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Sarcoplasmic reticulum calcium ATPase

Labeling of a putative Mg^{2+} site by reaction with a carbodiimide and a spin-label

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The sarcoplasmic reticulum Ca^{2+}-ATPase loses hydrolytic activity and the ability to be phosphorylated by P_i following incubation with EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide]. 4 nmol of tempamine per mg SR protein can be coupled to either a glu or an asp side chain through the EDC reaction. Mg^{2+} protects against loss of activity and tempamine labeling with a mid-point of about 3 mM in the absence of Ca^{2+}. This is similar to the K_d for a Mg^{2+} that serves as a cofactor in enzyme phosphorylation. The Mg^{2+} protection constant is lowered by an order of magnitude when Ca^{2+} is bound to the transport sites. It is suggested that control of the Mg^{2+} binding site affinity may be part of the mechanism of enzyme activation by Ca^{2+}.

ATPase; Sarcoplasmic reticulum; Carbodiimide; Spin-labeling; Magnesium site; Ion pump

1. INTRODUCTION

Relaxation of striated muscle cells requires removal of calcium ion from the cell's cytosol. An important part of this process is active transport of the ion into the lumen of SR membranes. This calcium pumping is carried out by a calcium ATPase, the membrane's principal intrinsic protein (for recent reviews, see [1–3]). The enzyme couples transport of two calciums to hydrolysis of one ATP, and undergoes phosphorylation (EP formation) during the transport cycle. In addition to the sites involved in binding of transported calcium, the CaATPase has at least one other metal site to which magnesium binds as a cofactor in EP formation from ATP or P_i [4–7]. Magnesium is also required for binding such putative P_i analogs as orthovanadate [8,9] and fluoride [10,11]; in the latter case, one magnesium becomes trapped on the CaATPase as the stable fluoride complex forms. Studies utilizing Cr(III)ATP as a substitution-inert substrate analog suggest that magnesium binds to the enzyme as a complex with ATP [12–14], and that the rate of this binding is increased when Ca^{2+} is bound to the transport sites [14].

Additional information on the magnesium site's location would be facilitated by the development of covalent labels. Since metal sites generally contain carboxyl groups, reagents that react with this group (e.g. carbodiimides) are likely candidates, particularly if they include a cationic group to direct the reagent to the negatively charged site. During a study of the affinity labeling behavior toward ATPases of an adduct of EDC and ATP [15–16], control experiments using EDC (a cationic group-containing carbodiimide) itself implied it inactivated the enzyme in a magnesium-protectable manner. These results, along with the fruitful use of EDC as a carboxyl group modifier of another ion pump [17], and the successful coupling of the spin-label tempamine to bacteriorhodopsin [18] prompted us to explore further the possibility of covalently labeling a magnesium site on the CaATPase. In this report we describe the use of EDC and tempamine toward this end.

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Abbreviations: SR, sarcoplasmic reticulum; EDC, 1-ethyl-3-(dimethylaminopropyl) carbodiimide; tempamine, 4-amino-tempo; MOPS, 3-(*N*-morpholino)propane sulfonate.

2. MATERIALS AND METHODS

SR vesicles were prepared from the white skeletal muscle of rabbit hind legs [19]. Vesicles were stored in a buffered sucrose medium (30% sucrose, 10 mM MOPS, pH 6.8) at 4°C and were used within 4–5 days of preparation. ATP, phosphoenolpyruvate, lactic dehydrogenase, pyruvate kinase, NADH, EDC, tempamine and ionophore A23187 were purchased from Sigma. $^{32}P_i$ was obtained from ICN.

Protein concentrations were obtained from the absorbance at 280 nm using an optical density of 1.05 cm^{-1} for 1 mg SR protein in 1 ml of 1% sodium dodecyl sulfate [20]. Ca-dependent ATPase activity was measured with a coupled enzyme assay system [21]. The amount of phosphoenzyme intermediate (EP) was determined by quenching an aliquot of the EDC reaction mixture into a phosphorylation medium containing 2 mM $^{32}P_i$, 10 mM $MgCl_2$, 2 mM EGTA, 16% Me_2SO, 15 μg/ml ionophore A23187, 100 mM MOPS, at pH 6.8 and 4°C, and worked up as described previously [16].

