AN ESTIMATE OF THE KINETICS OF CALCIUM BINDING AND DISSOCIATION OF THE SARCOPLASMIC RETICULUM TRANSPORT ATPase

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1. Introduction

The calcium transport protein in the sarcoplasmic reticulum membranes can be phosphorylated either by ATP or by inorganic phosphate [1,2–4]. Maximal phosphorylation by ATP takes place only when the high affinity calcium binding sites of the protein are saturated. Conversely, phosphorylation by inorganic phosphate is achieved only when these binding sites are depleted of calcium ions. A recent kinetic analysis of the incorporation of inorganic phosphate into the transport protein led to the conclusion that either calcium removal itself or a subsequent reaction step in the formation of the phosphate accepting protein conformation might be the rate determining step [5]. A decision between these alternatives could be made if it were possible to measure the dissociation rate of the calcium–protein complex. Since there is no way to determine the dissociation of the complex directly, an indirect procedure will be applied. The decay of the calcium–protein complex is monitored by determining the amount of phosphoprotein which is formed when a saturating concentration of ATP is added to the protein after it has reacted for different short periods with EGTA. ATP will transfer its terminal phosphate only to those transport molecules still combined with calcium ions. This method for monitoring the dissociation of the calcium complex would fail if the dissociation reaction would proceed rapidly leading to a calcium-free but still phosphate-accepting protein configuration. In this case, the decay of the phosphate accepting ability of the calcium free species would be measured. Yet, there exists no experimental support for the existence of a labile calcium-free state of the protein which can be phosphorylated by ATP. For the validity of the applied procedure it is important that the phosphorylation of the protein–calcium complex by ATP proceeds much more rapidly than the dissociation of the calcium–protein complex and other reactions leading to calcium losses. The results show that phosphoprotein formation proceeds sufficiently fast to establish a time course of calcium–protein decay. The rates of the slow decay are in good agreement with the rates found when phosphorylation by inorganic phosphate was started by calcium removal [5]. The slowly proceeding dissociation of calcium from the protein is taken as an indication for a multistep interaction between calcium and the transport protein.

2. Materials and methods

The vesicles of the sarcoplasmic membranes were prepared from rabbit skeletal muscles as in [6]. [γ-^{32}P]ATP was synthesized by the method in [7].

The rate of phosphoprotein formation was measured in a fast mixing device with three syringes and two mixing chambers. The apparatus was calibrated as in [5]. Calcium dissociation was monitored by mixing a vesicular suspension (1 mg/ml) containing 0.1 mM calcium with 20 mM EGTA. After 22 ms and 88 ms, 0.1 mM [γ-^{32}P]ATP and 2.0 mM $MgCl_2$ were added from the third syringe. Phosphorylation was terminated by 10% TCA in a test tube at

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Abbreviation: EGTA, Ethyleneglycol-2-(2-aminoethyl)-tetraacetic acid; TCA, trichloroacetic acid

the outlet of the mixing apparatus 28 ms and 112 ms, respectively, after starting phosphorylation.

To estimate the association rate of calcium, the protein was phosphorylated by ATP. Phosphorylation was started either by the addition of 0.2 mM [γ-^{32}P]-ATP, 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.1 mM EGTA to the vesicular protein (1.0 mg/ml) suspended in a solution containing the same components minus ATP or, vice versa, by adding 0.2 mM $CaCl_2$, 0.1 mM [γ-^{32}P]ATP, 1.0 mM $MgCl_2$ and 0.1 mM EGTA to the protein suspension with the same contents but without calcium. The reaction was quenched by ice-cold 10% TCA after various reaction times. The concentration of ionized calcium was calculated using the stability constant in [8]. The experiments were performed, at pH 6.0, using 40 mM Tris-maleate as buffer.

3. Results and discussion

Figure 1 illustrates that appreciable amounts of protein are phosphorylated by ATP when phosphorylation is started 22 ms and 88 ms after EGTA has been added to the calcium–protein to cause its dissociation at 30°C. At 20°C the dissociation of the calcium complex proceeds even more slowly so that phosphoprotein formation by ATP reaches ~ 80% and ~ 60%, respectively, of the values observed when no EGTA was added. The absolute values of the observed phosphoprotein amounts and the corresponding apparent dissociation rates are collected in table 1 together with the rates of inorganic phosphate incorporation previously observed. All reactions interfering with calcium–protein decay such as calcium transport tend to make the observed rate faster than the proper dissociation rates. Yet, the agreement between the rate of calcium–protein decay and the rate of phosphate incorporation induced by calcium removal at 20°C indicates that under these conditions the interfering reactions are of little significance. The very slow and temperature dependent dissociation rate can hardly be reconciled with the concept that the protein might chelate calcium like EGTA or EDTA do. The interaction of these chelating agents with calcium ions has been described by a one-step binding mechanism [9]. The decay of such complexes occurs in the range of milliseconds if they have stability constants $< \sim 10^5\ M^{-1}$ as they were found for the calcium–transport protein complex at pH 6.0 [10]. Furthermore, the decay of such complexes should only little depend on the temperature. We must, therefore, conclude that in analogy to the interaction of various cations with their corresponding ionophores [11,12], calcium interacts with the protein in at least a two-step sequence:

$$E + Ca \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1Ca \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_2Ca$$

The first reaction leading to the rapid formation or dissociation of an encounter complex is followed by a slow isomerization process. The second intermediate in this reaction chain is the one which can be phosphorylated by ATP. This conclusion derived from the slow dissociation rate of the calcium–protein com-

