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Characterization of CrATP-induced calcium occlusion in membrane-bound and soluble monomeric sarcoplasmic reticulum Ca^{2+}-ATPase

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Occlusion of Ca^{2+} induced by β,γ-bidentate CrATP in membrane bound and in soluble monomeric sarcoplasmic reticulum Ca^{2+}-ATPase was studied by previously developed filtration and HPLC techniques (Vilsen and Andersen (1986) Biochim. Biophys. Acta 855, 429–431). Activation of Ca^{2+} occlusion occurred at micromolar free Ca^{2+} and depended on the concentration of Ca^{2+}, H^+ and Mg^{2+} in a similar way as activation of Ca^{2+} transport and equilibrium Ca^{2+} binding to high-affinity Ca^{2+} transport sites. The slopes of the Ca^{2+} titration curves indicated that Ca^{2+} binding is a cooperative process both in membraneous and in soluble monomeric enzyme. At alkaline pH and absence of Mg^{2+}, occlusion of Ca^{2+} was inhibited by 1 mM Ca^{2+} in membrane-bound, but not in soluble monomeric Ca^{2+}-ATPase. Parallel studies of phosphorylation from [γ-^{32}P]CrATP indicated a stoichiometry of 2 mol Ca^{2+} occluded per mol Ca^{2+}-dependent EP formed, at saturating as well as at desaturating Ca^{2+} concentrations. Tryptic digestion of the CrATP induced Ca^{2+} occluded complex indicated that it belongs to the E_1 conformational class (E_1P). In the absence of Ca^{2+} and Mg^{2+}, but presence of CrATP the conformational state was E_2. When Mg^{2+} was added together with CrATP at alkaline pH the conformation was shifted in direction of E_1.

Introduction

The $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum is responsible for active uptake of Ca^{2+} from the myoplasm during muscle relaxation [1]. Under appropriate conditions at saturating calcium concentrations and neutral pH, two Ca^{2+} ions are transported per molecule of ATP hydrolyzed [2,3]. Several authors have demonstrated the existence of a phosphorylated transport intermediate containing occluded calcium ions, i.e. bound Ca^{2+}, which is unable to exchange with Ca^{2+} at either side of the membrane [4–10]. The nature of the Ca^{2+}-occluded form is largely uncharacterized. It is uncertain whether the calcium occluded complex belongs to one of the major conformational classes (E_1 and E_2) of Ca^{2+}-ATPase or it represents a third (intermediate?) state [3,11]. Moreover an unanswered aspect is whether one or more polypeptide chains participate in Ca^{2+} occlusion in the native mem-

Abbreviations: HPLC, high performance liquid chromatography; $C_{12}E_8$, octaethyleneglycol monododecyl ether; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; EP, phosphorylated Ca^{2+}-ATPase; E_1 and E_2, major conformational states of Ca^{2+}-ATPase; K_d^{app}, apparent dissociation constant; CrATP, β,γ-bidentate complex of chromium(III) with adenosine 5′-triphosphate.

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brane. The observation of a Hill number higher than 3 at alkaline pH in equilibrium Ca^{2+} binding studies, suggests that Ca^{2+} sites on different ATPase chains may interact [12].

Previously we have shown that detergent solubilized monomeric Ca^{2+}-ATPase, stabilized in a phosphorylated state by the β,γ-bidentate chromium(III) complex of ATP, occludes 6–7 nmol Ca^{2+} per mg ATPase at 123 μM free Ca^{2+} [13]. It was, however, not obvious whether this Ca^{2+} was bound at the high-affinity transport sites and whether monomerization by detergent affected stoichiometry and cooperativity of the reaction.

The purpose of the present study has been to investigate in more detail the nature of the Ca^{2+}-occluded complex stabilized by CrATP. We have examined the Ca^{2+} and Mg^{2+} dependences of rates of Ca^{2+} occlusion in membrane-bound and in soluble monomeric enzyme, as well as the relation between Ca^{2+} occlusion and phosphoprotein formation. The conformational state has been characterized by tryptic cleavage.

Materials and Methods

Ca^{2+}-ATPase preparations

Sarcoplasmic reticulum from rabbit skeletal muscle was isolated as described by De Meis and Hasselbach [14] and purified by extraction with a low concentration of deoxycholate according to Method 2 of Meissner et al. [15]. For solubilization of Ca^{2+}-ATPase 10 mg $C_{12}E_8$ (Nikko Chemicals) was added to 4 mg purified Ca^{2+}-ATPase protein in 1 ml of 20 mM Tes (pH 7.0 or 7.5), 20% glycerol, 100 mM NaCl, 0.4 mM $CaCl_2$ with or without 10 mM $MgCl_2$. The insoluble residue was removed by centrifugation in a Beckman airfuge at 130000 × g for 30 min.

Preparation of CrATP

The β,γ-bidentate chromium(III) complex of ATP (CrATP) as well as its γ-^{32}P derivative were prepared according to Method B of Dunaway-Mariano and Cleland [16]. CrATP was focused off the ionic-exchange resin (AG 50W-X2-H^+-form (100–200 mesh), Bio-Rad) by using 0.3 M aniline. The aniline was immediately removed from the eluate by extraction with 3 × 5 volumes ether, and the residual ether was evaporated in vacuo. When used, [γ-^{32}P]ATP (Amersham, Bucks., U.K.) was purified before CrATP synthesis as described by Nørby and Jensen [17].