2.1. *EDC-tempamine labeling*

6 mg SR protein/ml was incubated at 37°C with 3 mM EDC in 100 mM MOPS, pH 6.8, 80 mM KCl and concentrations of $CaCl_2$, EGTA, and $MgCl_2$ as specified for given experiments. To measure the decrease in catalytic activity (Fig. 1), 2 μl aliquots were added directly to 1 ml of the coupled assay medium at the specified times. To couple tempamine to the ATPase, tempamine was added 1.5 min after the addition of EDC to give a final concentration of 9.6 mM. The degree of tempamine labeling (Fig. 4) was determined by diluting a 1 ml aliquot into 10 ml of cold buffer at a specified time and pelleting the SR by centrifugation. Excess tempamine was removed by repeating this process twice, at which point free tempamine was no longer apparent in the EPR spectrum. In addition, the EPR spectrum of the immobilized component not did change with repeated washings, indicating that the label generating this signal was covalently bound. The final pellet was resuspended in a small amount of buffer (approximately a 20 mg SR protein/ml) for EPR measurement. The spectrum (taken as the first derivative of the absorption) was integrated twice and the integral area was compared to a plot of integral area vs. known tempamine concentration. A Bruker ER-200D EPR spectrometer (X-band) interfaced with an IBM S-9001 computer was used with IBM EPR application software.

2.2. *Fitting procedures*

Kinetic constants and binding constants were estimated with computer programs that use a nonlinear regression algorithm [22] to fit exponential or hyperbolic functions. Uncertainties are given as the standard error of the estimate.

3. RESULTS

When EDC is added to a suspension of SR vesicles, the hydrolytic activity of the Ca^{2+}-ATPase is slowly lost. A time course for the reaction is shown in Fig. 1. As is also shown in the figure, millimolar concentrations of Mg^{2+} in the reaction medium decrease the rate of loss in activity. The data are adequately described as a first-order complete inactivation with a single k_{obs} at each Mg^{2+} concentration. In Fig. 2, the observed inactivation rate constants are plotted as a function of the Mg^{2+} concentration.

The presence of Ca^{2+} in a concentration range that would saturate the transport sites did not protect against the loss of activity (the rate of inactivation was monitored between 0.1 and 50 μM free Ca^{2+} (data not shown); 16 μM was present in the experiments shown in Fig. 1). However, if Ca^{2+} was not added to the incubation medium, which contained 2 mM EGTA, a higher concentration of Mg^{2+} was needed to protect against the loss in activity (Fig. 2). The K_d estimated for the inactivation in the presence of 16 μM free Ca^{2+} was an order of magnitude lower than that obtained without added Ca^{2+}, suggesting that saturation of the Ca^{2+} transport sites substantially increases the affinity of the EDC-reactive site for Mg^{2+}.

It is well established that at least one Mg^{2+} must bind to the catalytic site before the enzyme can be phosphorylated by P_i. Through this pathway the transport sites must be Ca^{2+} free for phosphorylation to occur and the K_d estimated in Fig. 2 from the data without the addition of Ca^{2+} is similar to the K_d for the site that supports phosphorylation with P_i (5.6 mM, [23]). In Fig. 3 we show that the ATPase loses the ability to be phosphorylated by P_i as the EDC reaction progresses. There was no added Mg^{2+} in the EDC reaction mixture, but 10 mM Mg^{2+} was in the phosphorylation medium, demonstrating that once the EDC reaction has taken place, subsequent additions of Mg^{2+} do not reverse the inhibition. Moreover, Ca^{2+} could be in the

