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Occlusion of Ca^{2+} in soluble monomeric sarcoplasmic reticulum Ca^{2+}-ATPase

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Sarcoplasmic reticulum Ca^{2+}-ATPase solubilized in monomeric form by nonionic detergent was reacted with CrATP in the presence of $^{45}Ca^{2+}$. A Ca^{2+}-occluded complex formed, which was stable during high performance liquid chromatography in the presence of excess non-radioactive Ca^{2+}. The elution position corresponded to monomeric Ca^{2+}-ATPase. It is concluded that a single Ca^{2+}-ATPase polypeptide chain provides the full structural basis for Ca^{2+} occlusion.

Definition of the minimal functional unit of the Ca^{2+} pump protein (Ca^{2+}-ATPase) of sarcoplasmic reticulum is an important issue in relation to the mechanism of active Ca^{2+} transport. It has been demonstrated that a single Ca^{2+}-ATPase polypeptide chain (M_r 110000) is able to undergo the conformational transitions involved in energy transduction [1–3]. It is, however, not known if vectorial Ca^{2+} translocation requires participation of two or more polypeptide chains per pump unit. The large Hill coefficient (> 3) measured in Ca^{2+}-binding experiments at alkaline pH suggests that Ca^{2+} sites on different ATPase chains may interact [4]. Furthermore a number of structural studies indicate that the Ca^{2+}-ATPase polypeptide chains are in close contact in the native membrane [5–8].

The initial Ca^{2+} occlusion occurring in relation to phosphorylation is likely to be a key event in the translocation process [9–11]. In the occluded state there is no exchange of bound Ca^{2+} with cytoplasmic or luminal free Ca^{2+}. The structural basis for this very tight binding has not yet been elucidated. In the present report we demonstrate that monomeric Ca^{2+}-ATPase peptide solubilized by the non-ionic detergent octaethyleneglycol monododecyl ether ($C_{12}E_8$) is able to form a stable occluded state.

Previous studies on membrane bound Ca^{2+}-ATPase suggest that the Ca^{2+} occluded phosphorylated intermediate is identical to the ADP sensitive phosphoenzyme species (E_1P) [9,10]. After solubilization in monomeric form with $C_{12}E_8$ the transition from E_1P (formed from ATP) to E_2P is much accelerated [3], thus precluding examination of Ca^{2+} occlusion. We have therefore taken advantage of the finding by Serpersu et al. [11], that phosphorylation from β,γ-bidentate CrATP gives rise to an E_1P form which converts only very slowly to E_2P. Ca^{2+}-ATPase was solubilized in $C_{12}E_8$ at a detergent to protein concentration ratio of 10:1, which according to our large zone gel filtration experiments and analytical ultracentrifugation studies monomerizes all active enzyme. The monomeric Ca^{2+}-ATPase was incubated with 1 mM CrATP in the presence of 123 μM $^{45}Ca^{2+}$ and 10 mM $MgCl_2$ to bind $^{45}Ca^{2+}$ only to specific high-affinity transport sites. After 4 h the sample was subjected to molecular sieve high performance liquid chromatography (HPLC) as previously described [13]. It can be seen from Fig. 1 that $^{45}Ca^{2+}$

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; SDS, sodium dodecyl sulfate.

remained bound to the soluble monomeric Ca^{2+}-ATPase protein even in the presence of 1.5 mM non-radioactive Ca^{2+} in the eluant. When 1% SDS was included in the eluant, or when CrATP was not present during incubation with $^{45}Ca^{2+}$, no $^{45}Ca^{2+}$ was associated with the Ca^{2+}-ATPase peak. Therefore the CrATP induced occlusion of $^{45}Ca^{2+}$ depends on the intact structure of the Ca^{2+}-ATPase polypeptide, but does not require participation of more than one chain.

