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Phosphorylated Ca^{2+}-ATPase Stable Enough for Structural Studies*

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The atomic structure of sarcoplasmic reticulum Ca^{2+}-ATPase, in a Ca^{2+}-bound conformation, has recently been elucidated (Toyoshima, C., Nakasako, M., Nomura, H. & Ogawa, H. (2000) *Nature* 405, 647–655). Important steps for further understanding the mechanism of ion pumps will be the atomic structural characterization of different key conformational intermediates of the transport cycle, including phosphorylated intermediates. Following our previous report (Champeil, P., Henao, F., Lacapère, J.-J. & McIntosh, D. B. (2000) *J. Biol. Chem.* 276, 5795–5803), we show here that it is possible to prepare a phosphorylated form of sarcoplasmic reticulum Ca^{2+}-ATPase (labeled with fluorescein isothiocyanate) with a week-long stability both in membranes and in mixed lipid-detergent micelles. We show that this phosphorylated fluorescein isothiocyanate-ATPase can form two-dimensional arrays in membranes, similar to those that were used previously to reconstruct from cryoelectron microscopy images the three-dimensional structure of Ca^{2+}-free unphosphorylated ATPase. The results also provide hope that crystals of phosphorylated Ca^{2+}-ATPase suitable for x-ray crystallography will be achieved.

The detailed architecture of a membranous ion-pump protein, sarcoplasmic reticulum Ca^{2+}-ATPase, in one of its various conformations, has recently been deduced from x-ray crystallography (see Ref. 1, and for commentaries see Refs. 2 and 3). However, the catalytic cycle of this Ca^{2+} pump, like for all P-type membrane ATPases, comprises several intrinsically transient autophosphorylated forms, the processing of which is tightly coupled to the binding or dissociation of calcium and hydrogen ions at distant transport sites. Thus, the next important step for understanding the mechanism of the pump will be obtaining the atomic structures of several of the key conformational intermediates within the transport cycle. Up to now, however, the instability of most intermediate forms has prevented their use for biophysical purposes, especially in the presence of solubilizing concentrations of detergent.

We recently found that after covalent modification of Ca^{2+}-ATPase with fluorescein isothiocyanate (FITC),[1] and under conditions that were in fact already mentioned 20 years ago (4), it is possible to form a phosphorylated species of FITC-ATPase with very unusual characteristics (5), among which is a very low fluorescence of the bound FITC. We now report that this phosphorylated species has an amazing stability, even in the presence of detergent, as well as the ability to form two-dimensional crystalline arrays in the membrane. This form of the Ca^{2+} pump might be appropriate for obtaining three-dimensional crystals and elucidating the atomic structure of a different intermediate of the catalytic cycle of the pump.

RESULTS AND DISCUSSION

A phosphorylated form of FITC-labeled ATPase in sarcoplasmic reticulum vesicles can be formed by acetyl phosphate-induced Ca^{2+} loading of the vesicles followed by calcium chelation (4, 5). This FITC-ATPase species has a low fluorescence (4, 5). We found that the low fluorescence of this species remained stable in the presence of thapsigargin and decavanadate (Fig. 1*a*). The decavanadate-induced fast drop in FITC fluorescence (followed by a slower rise, because the added decavanadate also contained trace amounts of orthovanadate) is essentially due to true fluorescence quenching, as also described for non-phosphorylated FITC-labeled ATPase (6).

Because the same phosphorylated form can be formed from P_i and passively loaded vesicles, at low enough free Ca^{2+} (4, 5), this made it possible to evaluate the stability of the above low fluorescence phosphorylated form on a much longer time scale. We found that the stability of this phosphoenzyme at 20 °C was increased from a few tens of minutes to more than 1 week in the presence of thapsigargin (TG), and the increased stability provided by TG was not impaired by the additional presence of decavanadate (Fig. 1*b*). This was also the case, remarkably, in the presence of a solubilizing concentration of dodecylmaltoside. In the latter case, the increased stability was associated with a slow loss of the sensitivity to Ca^{2+}, because after a few hours the solubilized phosphoenzyme no longer experienced rapid dephosphorylation after addition of Ca^{2+} (data not shown).

We then found that in its membranous state, this special type of phosphorylated FITC-ATPase easily formed two-dimensional arrays in the presence of decavanadate (Fig. 1*c*), with crystalline networks being formed on most vesicles (more than 90%). These arrays accounted for such a large proportion of the ATPases that their formation by the few residual non-phosphorylated ATPases (7–10) is excluded. Nevertheless, in contrast with the two-dimensional crystals formed from phosphorylated scallop ATPase (11), which exhibited a p1 symmetry, our crystals of phosphorylated rabbit ATPase exhibited a p2 symmetry (12), presumably because of the presence of decavanadate-dependent intermolecular links. We are currently defining conditions that allow fusion of vesicles to larger structures reproduc-

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[1] The abbreviations used are: FITC, fluorescein isothiocyanate; TG, thapsigargin; MOPS, 4-morpholinepropanesulfonic acid.