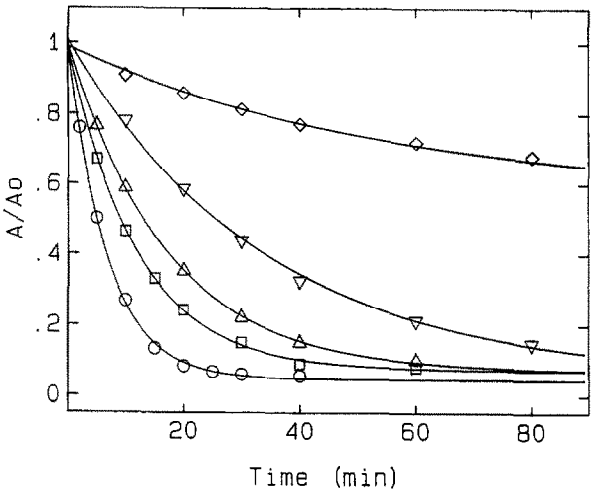

Fig. 1. Time course of inactivation of the SR Ca^{2+}-ATPase by EDC at various magnesium concentrations. SR vesicles (6 mg/ml) were incubated with 3 mM EDC (37°C, 2 mM EGTA, 1.8 mM $CaCl_2$, 100 mM MOPS, pH 6.8, 80 mM KCl, $MgCl_2$ as specified). At the indicated times aliquots were diluted into 1 ml of assay buffer for measurement of the Ca^{2+}-ATPase activity. The $MgCl_2$ in the incubation medium was: ○, no added Mg^{2+}; □, 0.334 mM; △, 1 mM; ▽, 2.5 mM; ◇, 7 mM. The lines are drawn using parameters obtained from computer fitting the data to a single exponential function. The hydrolytic activity at time zero (Ao) was 4.2 μM $mg^{-1} \cdot min^{-1}$.

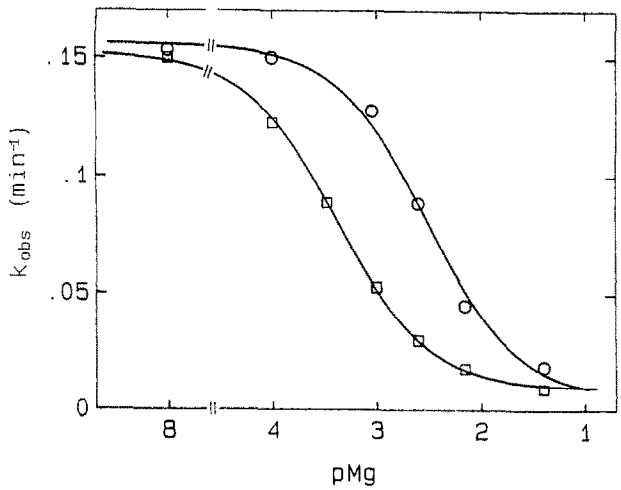

Fig. 2. Mg^{2+} concentration dependence of the rate constant of inactivation by EDC. Values of k_{obs} were obtained from fits to the time courses given in Fig. 1 (□) and from a set of experiments that were identical in all respects except that $CaCl_2$ was omitted (○). Lines are drawn using parameters obtained from computer fitting the data to a saturation function with respective K_d values of 0.42 mM (10 μM free Ca^{2+}) and 3.1 mM (no added Ca^{2+}).

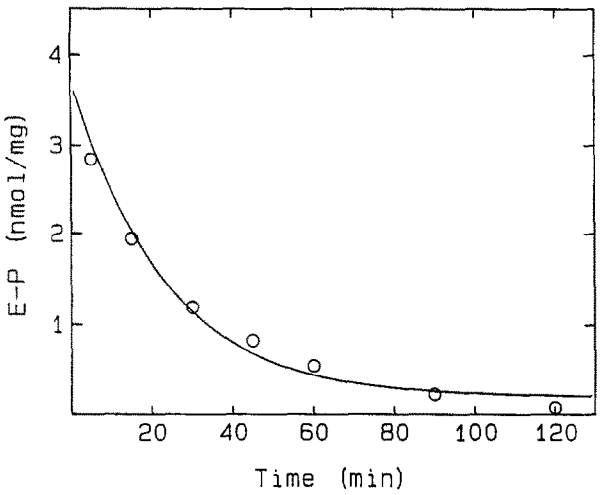

Fig. 3. Time courses of the ability to form E-P during incubation of SR Ca^{2+}-ATPase with EDC. Aliquots were taken from a EDC - SR reaction mixture containing no added Mg^{2+}, as described in Fig. 1, and diluted into phosphorylation medium at the specified times (it was assumed that the dilution factor and the excess Mg^{2+} and P_i quenched the EDC reaction).