In Fig. 2 the rate and stoichiometry of Ca^{2+} occlusion in the soluble monomer has been examined by the same technique as used in Fig. 1. It is seen that CrATP induced Ca^{2+} occlusion is a slow process, in accordance with the original study by Serpersu et al. [11] on membrane bound Ca^{2+}-ATPase. After 8 h incubation a steady state is reached in which 6–7 nmol Ca^{2+}/mg ATPase protein has become occluded. Fig. 2 also shows that Ca^{2+} occlusion can be reversed by addition of EGTA which chelates free Ca^{2+}, thereby stopping new formation of phosphoenzyme. The rate constant for deocclusion is 0.5–1 per hour.

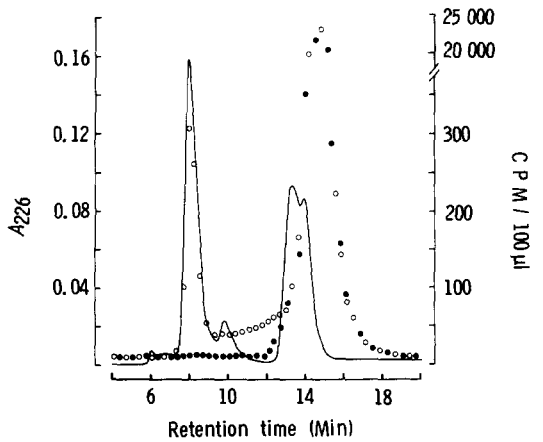

Fig. 1. CrATP-induced Ca^{2+} occlusion in soluble monomeric Ca^{2+}-ATPase. Purified Ca^{2+}-ATPase vesicles (0.5 mg), prepared from rabbit skeletal muscle as in Ref. 1, were solubilized in 1 ml buffer containing 5 mg $C_{12}E_8$/ml, 0.1 M NaCl, 20 mM Tes (pH = 7.0), 123 µM Ca^{2+} (with $^{45}Ca^{2+}$), 10 mM $MgCl_2$ and 5 mM dithiothreitol. The sample was centrifuged for 30 min in a Beckman Airfuge at 130000 × *g*, and the supernatant was incubated at 20°C with 1 mM β,γ-bidentate Cr(III)ATP synthesized and purified as described in Ref. 14. After 4 h 200 µl sample was injected into a TSK G 3000 SW column (7.5 mm × 30 cm) operated at a flow rate of 0.8 ml/min (pressure 8–10 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 $MgCl_2$ and 5 mM dithiothreitol. The collected fractions were analyzed for protein content and radioactivity. The line shows absorbance read at 226 nm. Points show $^{45}Ca^{2+}$ radioactivity; ○, soluble Ca^{2+}-ATPase incubated with CrATP; ●, soluble Ca^{2+}-ATPase incubated in the absence of CrATP. The peak emerging after 8 min corresponds to monomeric Ca^{2+}-ATPase as determined by analytical ultracentrifugation and calibration of the column with standard proteins (cf. Ref. 13). The small peak at 10 min retention time contains mixed micelles of phospholipid and detergent. The peaks at approx. 14 min contain free CrATP and degradation products. The radioactivity associated with the Ca^{2+}-ATPase peak corresponds to 5–6 nmol Ca^{2+}/mg protein.