Measurement of Ca^{2+}-occlusion

Soluble Ca^{2+}-ATPase (0.25 mg/ml) was incubated at 20 °C with 0.8 mM CrATP in 5 mg $C_{12}E_8$/ml, 5% glycerol, 20 mM Tes (pH 7.0 or 8.0), 100 mM NaCl, 0.123–1.0 mM $CaCl_2$ (with ^{45}Ca), 0–1.0 mM EGTA and 0–20 mM $MgCl_2$ to produce various free Ca^{2+} and Mg^{2+} concentrations, as indicated in the figures. In control experiments the medium had the same composition except that CrATP was omitted. For measurements of the initial rate of Ca^{2+} occlusion the duration of incubation was 0–30 min. The reaction was terminated by addition of 1 mM EGTA and 2.5 mM $^{40}CaCl_2$ followed by injection of 600 μl sample into a TSK G 3000 SW HPLC column (7.5 mm × 30 cm, Toyo Soda). The flow rate was 0.8 ml/min (pressure 4–5 bar). The eluant contained 5 mg $C_{12}E_8$/ml, 0.1 M NaCl, 20 mM Tes (pH 7.0). 1.5 mM $^{40}CaCl_2$, 1.0 mM EGTA, 10 mM $MgCl_2$. Absorbance was read continuously at 226 nm. The major protein peak, which was well separated from unbound $^{45}Ca^{2+}$ and CrATP [13], was collected and analyzed for protein content and radioactivity.

Membrane-bound Ca^{2+}-ATPase (0.25 mg/ml) was incubated as described for soluble Ca^{2+}-ATPase except for the absence of detergent and glycerol. The reaction was terminated by transfer of 400 μl sample to 5 ml washing medium containing 20 mM Tes (pH 8.0), 100 mM NaCl and 2 mM EGTA. The mixture was filtered through a Millipore filter (0.22 μm) and the protein deposit was washed with 4 × 5 ml washing medium. Radioactivity was measured by liquid scintillation counting.

Measurement of phosphorylation from [γ-^{32}P]CrATP

Phosphorylation of soluble Ca^{2+}-ATPase was measured by two different procedures. The first method, which avoided acid precipitation was identical to the HPLC technique described above under 'Measurement of Ca^{2+} occlusion', except that [γ-^{32}P]CrATP and $^{40}CaCl_2$ were used and that Ca^{2+}-independent phosphorylation was mea-

sured in presence of 20 mM EGTA. In the second method soluble Ca^{2+}-ATPase (0.5 mg/ml) was incubated at 20 °C with 1.0 mM [γ-^{32}P]CrATP in 5 mg $C_{12}E_8$/ml, 20 mM Tes (pH 7.0), 100 mM NaCl, 10 mM $MgCl_2$ with either 123 μM $^{40}CaCl_2$ (for determination of the Ca^{2+}-dependent phosphorylation) or with 20 mM EGTA (Ca^{2+}-independent phosphorylation). At various times the reaction was terminated by transfer of 200 μl sample to 500 μl ice-cold 8% (w/v) perchloric acid with 10 mM pyrophosphate. Precipitation was allowed to occur for 30 min at 0 °C. The precipitate was washed by centrifugation and resuspended as previously described [18], and aliquots were taken for determination of radioactivity and protein content. Identical phosphorylation levels were measured by the two procedures described.

Phosphorylation of membrane bound Ca^{2+}-ATPase was determined following the second method described for soluble Ca^{2+}-ATPase, except that detergent was omitted in the incubation medium and that the precipitate was washed on a Gelman glassfiber filter. To demonstrate ADP sensitivity of the phosphoprotein, CrATP was removed by rapid gelfiltration in Sephadex G-25 columns (1 cm × 25 cm) prior to addition of 2 mM EGTA with 2 mM ADP.

Tryptic cleavage of Ca^{2+}-ATPase

For tryptic digestion membranes (0.5 mg protein/ml) were incubated at 20 °C with 5 μg TPCK-trypsin (Merck) per 100 μg protein in buffer media containing 50 mM Tes-Tris (pH 8.0) or 20 mM Tes (pH 7.0) with various additions of NaCl, $CaCl_2$, $MgCl_2$, EGTA, ATP, CrATP and Na_3VO_4 as described in the figure legends. Digestion was terminated by addition of soybean trypsin inhibitor (4 μg/μg trypsin) and subsequent cooling at 0 °C. The digested Ca^{2+}-ATPase was reduced in 1% mercaptoethanol and 2% SDS at 100 °C for 3 min and subjected to SDS-polyacrylamide gel electrophoresis in 5–15% gradient slab gels prepared as previously [19].

Other methods

The sedimentation velocity of soluble Ca^{2+}-ATPase, incubated with CrATP, was examined at 44000 rpm and 20 °C in a Beckman model E analytical ultracentrifuge as before [20].

Protein concentrations were measured by the method of Lowry et al. as previously described [20].

Free concentrations of Ca^{2+} were calculated using published stability constants for CaEGTA and MgEGTA [21,22]. These calculations resulted in the following apparent association constants for CaEGTA at 20 °C: $1.7 \cdot 10^6$ M^{-1} (pH 7.0, 10 mM Mg^{2+}), $2.1 \cdot 10^8$ M^{-1} (pH 8.0, 0 Mg^{2+}) and $2.0 \cdot 10^7$ M^{-1} (pH 8.0, 10 mM Mg^{2+}). The Ca^{2+} concentration was adjusted by taking into consideration the amount of contaminant Ca^{2+} present in the Ca^{2+}-ATPase preparation (18 μM Ca^{2+}/mg protein) and in buffer solutions (5 μM) as determined by atomic absorption spectrometry.