EDC reaction mixture if it was subsequently chelated by EGTA in the phosphorylation medium, demonstrating that the modification done in the presence of Ca^{2+} also results in the inhibition of EP formation. EDC modification also inhibited EP formation from ATP (data not shown).

An advantage of using a carbodiimide to modify the enzyme is that an amine with a reporter group can be coupled to the modified residue and the stoichiometry of the labeling reaction can be followed. We added tempamine, a spin-label, to the reaction medium and as shown in Fig. 4a approximately 4 nmol of tempamine could be attached to one mg SR protein (an average of 4.0 ± 0.2 was obtained from 5 experiments). This is very close to a 1:1 ratio between the tempamine and the ATPase. The leveling of the time course implies that the reaction went to completion (i.e. that there was little partial labeling of slower reacting residues). As a control, time courses were obtained at higher tempamine concentrations (up to 50 mM) and there was no evidence of additional labeling. Further additions of EDC during the reaction had no effect on either the rate or the stoichiometry, indicating the reagent was not being consumed during the reaction. The EPR spectrum of the bound tempamine is given in Fig. 4b. The spectrum is that of a physically constrained label, which is typically observed with spin-labeled proteins. The spectrum did not vary with the degree of labeling.

Additions of millimolar Mg^{2+} inhibited the tempamine reaction (Fig. 4a) and the concentration dependence of the rate constant for tempamine incorporation (from the fit to the data given in the figure) was similar to that for the inactivation with a midpoint at about 0.3 mM Mg^{2+}. It should also be noted that there was no evidence of a population of residues that was not protected by the Mg^{2+}. At high Mg^{2+} concentrations, where the reaction rate is decreased by more than a factor of 10, an unprotected population would be quite apparent in the early part of the time course. 16 μM free Ca^{2+} was present in the experiments shown in Fig. 4; however, when Ca^{2+} was eliminated from the medium, the stoichiometry, the reaction rate, and the EPR spectrum were not significantly altered.