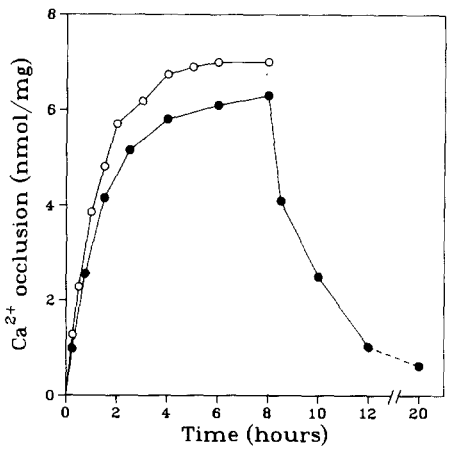

Fig. 2. Time dependence of CrATP-induced Ca^{2+} occlusion in soluble and membrane bound Ca^{2+}-ATPase. Soluble monomeric Ca^{2+}-ATPase (●) was incubated with 1 mM CrATP as described for Fig. 1. After various times aliquots were chromatographed as in Fig. 1 and Ca^{2+} occlusion determined from the radioactivity associated with the protein peak. After 8 h 2 mM EGTA was added to the sample to induce deocclusion. Membrane bound Ca^{2+}-ATPase (○) was incubated likewise but in the absence of detergent. Aliquots were washed with 20 ml buffer (0.1 M NaCl, 20 mM Tes (pH 7.0), 1.5 mM $^{40}CaCl_2$, 1.0 mM EGTA, 10 mM $MgCl_2$) on Millipore filters (0.22 µm) and the radioactivity deposited on the filters was measured.

In addition Millipore filtration experiments performed with membrane bound Ca^{2+}-ATPase are shown in Fig. 2. The results are very similar to those obtained with the soluble monomer (7 nmol Ca^{2+} occluded per mg protein). The small difference can be ascribed to higher stability of membrane bound Ca^{2+}-ATPase during the long incubation period [12].

To obtain the high steady state levels of occluded Ca^{2+} shown in Fig. 2, we found it essential, that our CrATP concentration was kept relatively high (1 mM). At a lower concentration (0.1 mM) the same initial rate of occlusion was observed, but

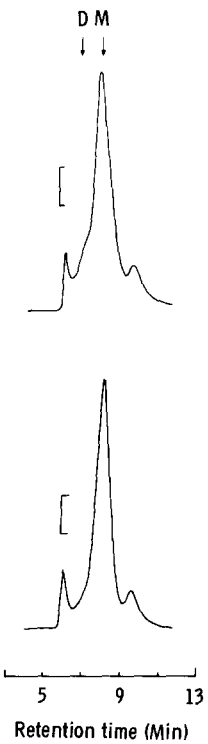

Fig. 3. Effect of CrATP-induced Ca^{2+} occlusion on monomer-dimer equilibrium in soluble Ca^{2+}-ATPase at low detergent concentration. Ca^{2+}-ATPase was solubilized at a detergent to protein concentration ratio of 2:1 and incubated as in Fig. 1. The HPLC was run as in Fig. 1 except that the eluant contained 0.1 mg $C_{12}E_8$/ml and 500 μl protein was injected. Vertical bars indicate ΔA_{226} values corresponding to 0.1 absorbance unit. The upper chromatogram shows Ca^{2+}-ATPase incubated in absence of CrATP. The lower chromatogram shows Ca^{2+}-ATPase incubated in presence of CrATP. D and M indicate elution positions of pure dimers and monomers of Ca^{2+}-ATPase, respectively (cf. [13]).

a steady state of only 3–4 nmol/mg was reached within 2 h. This is probably due to chemical decomposition of CrATP, which is not negligible at pH 7.0.

Equilibria between soluble monomers and oligomers of Ca^{2+}-ATPase can be studied by HPLC at a low detergent concentration [13]. Fig. 3 shows the effect of CrATP induced Ca^{2+} occlusion on the monomer-dimer equilibrium. The presence of dimer in absence of CrATP is indicated by the 'shoulder' on the major peak which furthermore elutes a little in front of the monomer position. Most of the dimer is seen to dissociate into monomer when CrATP has reacted with the soluble protein. This indicates that the association constant for dimer formation is lower in the Ca^{2+} occluded E_1P form relative to the non-occluded E_1 state, present in absence of CrATP.

From these data we conclude, that the minimal functional unit in Ca^{2+} occlusion is constituted by a single Ca^{2+}-ATPase polypeptide chain. Further studies on the cooperativity between the sites involved in Ca^{2+} occlusion are in progress in this laboratory.

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