Results

Ca^{2+} dependence of the initial rate of Ca^{2+} occlusion

Earlier investigations have indicated that a stable Ca^{2+} occluded complex is obtained with CrATP as phosphorylating substrate [9,13]. Fig. 1 shows the Ca^{2+} dependence of the initial rate of CrATP induced Ca^{2+} occlusion at various Mg^{2+} and H^+ concentrations for membrane bound as well as for soluble Ca^{2+}-ATPase. The previously developed HPLC technique [13] was applied to soluble enzyme, whereas Millipore filtration was used in the case of membranous Ca^{2+}-ATPase. The data have been fitted according to the Hill equation resulting in the curves shown.

For membrane bound Ca^{2+}-ATPase at pH 7.0 and 10 mM Mg^{2+} activation of CrATP induced Ca^{2+} occlusion takes place between 0.2 μM and 2 μM free Ca^{2+}. This range is similar to the activation range for ATP hydrolysis and Ca^{2+} transport [1,23]. In the soluble enzyme the activation occurs between 0.5 μM and 5 μM free Ca^{2+}. A similar shift induced by solubilization has been observed previously in studies of Ca^{2+}-ATPase activity by Møller et al. [24]. The activation process is characterized by a Hill coefficient close to 2 for both membrane bound and solubilized Ca^{2+}-ATPase, consistent with the existence of two interacting Ca^{2+} binding sites per peptide chain [20,25].

At pH 8.0 the activation range is displaced towards lower free Ca^{2+} concentrations. A further decrease in apparent K_d for Ca^{2+} is observed in

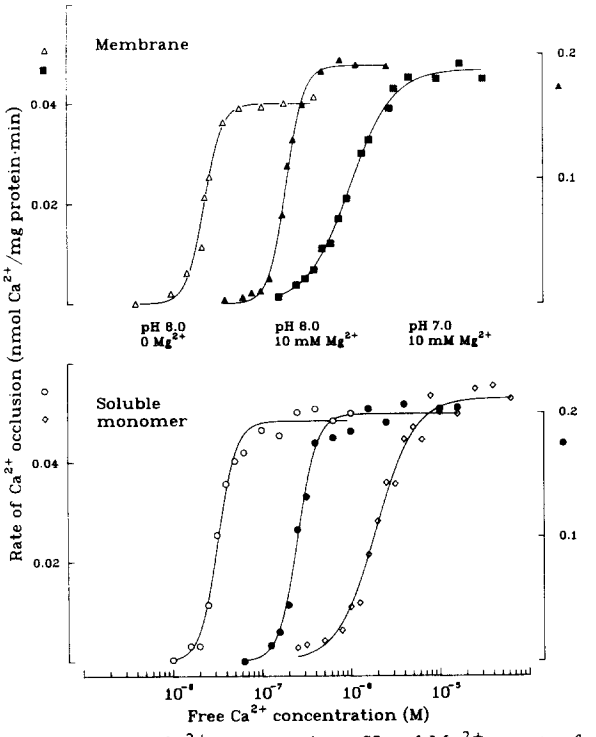

Fig. 1. Effect of Ca^{2+} concentration, pH and Mg^{2+} on rate of Ca^{2+} occlusion in membrane-bound and soluble Ca^{2+}-ATPase. Membrane-bound Ca^{2+}-ATPase (0.25 mg/ml) (△, ▲, ■) was incubated at 20 °C for 7 min (▲) or 30 min (△, ■) with 0.8 mM CrATP in 20 mM Tes (pH 7.0 or 8.0), 100 mM NaCl, 0 (△) or 10 mM (▲, ■) $MgCl_2$, 123 μM $^{45}CaCl_2$ and various concentrations of EGTA to produce the indicated free Ca^{2+} concentrations. The reaction was terminated by transfer of 400 μl sample to 5 ml washing medium, and the amount of occluded Ca^{2+} was determined after Millipore Filtration as described in Materials and Methods. Soluble Ca^{2+}-ATPase (0.25 mg/ml) (○, ●, ◇) was incubated at 20 °C for 7 min (●) or 30 min (○, ◇) with 0.8 mM CrATP in 5 mg $C_{12}E_8$/ml, 5% glycerol, 20 mM Tes (pH 7.0 or 8.0), 100 mM NaCl, 0 (○) or 10 mM (●, ◇) $MgCl_2$, 123 μM $^{45}CaCl_2$ and various concentrations of EGTA to produce the indicated free Ca^{2+} concentrations. The reaction was terminated by addition of $^{40}CaCl_2$ followed by injection of 600 μl sample into a TSK G 3000 SW HPLC column, and the amount of occluded Ca^{2+} was determined as described in Materials and Methods. Experimental points (only average values of 2–5 determinations are shown) were fitted according to the Hill equation by non-linear least-squares regression (lines). The following Hill numbers and K_d^{app} values were obtained: (△) $n_H = 4.3$, $K_d^{app} = 0.0236$ μM; (○) $n_H = 4.2$, $K_d^{app} = 0.0323$ μM; (▲) $n_H = 4.4$, $K_d^{app} = 0.195$ μM; (●) $n_H = 4.0$, $K_d^{app} = 0.254$ μM; (■) $n_H = 1.8$, $K_d^{app} = 1.02$ μM; (◇) $n_H = 2.0$, $K_d^{app} = 1.92$ μM.