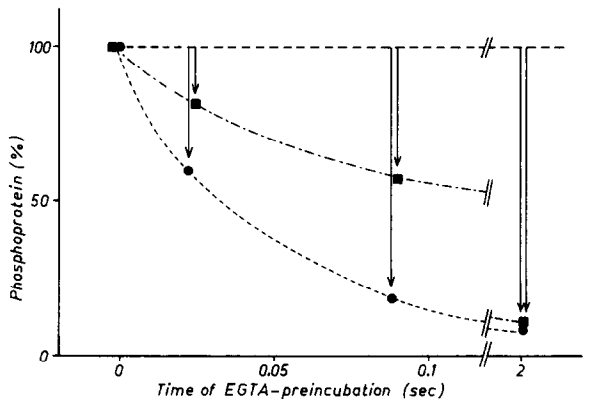

Fig.1. Time course of the decay of the calcium–transport protein complex monitored by ATP-supported phosphorylation of the residual calcium–protein complex. The figure shows the amounts of phosphoprotein which were formed by the addition of 0.1 mM ATP and 2.0 mM $MgCl_2$ from the third syringe (13 ml), after the vesicular suspension (1 mg/ml) containing 0.1 mM Ca^{2+} in the first syringe (1.3 ml) was mixed with 20 mM EGTA from the second syringe (1.3 ml) for different time intervals at 20°C (■) and 30°C (●). All solutions were buffered with 40 mM Tris-maleate, pH 6.0. Phosphorylation was only measured at three different times after the addition of EGTA, since the volume of the third syringe of our device is quite large and, therefore, needs considerable quantities of [γ-^{32}P]ATP. The rate constant for the decay of the calcium–enzyme complex can be approximated from the half-time of phosphoprotein decay.

Table 1
Absolute values anf half-times of phosphoprotein formation after different periods of EGTA being preincubated in the medium containing calcium and sarcoplasmic membranes as indicated in section 2 and in fig.1

		20°C	30°C
E–P formation without adding EGTA (nmol/mg protein)		1.40–1.45	1.65–1.80
E–P formation started after different periods of Ca removal initiated by EGTA (nmol/mg protein)	22 ms	1.18–1.45	0.93–1.80
	88 ms	0.81–0.87	0.29–0.32
	2 s	0.12–0.14	0.07–0.16
Calcium–transport half-times (ms) protein complex decay monitored by ATP-supported residual calcium protein phosphorylation		100	28
E–P formation by inorganic phosphate half-times (ms) (reaction started by Ca removal)		80–130	from [5]

Phosphoprotein formation by inorganic phosphate was measured at pH 6.0 and 20°C with 30 mM phosphate, 20 mM $MgCl_2$, 40 mM Tris maleate, 5 mM EGTA and 0.5 mg/ml sarcoplasmic vesicles in the reaction medium. The reaction was started by the addition of magnesium and EGTA

plex is supported by experiments in which the rate of calcium association was determined. Phosphorylation was started either by the addition of calcium to the ATP containing but calcium–free assay or by the addition of ATP to the calcium containing medium. Figure 2 shows that the calcium–protein complex is phosphorylated by ATP (○) curve a) much faster than the ATP–protein complex after the addition of calcium ions ((●) curve b). The phosphorylation rate of the calcium–protein complex at pH 6.0 and 30°C reaches a value of ~70 s^{-1} which is somewhat lower than the rate observed [14] at pH 7.0 and 20°C. Since the experiments were performed in assay media containing concentrations of ATP and calcium sufficiently high to saturate their binding sites [13] and since the phosphorylation of the calcium–protein complex is not rate limiting, curve b in fig.2 allows to estimate the rate of formation of the calcium-containing protein complex. From the observed half-times for phosphoprotein formation (fig.2, curve b) and decay (fig.1) the rate constants of the slow isomerization step of the assumed two-step mechanism for calcium binding can be approximated. The obtained values for k_2 and k_{-2} which are $< 20\ s^{-1}$ at 30°C are considerably slower than those found for ionophore complexes. Since the rate of calcium turnover during calcium uptake and release by sarcoplasmic reticulum vesicles corresponds approximately to the approximated isomerization rate, it may, therefore, be considered to represent a rate-determining step in the energy-dependent calcium translocation.

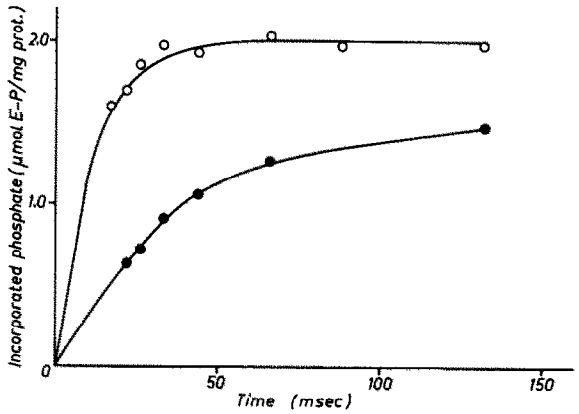

Fig.2. Time course of calcium-dependent phosphorylation of the calcium–transport protein by ATP. Phosphorylation was started by mixing a suspension of vesicles (1 mg/ml) with an equal volume of a solution containing either 0.2 mM [γ-^{32}P]ATP (○), curve (a), or 0.2 mM $CaCl_2$ (●), curve (b). The concentrations of the other reagents are given in section2.

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