained using purified ATPase, proving that the enzyme is responsible for the CD changes.

The reaction of detergent with vesicles was followed by recording the ellipticity at 222 nm for 30 min. As shown in fig. 2, the monitoring was started 30 s after addition of $C_{12}E_8$. Complete clarification of the samples occurred in less than 5 s, therefore the tracings monitored changes which were independent of turbidity, and represented the kinetics of the loss of ellipticity of SR ATPase

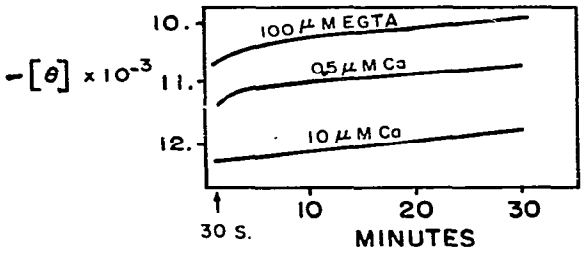

Fig. 2. Variation with time of the ellipticity of SR vesicles after addition of $C_{12}E_8$. The dead time of the reaction was 30 s. Final concentration of $C_{12}E_8$ was 15 mg/ml; in 10 mM Mops, 200 mM sucrose, at pH 7.0; protein concentration, 0.154 mg/ml.

produced by the interaction of the vesicles with $C_{12}E_8$. The reaction of the detergent with the vesicles appeared to be dependent on the amount of free Ca^{2+} present in the system. In the presence of 10 μM Ca^{2+}, at pH 7.0, the vesicles were completely saturated and the reaction was monophasic, as indicated by the slow decrease in ellipticity during the entire period of observation. In the presence of 0.5 μM Ca^{2+}, the vesicles were partially saturated and a component reacting at a higher rate with the detergent was present, as indicated by the sudden decrease in ellipticity during the first 1–2 min of the reaction. In the absence of Ca^{2+} (100 μM EGTA), the amount of the fast reacting species increased to a maximum. This would indicate that the ATPase in the SR is distributed in at least two different conformations which interact with the detergent at different rates. This distribution seems to be controlled by the amount of free Ca^{2+} present. The initial fast reaction of the vesicles with the detergent could not be analyzed in detail, because too much of it was masked by the dead time of the experiment (30 s). The amount of the enzyme present in the slowly reacting conformation 'y' was evaluated by monitoring the ellipticity at 222 nm, 4 min after the addition of the detergent, using

$$y = \frac{\theta_{obs} - \theta_{EGTA}}{\theta_{Ca} - \theta_{EGTA}} \qquad (1)$$

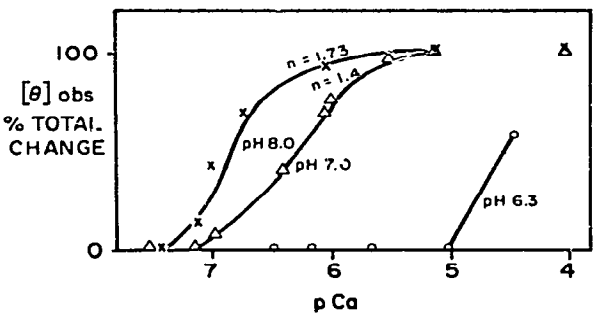

Fig. 3. Perturbation of SR vesicles by $C_{12}E_8$ in the presence of different amounts of Ca^{2+} at various pH values. The quantity y was evaluated from measurements of ellipticity 4 min after addition of detergent, using eq. 1, and represents the fraction of the slow reacting species. In 10 mM Hepes (pH 8.0), Mops (pH 7.0), Mes (pH 6.3), 200 mM sucrose, 100 μM EGTA. The amounts of free Ca^{2+} produced by addition of $CaCl_2$ were estimated by the method of Fabiato and Fabiato [9].

where θ_{EGTA} is the ellipticity in the presence of excess EGTA and θ_{Ca} that in the presence of excess Ca^{2+}.

The resulting titrations are shown in fig. 3. They resemble those obtained by Watanabe et al. [5] using chromatographic procedures for measuring the amount of Ca^{2+} bound to SR vesicles. The pH dependence was very similar and Hill plots at pH 7.0 and 8.0 gave n values of 1.4 and 1.73, respectively, consistent with the expected binding cooperativity of the system [3].

4. Discussion

As shown in table 1 and fig. 1, solubilization of SR vesicles with $C_{12}E_8$ produced a partial denaturation of ATPase, which was inhibited by the presence of Ca^{2+}. Our experiments show that the kinetics of the denaturation produced by the detergent was biphasic, indicating the presence of at least two components, representing two different conformational states of the protein.

One component reacted so fast that its denaturation was almost complete within the first 30 s of the reaction, so that the ellipticity at 4 min after addition of $C_{12}E_8$ could be taken as the measurement of the fraction of protein in the slow reacting conformation which was proportional to the amount of free Ca^{2+} present in the system.

It is well known that the enzymatic activity of SR ATPase is calcium dependent. Chaloub et al. [13] proposed a model in which a conformational switch of the system is produced by Ca^{2+}. Much experimental evidence based on fluorescence spectroscopy, SH-group reactivity, calcium-binding cooperativity and ESR spectroscopy [3,5,13–16] supports this hypothesis.

Our findings are consistent with the presence of a two-state system. Binding of Ca^{2+} changes the conformation of the enzyme into a form more resistant to denaturation by $C_{12}E_8$.

This interpretation is also supported by data of Møller et al. [12] which show that the presence of Ca^{2+} preserves the enzymatic activity of ATPase exposed to $C_{12}E_8$.

This assumption is strengthened by the observation that titration of the slow reacting species with

increasing amount of Ca^{2+} gave results very similar to calcium-binding curves measured directly, by chromatographic procedures [5].

The following model can be proposed

$$E^*Ca \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \xrightarrow{k_2} ED \qquad (2)$$

where E*Ca is the Ca^{2+}-enzyme complex with the enzyme in the conformation resistant to denaturation, E the free enzyme and ED the enzyme denatured by the detergent. As expected from the high affinity of the enzyme for Ca^{2+}, we have obtained $k_1 \ll k_{-1}$. We found our data can be explained assuming $k_2 \gg k_1$, so that the relative amounts of E*Ca in the system could be measured monitoring ellipticities 4 min after addition of $C_{12}E_8$.

The model proposed in eq. 2 is probably an oversimplification which does not take into consideration multiple binding sites for Ca^{2+} and possible additional conformational components of the system.

Better models will be formulated when detailed analyses of the kinetics of the interaction with detergent become possible, starting at times shorter than 30 s. With this goal in mind we are now implementing stopped-flow equipment in our spectropolarimeter.

It should be stressed that even in the absence of rapid mixing devices measurements of ellipticity at a fixed wavelength can be used for monitoring bind of Ca^{2+} to SR vesicles.

Our data support the view that solvent perturbation techniques may be very useful for exploring the conformational states of membrane-bound proteins.

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