the absence of Mg^{2+}. This data is in accordance with other evidence of competition between Mg^{2+}, Ca^{2+} and H^+ at the transport sites [23,26,27]. At pH 8.0 detergent solubilization shifts the activation range towards higher free Ca^{2+} concentrations in a similar way as observed at pH 7.0. The Hill coefficients calculated for the four curves obtained at pH 8.0, with and without Mg^{2+}, in presence and absence of detergent, do not differ significantly and are close to 4, suggesting that Ca^{2+} occlusion is a highly cooperative process under these circumstances. The maximum initial rate of Ca^{2+} occlusion as measured in presence of a saturating Ca^{2+} concentration (p*Ca* 4–5) also depends on pH. At pH 8.0 and 10 mM Mg^{2+} it is seen to be about 4-fold higher than at pH 7.0, 10 mM Mg^{2+} (compare ordinate scales). At pH 8.0, 10 mM Mg^{2+} a maximum level of 6–7 nmol Ca^{2+} occluded per mg protein was attained within 2–3 hours at p*Ca* 4. Total Ca^{2+} binding (including freely exchangeable Ca^{2+}) measured by Millipore filtration of membrane bound Ca^{2+}-ATPase in presence of $^{45}Ca^{2+}$, amounted to 7–8 nmol/mg (data not shown). Hence there is no evidence under these conditions for modulation of the reaction rate by Ca^{2+} bound at sites distinct from those involved in Ca^{2+} occlusion.

Effect of high Ca^{2+} concentrations and Mg^{2+}

Table I shows the effect on the initial rate of Ca^{2+} occlusion of increasing the free Ca^{2+} concentration from 0.1 mM to 1 mM. For soluble

TABLE I

EFFECT OF HIGH Ca^{2+} CONCENTRATION ON CrATP INDUCED Ca^{2+} OCCLUSION IN MEMBRANOUS AND SOLUBLE MONOMERIC Ca^{2+}-ATPase

Measurements were performed as in Fig. 1. At 1.0 mM Ca^{2+} no EGTA was present during incubation. Figures are presented as means ± S.D. of three experiments.

Ca^{2+}-ATPase	Initial rate of Ca^{2+} occlusion (nmol/mg per min)	
	at 0.1 mM Ca^{2+}	at 1.0 mM Ca^{2+}
Soluble		
pH 7.0, 10 mM Mg^{2+}	0.056 ± 0.008	0.068 ± 0.007
pH 8.0, 10 mM Mg^{2+}	0.244 ± 0.031	0.245 ± 0.041
pH 8.0, no Mg^{2+}	0.073 ± 0.012	0.074 ± 0.010
Membrane-bound		
pH 7.0, 10 mM Mg^{2+}	0.062 ± 0.009	0.063 ± 0.011
pH 7.0, no Mg^{2+}	0.019 ± 0.005	0.019 ± 0.004
pH 8.0, 10 mM Mg^{2+}	0.210 ± 0.025	0.131 ± 0.022
pH 8.0, no Mg^{2+}	0.061 ± 0.010	0.014 ± 0.008

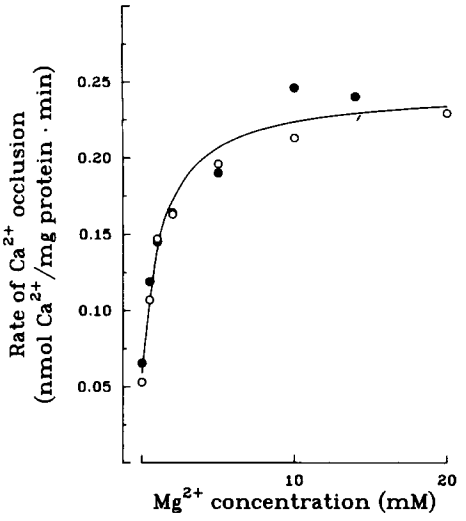

Fig. 2. Effect of Mg^{2+} concentration on rate of Ca^{2+} occlusion in membrane-bound and soluble Ca^{2+}-ATPase. Membrane-bound Ca^{2+}-ATPase (0.25 mg/ml) (○) was incubated at 20 °C for 7 min with 0.8 mM CrATP in 20 mM Tes (pH 8.0), 100 mM NaCl, 123 μM $^{45}CaCl_2$ and 0–20 mM $MgCl_2$. The reaction was terminated by transfer of 400 μl sample to 5 ml washing medium and the amount of occluded Ca^{2+} was determined after Millipore filtration as described in Materials and Methods. Soluble Ca^{2+}-ATPase (0.25 mg/ml) (●) was incubated at 20 °C for 7 min with 0.8 mM CrATP in 5 mg $C_{12}E_8$/ml, 5% glycerol, 20 mM Tes (pH 8.0), 100 mM NaCl, 123 μM $^{45}CaCl_2$ and 0–14 mM $MgCl_2$. The reaction was terminated by addition of $^{40}CaCl_2$ followed by injection of 600 μl sample into a TSK G 3000 SW HPLC column, and the amount of occluded Ca^{2+} was determined as described in Materials and Methods. Experimental points were fitted by non-linear least-squares regression (line) to the equation:

rate of Ca^{2+} occlusion

$$= (\alpha[Mg^{2+}] + \beta)/([Mg^{2+}] + \gamma)$$

resulting in the following constants: $\alpha = 0.24$ nmol/mg per min; $\beta = 7.65 \cdot 10^{-2}$ (nmol/mg per min)·mM; $\gamma = K_d^{app} = 1.29$ mM.

Ca^{2+}-ATPase the rates measured at the two Ca^{2+} concentrations are almost identical irrespective of variation in pH and Mg^{2+} concentration. By contrast the rate of Ca^{2+} occlusion for membrane bound Ca^{2+}-ATPase at pH 8.0 declines at 1 mM free Ca^{2+} to 23% of the value at 0.1 mM free Ca^{2+}. In presence of 10 mM Mg^{2+} a less pronounced effect of 1 mM free Ca^{2+} is observed at pH 8.0 (i.e. a decline to 62% of the value at 0.1 mM free Ca^{2+}), and at pH 7.0 the inhibition by high Ca^{2+} has completely vanished. These data resemble results obtained by Møller et al. [24] and Andersen et al. [18], showing that inhibition by high Ca^{2+} concentration of ATPase activity and of the $E_1P \rightarrow E_2P$ conformational transition is much more pronounced for membrane-bound than for $C_{12}E_8$-solubilized Ca^{2+}-ATPase, and that the Ca^{2+} inhibition is antagonized by Mg^{2+}.