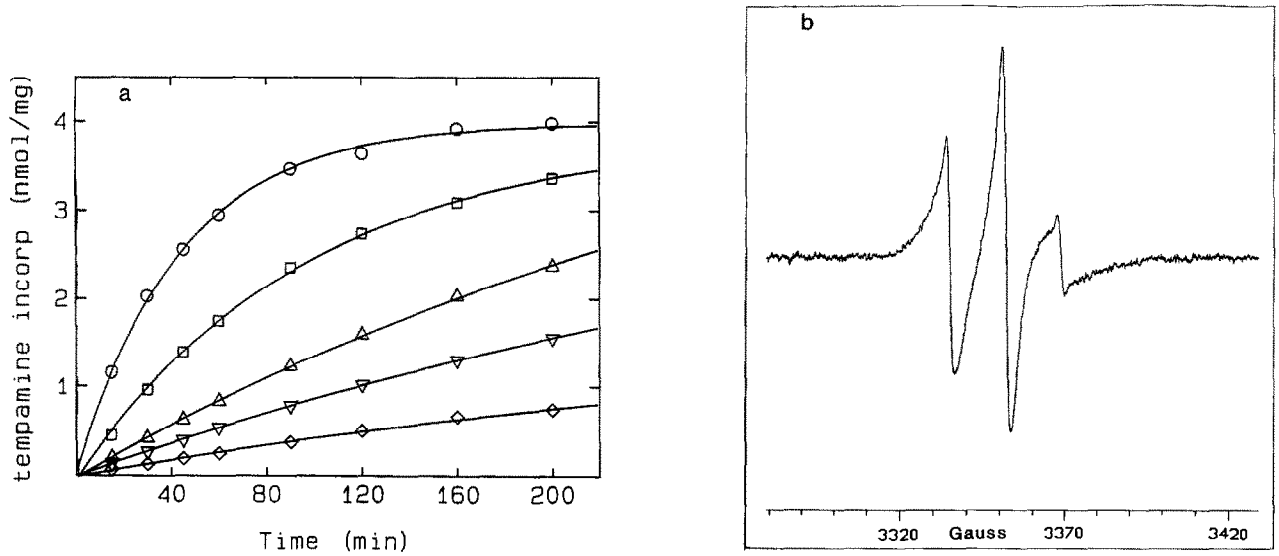

Fig. 4. Tempamine Labeling of the SR Ca^{2+}-ATPase. (a) Time course of the incorporation of tempamine into the SR Ca^{2+}-ATPase. Tempamine was added to an EDC–SR reaction mixture 1 min after the addition of EDC. Reaction conditions were as given in Fig. 1. Aliquots were taken at the specified times, and following extensive washing an EPR spectrum was obtained. Double integration of the spectrum provided the stoichiometry (see Section 2). The $MgCl_2$ in the incubation mixture was: ○, no added $MgCl_2$; □, 0.33 mM; △, 1 mM; ▽, 2.5 mM; ◇, 22 mM. Solid lines are drawn using parameters obtained from computer fitting the data to a single exponential function. (b) The EPR spectrum of tempamine-ATPase. Modulation frequency, 2 G; time constant, 0.16 s; sweep time 500 s; sweep width, 100 G.

4. DISCUSSION

There are two main conclusions that can be derived from the experiments presented here: the affinity of the enzyme for Mg^{2+} increases by an order of magnitude when Ca^{2+} binds to the transport sites, and a carboxyl residue at (or very near) the putative Mg^{2+} site is reactive toward EDC and can be selectively labeled with tempamine. There is no reason to assume that EDC reacts with only one residue; the *O*-acylisourea reaction intermediate can proceed through several pathways. However, it appears that only one residue preferentially reacts with the added tempamine (Fig. 4), and, as only a carboxylate group can proceed through the pathway that forms an amide, the reactive residue is likely to be either a glutamic or an aspartic acid.

In view of evidence for calcium site reactivity toward nonpolar carbodiimides [24–26], it is noteworthy that calcium bound to the transport sites conferred no protection from inactivation by EDC. This suggests that calcium site labeling is not significantly involved in reaction with the polar EDC.

Here, a correlation can be made between the loss of activity and tempamine labeling by the protective effect of Mg^{2+}. There is no evidence that more than one Mg^{2+} site is involved in the protection, and there is no residual population of residues that cannot be protected from either the inactivation or the spin-labeling. (The latter point is important in that a constrained spectrum can be generated when a spin label partitions into a membrane. The protection afforded by Mg^{2+} and the ability to endure extensive washing indicate that contributions from noncovalently bound label are minimal.) Thus, it would appear that the carboxyl residue that is labeled by tempamine is responsible for the loss of activity, and it is likely that this residue either comprises one of the Mg^{2+} ligands, or is sufficiently close to the Mg^{2+} site that cation binding inhibits the labeling.

There is considerable evidence that a Mg^{2+} binding site comprises part of a larger catalytic site [5,23,27,28] and that Mg^{2+} can bind independently, in a random mechanism, with either P_i [5,23] or ATP [28] to function as a cofactor for phosphorylation. There are no properties of Mg^{2+} that facilitate measurement of its binding, and most estimations of binding properties come from following the Mg^{2+} dependence of EP formation. For the most part P_i has been used because of complications from the hydrolytic activity of ATP, and this has precluded measurements with Ca^{2+} on the transport sites. A recent exception is the measurement of the concentration dependence of the Mg^{2+} requirement for reaction of an adduct of ATP and EDC with the Ca^{2+}-ATPase; the results showed a midpoint at 3 mM magnesium [16]. Another is the study by Reinstein and Jencks in which competition between Ca^{2+} and Mg^{2+} at the catalytic site was used to estimate cation binding constants with ATP as the phosphorylating substrate. Estimations of the K_d from the latter study, with and without Ca^{2+} on the transport sites, was similar to the those obtained from the data in Fig. 2 [28].