Table I also shows that Mg^{2+} accelerates Ca^{2+} occlusion 3–4-fold at optimum Ca^{2+} concentration. This effect has been further investigated in Fig. 2, which shows titration of the rate of Ca^{2+} occlusion with Mg^{2+} at 123 μM Ca^{2+} and pH 8.0 for membrane bound as well as for soluble Ca^{2+}-ATPase. Both sets of data are satisfactory fitted by a simple hyperbolic dependence on Mg^{2+} concentration with an apparent K_d for Mg^{2+} of 1.29 mM. At pH 7.0 the apparent K_d for Mg^{2+} was found to be 6.0 mM (not shown). The stimulating effect of Mg^{2+} cannot be explained by Mg^{2+} binding in a complex with ATP since the metal binding site at the β- and γ-phosphates is already occupied by Cr^{3+}.

Analytical ultracentrifugation

The experiments with soluble Ca^{2+}-ATPase shown in the previous figures were performed at a detergent to protein concentration ratio of 20:1 (w/w). This ratio has been demonstrated to result in complete monomerization of active Ca^{2+}-ATPase in the absence of substrate [18,20]. In order to examine if the protein remains mono-

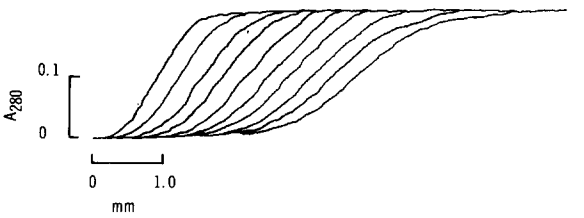

Fig. 3. Sedimentation of detergent-solubilized Ca^{2+}-ATPase incubated 6 h with CrATP. Soluble Ca^{2+}-ATPase (0.25 mg/ml) was incubated with 0.5 mM CrATP in 5 mg $C_{12}E_8$/ml, 20 mM Tes (pH 7.0), 100 mM NaCl, 10 mM $MgCl_2$ and 123 μM Ca^{2+} for 6 h, at 20 °C before sedimentation in the analytical ultracentrifuge at 44000 rpm as described in Materials and Methods. The reference cell contained an identical medium except for the absence of enzyme. Scans (at 280 nm) were taken with 8 min intervals. The sedimentation coefficient ($s_{20,w}$) calculated from the data was 4.76 S.

meric during incubation with CrATP, we have studied the sedimentation velocity in the analytical ultracentrifuge at various times after initiation of Ca^{2+} occlusion The sedimentation rate was found to be independent of the duration of incubation with CrATP. Fig. 3 shows sedimentation of Ca^{2+}-ATPase (0.25 mg/ml) in presence of 0.5 mM CrATP after 6 h incubation in 123 μM Ca^{2+}, 10 mM Mg^{2+} and pH 7.0. The scans were taken at 8-min intervals. It can be seen that a single homogeneous boundary formed. The sedimentation coefficient ($s_{20,w}$) calculated from the data is 4.76 S, i.e. slightly lower than for $C_{12}E_8$ solubilized monomeric Ca^{2+}-ATPase in the absence of CrATP [20,24]. When the protein concentration was varied from 0.05 mg/ml to 1.0 mg/ml under otherwise similar conditions as in Fig. 3, the sedimentation coefficient was found to be constant within experimental error. This data indicates that CrATP does not induce dimerization. Furthermore irreversible time dependent aggregation [20] seems to be prevented by CrATP.

Phosphorylation by [γ-^{32}P]CrATP

In order to evaluate the stoichiometry of Ca^{2+} occlusion we have studied the phosphorylation of the enzyme by γ-^{32}P labelled CrATP. Fig. 4A (upper curve) shows time dependence of the amount of phosphoprotein formed from [γ-^{32}P]CrATP in soluble monomer and in membrane bound Ca^{2+}-ATPase at pH 7.0, 10 mM Mg^{2+}, 123 μM Ca^{2+} (i.e., the same conditions as applied in the previous study of Ca^{2+} occlusion, Fig. 2 of Ref. 13). The lower curve indicates that some phosphoenzyme is formed also in the absence of Ca^{2+}. This Ca^{2+}-independent phosphorylation is only seen when CrATP is used as substrate, but not with ATP. Ca^{2+}-independent phosphorylation by CrATP has also been observed by Serpersu et al. [9]. We found that this phosphoprotein is stable at acid pH in the presence of 1% SDS, and that it increases after thermal denaturation of Ca^{2+}-ATPase membranes.

The Ca^{2+}-dependent phosphorylation associated with Ca^{2+} occlusion can be calculated by subtraction of the Ca^{2+}-independent phosphorylation from the total phosphorylation. After 4–5 h the curves for total phosphorylation and the Ca^{2+}-independent phosphorylation have identical

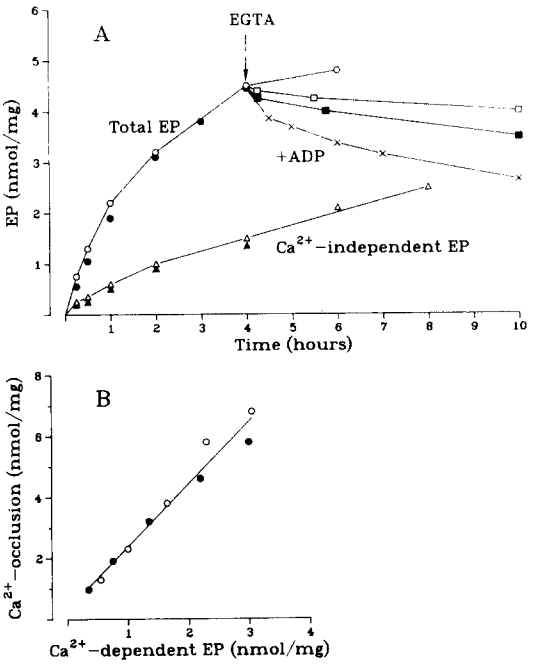

Fig. 4(A) Time dependence of phosphorylation of membrane bound and soluble Ca^{2+}-ATPase by [γ-^{32}P]CrATP. Membrane bound Ca^{2+}-ATPase (0.5 mg/ml, ○, △, □, x) was incubated with 1.0 mM [γ-^{32}P]CrATP in 20 mM Tes (pH 7.0), 100 mM NaCl, 10 mM $MgCl_2$ with either 123 μM $CaCl_2$ (○) or with 20 mM EGTA (△), at 20 °C. Soluble Ca^{2+}-ATPase (0.5 mg/ml, ●, ▲, ■) was incubated likewise but in presence of 5 mg $C_{12}E_8$/ml. After 4 h (arrow) 2 mM EGTA (□, ■) or 2 mM EGTA with 2 mM ADP (x) was added to the samples to induce dephosphorylation. In the experiment with ADP, CrATP had been removed prior to dephosphorylation by rapid gelfiltration after 4 h incubation. Phosphoprotein was measured by the second method described in Materials and Methods. (B) Relation between Ca^{2+} occlusion and Ca^{2+}-dependent EP. (○), membrane-bound Ca^{2+}-ATPase; (●), soluble Ca^{2+}-ATPase. Data from (A) were plotted together with data obtained previously from measurement of Ca^{2+} occlusion under the same incubation conditions as in (A), (Ref. 13, Fig. 2).

slopes, indicating that the Ca^{2+}-dependent phosphorylation has reached a steady state. This corresponds to 3–3.5 nmol EP per mg Ca^{2+}-ATPase. Fig. 4A also indicates that after addition of EGTA, the amount of total phosphoenzyme declines towards the same level as that of Ca^{2+}-independent phosphorylation, at a rate which is higher for soluble monomeric Ca^{2+}-ATPase than for membrane-bound enzyme. In both enzyme preparations the rate of dephosphorylation is lower than deocclusion of Ca^{2+} (cf. Ref. 13), suggesting that

the occluded sites are only incompletely removed from contact with the aqueous phase, or that E_2P may accumulate after dissociation of Ca^{2+}. As also shown in Fig. 4A (x), ADP accelerates EGTA induced dephosphorylation as expected from the definition of the Ca^{2+}-occluded form as ADP-sensitive phosphoenzyme [7]. The ADP induced enhancement of the rate of dephosphorylation was observed only after removal of unbound CrATP by gelfiltration (see Materials and Methods), probably because of competition between CrATP and ADP for binding at the nucleotide site.

Fig. 4B shows that there is a linear relation between the Ca^{2+}-dependent phosphorylation and the amount of occluded Ca^{2+}. The slope indicates that two Ca^{2+} ions are occluded per phosphorylated site both in membrane bound and in soluble monomeric Ca^{2+}-ATPase.

In order to explore whether this Ca^{2+}/EP ratio of 2 is maintained also at low free calcium concentrations, where the calcium binding sites of the pump are desaturated, we have performed phosphorylation experiments at pH 7.0 and 10 mM $MgCl_2$ with soluble monomeric enzyme, in the same p*Ca* range as already examined in the Ca^{2+} occlusion experiments of Fig. 1 (see curve (◇)). Results are shown in Fig. 5. The small difference between the curve representing the phosphoryla-

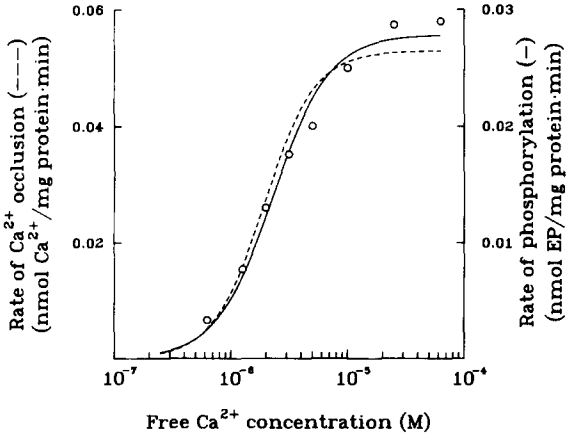

Fig. 5. Relation between Ca^{2+} occlusion and Ca^{2+} dependent phosphorylation at desaturating Ca^{2+} concentrations. The broken line representing Ca^{2+} occlusion was transferred from Fig. 1 (soluble enzyme, pH 7.0, 10 mM Mg^{2+} (◇)). The unbroken line fitted to the points represents Ca^{2+}-dependent phosphorylation from [γ-^{32}P]CrATP measured under the same conditions as Ca^{2+} occlusion by the first method described in Materials and Methods. Curve fitting was carried out as described for Fig. 1. The resulting parameters were $K_d^{app} = 2.30$ μM, $n_H = 1.8$.