An advantage of the method used here is that it does not require the presence of a phosphorylating substrate, and provides a fairly direct means of measuring the Mg^{2+} affinity, irrespective of other ligands. In turn, the Ca^{2+} dependence of the Mg^{2+} K_d demonstrates that the two metal sites can communicate, independently of the substrate, although they are quite distal from each other. The stringent requirement of Ca^{2+} binding to the transport sites for the utilization of ATP is well established. Phosphorylation is a key step in this utilization. It would seem, therefore, that an increase in the affinity of the enzyme for Mg^{2+} may be part of the activation process.

REFERENCES

[1] Jencks, W.P. (1989) Methods Enzymol. 171, 145–164.
[2] MacLennan, D.H. (1990) Biophys. J. 58, 1355–1365.
[3] Inesi, G. and Kirtley, M.R. (1992) J. Bioenerg. Biomembr. 24, 271–283.
[4] Panet, R., Pick, U. and Selinger, Z. (1971) J. Biol. Chem. 246, 7349–7356.
[5] Punzengruber, C., Prager, R., Kolassa, N., Winkler, F. and Suko, J. (1978) Eur. J. Biochem. 92, 349–359.
[6] Champeil, P., Gingold, M.P., Guillain, F. and Inesi, G. (1983) J. Biol. Chem. 258, 4453–4459.
[7] Wakabayashi, S. and Shigekawa, M. (1984) J. Biol. Chem. 259, 4427–4436.
[8] Dupont, Y. and Bennett, N. (1982) FEBS Lett. 139, 237–240.
[9] Pick, U. (1982) J. Biol. Chem. 257, 6111–6119.
[10] Murphy, A.J. and Coll, R.J. (1992) J. Biol. Chem. 267, 5229–5235.
[11] Coll, R.J. and Murphy, A.J. (1992) J. Biol. Chem. 267, 21584–21587.
[12] Serpersu, E.H., Kirch, U. and Schoner, W. (1982) Eur. J. Biochem. 122, 347–354.
[13] Vilsen, B. and Andersen, J.P. (1987) Biochim. Biophys. Acta 898, 313–322.
[14] Chen, Z.D., Coan, C., Fielding, L. and Cassafer, G. (1991) J. Biol. Chem. 266, 12386–12394.
[15] Murphy, A.J. (1990) FEBS Lett. 263, 175–177.
[16] Murphy, A.J. (1990) Biochemistry 29, 11236–11242.
[17] Pedemonte, C.H. and Kaplan, J.H. (1986) J. Biol. Chem. 261, 16660–16665.
[18] Herz, J.M., Melhorn, R.J. and Packer, L. (1983) J. Biol. Chem. 258, 9899–9907.
[19] Eletr, S. and Inesi, G. (1972) Biochim. Biophys. Acta 282, 174–179.
[20] Thorley-Lawson, D.A. and Green, N.M. (1973) Eur. J. Biochem. 40, 403–413.
[21] Anderson, K.W. and Murphy, A.J. (1983) J. Biol. Chem. 258, 14276–14278.
[22] Bevington, P.R. (1969) Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York.
[23] Inesi, G., Lewis, D. and Murphy, A.J. (1984) J. Biol. Chem. 259, 996–1003.
[24] Pick, V. and Racker, E. (1979) Biochemistry 18, 108–113.
[25] Murphy, A.J. (1981) J. Biol. Chem. 256, 12046–12050.
[26] Scofano, H.M., Barribin, H. and Inesi, G. (1985) Biochemistry 24, 1025–1029.
[27] Highsmith, S. and Murphy, A.J. (1984) J. Biol. Chem. 259, 14651–14656.
[28] Reinstein, J. and Jencks, W.P. (1993) Biochemistry 32, 6632–6642.