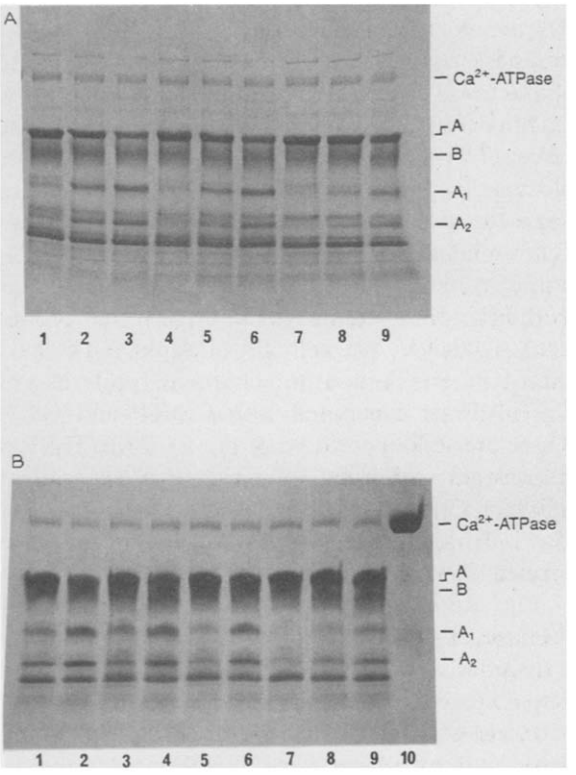

Fig. 6. Effect of CrATP induced Ca^{2+} occlusion on tryptic cleavage of Ca^{2+}-ATPase. (A) Digestion of purified Ca^{2+}-ATPase membranes with trypsin (5 μg/100 μg Ca^{2+}-ATPase) was performed at 20 °C in 20 mM Tes (pH 7.0), 100 mM NaCl for 1 min (lanes 1, 4, 7); 5 min (lanes 2, 5, 8) and 10 min (lanes 3, 6, 9) as described in Materials and Methods. Further additions are indicated below. Lanes 1, 2, 3: 0.1 mM $CaCl_2$. Lanes 4, 5, 6: membranes were preincubated 5 h in presence of 0.1 mM $CaCl_2$ and 0.8 mM CrATP before addition of trypsin. Lanes 7, 8, 9: preincubated as lanes 4, 5, 6 but in presence of 2 mM EGTA and 0.8 mM CrATP.
(B) Digestion was performed as described in Materials and Methods in 50 mM Tes/Tris (pH 8.0), 30 mM NaCl for 2 min (lanes 1, 3, 5, 7) and 5 min (lanes 2, 4, 6, 8, 9). Further additions are indicated below. Lanes 1, 2: 50 μM $CaCl_2$, 10 mM $MgCl_2$. Lanes 3, 4: Ca^{2+}-ATPase was preincubated 2 h in presence of 50 μM $CaCl_2$, 10 mM $MgCl_2$ and 0.8 mM CrATP before addition of trypsin. Lanes 5, 6: preincubated 2 h in presence of 2 mM EGTA, 10 mM $MgCl_2$, 0.8 mM CrATP before addition of trypsin. Lanes 7, 8: preincubated 2 h in presence of 2 mM EGTA, 500 μM Na_3VO_4, 10 mM $MgCl_2$, 0.8 mM CrATP before addition of trypsin. Lane 9: preincubated 10 s in presence of 5 mM ATP, 50 μM $CaCl_2$, 10 mM $MgCl_2$ before addition of trypsin. Lane 10: Ca^{2+}-ATPase not digested with trypsin.

tion data and the curve transferred from Fig. 1 (◇) after adjustment of the ordinate scale by a factor 2 is within experimental error.

Tryptic cleavage patterns

These experiments were designed in order to characterize the conformational state of the Ca^{2+}-occluded phosphoenzyme. Fig. 6A shows the effect of CrATP-induced Ca^{2+} occlusion on tryptic cleavage of membrane bound Ca^{2+}-ATPase incubated at pH 7.0. In the first three lanes Ca^{2+}-ATPase has been treated with trypsin for 1, 5 and 15 min in the presence of 0.1 mM Ca^{2+}, i.e. in the E_1 conformation. It can be seen that prominent A_1 and A_2 fragments form rapidly. The next three lanes show the cleavage patterns of Ca^{2+}-ATPase incubated with CrATP and Ca^{2+}. These are similar to those of the E_1 form. The last three lanes on the gel show a control experiment in which CrATP has been added in the absence of Ca^{2+}. In this case the A_1 and A_2 bands are formed slowly indicating an E_2 form.

Fig. 6B shows cleavage patterns of Ca^{2+}-ATPase after digestion for 1 and 5 min with trypsin at low salt concentration and pH 8.0. These cleavage patterns are seen to be identical with and without CrATP in presence of Ca^{2+} and Mg^{2+} (compare lanes 1 and 2 with lanes 3 and 4). The A_1 and A_2 peptides are very prominent as was also observed at pH 7.0. By contrast little of these fragments are formed during steady-state phosphorylation with ATP in presence of Mg^{2+} (compare lanes 2 and 4 with lane 9). Furthermore, when the reaction mixture contained vanadate together with EGTA and CrATP the A_1 peptide was barely detectable, indicating that the prevalent enzyme form was E_2V (lanes 7 and 8). In the absence of Ca^{2+} but presence of Mg^{2+} and CrATP the amounts of A_1 and A_2 peptides formed were almost as high as in the E_1 form stabilized by Ca^{2+} (lanes 5 and 6). This result indicates that simultaneous binding of CrATP and Mg^{2+} shifts the conformational equilibrium towards E_1, even in the absence of Ca^{2+}.

Discussion

The present study demonstrates that CrATP induced Ca^{2+} occlusion occurs at the high-affinity calcium transport sites. This conclusion is based on the following findings: (a) The activation of Ca^{2+} occlusion takes place in the same Ca^{2+} concentration range as activation of ATP hydrolysis and Ca^{2+} transport and is influenced by H^+ and Mg^{2+} concentrations in the same way as equilibrium Ca^{2+} binding to high-affinity transport sites [12,26]. (b) The linear relation between the amounts of Ca^{2+}-dependent phosphoenzyme and occluded Ca^{2+} corresponding to 2 mol Ca^{2+} per mol EP. This ratio is in keeping with the established transport stoichiometry of two Ca^{2+} ions transferred across the membrane per ATP molecule hydrolyzed [1,2]. We did not find evidence for a lower coupling ratio at desaturating Ca^{2+} concentrations [28]. (c) At saturating Ca^{2+} concentrations binding of Mg^{2+} increases the rate of Ca^{2+} occlusion. This shows that Ca^{2+} occlusion takes place at divalent cation sites distinct from the site(s) normally occupied by Mg^{2+} [10,29–31].

The activation of Ca^{2+} occlusion by Mg^{2+} is of interest in connection with the current debate regarding the role of Mg^{2+} in the Ca^{2+}-ATPase reaction [29–31]. Since ATP is complexed with Cr^{3+} in our experiments, activating Mg^{2+} must be bound directly to a site on the protein. A similar conclusion has been reached in case of the (Na^+ + K^+)-ATPase, also based on studies with CrATP [32,33]. The apparent K_d for Mg^{2+} determined by Scheiner-Bobis and Schoner (0.7 mM) for (Na^+ + K^+)-ATPase [33] is close to the value of 1.3 mM measured in the present study. At alkaline pH a millimolar Ca^{2+} concentration was found to inhibit Ca^{2+} occlusion in membranous Ca^{2+}-ATPase, but this effect was antagonized by high Mg^{2+} concentration. It is possible that the inhibitory effect of Ca^{2+} is due to competition with activating Mg^{2+} at the low-affinity metal binding site referred to above.

Our data indicate that Ca^{2+} occlusion in membrane-bound and in soluble Ca^{2+}-ATPase have similar characteristics with respect to H^+ and Mg^{2+} dependences of the Ca^{2+} concentration range of activation, the slopes of the Ca^{2+} titration curves, the Ca^{2+}/EP coupling ratio and the apparent K_d for the activating effect of Mg^{2+}. On the other hand inhibition of Ca^{2+} occlusion by millimolar Ca^{2+} concentrations in the absence of Mg^{2+} was not observed in the soluble preparation.

A decrease in apparent affinity for inhibitory Ca^{2+}, induced by detergent solubilization, was previously noted in studies of ATP hydrolysis and of the $E_1P \rightarrow E_2P$ conformational transition [18,24].

The $C_{12}E_8$ solubilized Ca^{2+}-ATPase was found to be monomeric throughout the incubation with CrATP, as evidenced by its sedimentation velocity (Fig. 3). Furthermore the elution position in HPLC indicated that the monomeric state was retained during the process of measurement of Ca^{2+} occlusion [13]. At pH 7.0 the slopes of the Ca^{2+} titration curves (Fig. 1) were consistent with a cooperative binding of two Ca^{2+} per peptide chain [20,25]. However, at alkaline pH the curves were much steeper with Hill numbers close to 4 both for membrane bound and for soluble monomeric Ca^{2+}-ATPase. We did not observe a corresponding high-affinity Ca^{2+} binding in excess of the two Ca^{2+} ions occluded per phosphorylation site.

Our data suggest that cooperative interactions take place only between the two high-affinity Ca^{2+} binding sites localized on the same peptide chain. Some authors have reported a decrease in cooperativity of Ca^{2+} binding after solubilization in detergent [12,34], but this seems to be attributable to denaturation of the soluble Ca^{2+}-ATPase in the E_2 state [35,36]. In the present study the soluble enzyme was stabilized by CrATP and E_2 forms were not formed during measurement, due to the slow rate of dephosphorylation (Fig. 4A).

In order to characterize the conformational state of the Ca^{2+}-occluded complex we used tryptic digestion. It was previously shown that tryptic cleavage patterns differ in the two major conformational states E_1 and E_2 [19,37]. The conformation sensitive split has been localized to the bond at Arg-198 (T_2 site). In the CrATP induced Ca^{2+}-occluded phosphorylated form this bond was exposed to the same extent as in the unphosphorylated E_1 state (Fig. 6A). This was true also at pH 8.0, low salt concentration (Fig. 6B), where the major phosphoenzyme species, accumulated in steady-state during hydrolysis of MgATP, is E_2P (Fig. 6B, lane 9 and Ref. 18). Hence the Ca^{2+} occluded complex stabilized by CrATP, must be classified as an E_1 form (E_1P) as seen by tryptic digestion. The conformational change occurring in relation to phosphorylation and Ca^{2+} occlusion is probably of limited extent, since the bond at Arg-198 appears not to be involved, in spite of its localization close to putative Ca^{2+} binding domains [38]. In the absence of Ca^{2+} and Mg^{2+} and in presence of CrATP the tryptic cleavage pattern indicated an E_2 state (Fig. 6A). However, addition of Mg^{2+} together with CrATP at pH 8.0 resulted in an increased exposure of the T_2 site (Fig. 6B). Hence the conformation stabilized by CrATP and Mg^{2+} in absence of Ca^{2+} appears to be similar to E_1. This result is in accordance with kinetic evidence of stabilization by Mg^{2+} and ATP of an enzyme form which readily phosphorylates on addition of Ca^{2+} [39].

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