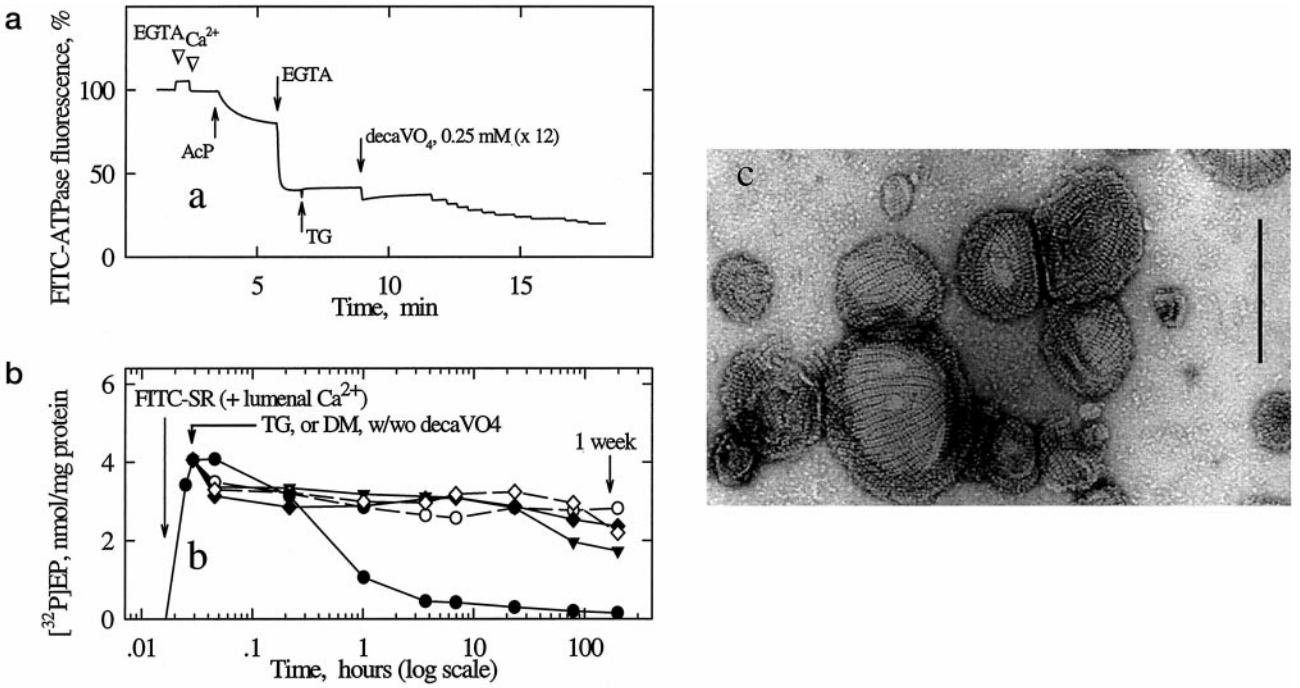

FIG. 1. **A low fluorescence phosphorylated form of FITC-ATPase is stabilized by thapsigargin and/or dodecylmaltoside; if decavanadate is added to the stabilized phosphorylated FITC-ATPase in its membranous form, two-dimensional arrays of ATPase form.** *a*, formation of the low fluorescence phosphorylated form of FITC-ATPase was induced by first actively loading FITC-labeled sarcoplasmic reticulum vesicles (0.1 mg/ml) in the presence of AcP (10 mM; *arrow*) and then adding EGTA (2 mM). The medium also contained 100 mM KCl, 5 mM Mg^{2+} and 50 mM MOPS-Tris (pH 7 and 20 °C), to which 40 μM EGTA followed by 50 μM Ca^{2+} (upside down triangles) had been added in a preliminary step. TG (1 μg/ml) and decavanadate (*decaVO4*; several additions up to 3 mM) were subsequently added. *b*, the stability of the same phosphorylated species, now formed from $[^{32}P]P_i$, was tested over extended periods of time. For this purpose, 1 min after the (nominal) zero time of the experiment (*i.e.* at $t = 0.016$ h), Ca^{2+}-loaded FITC-labeled sarcoplasmic reticulum (*FITC-SR*) vesicles were diluted to 1 mg/ml in a final medium containing 0.25 mM Ca^{2+}, 10 mM EGTA, and 1 mM $[^{32}P]P_i$. *Closed circles*, control experiments. 45 s later (*i.e.* at $t = 0.028$ h), various additions were made to the samples as follows: 10 μg/ml TG (*upside down closed triangles*), 10 μg/ml TG followed within a few seconds by 1 mM decavanadate (*decaVO$_4$*; *closed diamonds*), 5 mg/ml dodecylmaltoside (*DM*; *open circles*), or 10 μg/ml TG followed by 1 mM $decaVO_4$ and 5 mg/ml DM (*open diamonds*). In all cases, 40-μl aliquots were acid-quenched after various periods. *c*, typical two-dimensional arrays of phosphorylated membranous FITC-ATPase were formed in the presence of EGTA, thapsigargin, and decavanadate (typically 1 mM), following either the protocol in *a* or the one in *b* (but without detergent). Arrays of ATPase appeared after incubation at room temperature for 1–3 h. For electron microscopy after negative staining, carbon-coated grids were glow-discharged, 5-μl aliquots of the vesicle suspension were pipetted onto them, and the grids were rinsed with 50 μl of uranyl acetate (1–2%). Grids were then examined in a JEOL 1200EX electron microscope at a magnification of × 50,000. The *bar* corresponds to 200 nm.

ibly and have already obtained micrometer-long tubes appropriate for two-dimensional crystallographic analysis.[2]

As discussed elsewhere, the phosphorylated FITC-ATPase in these arrays should probably be classified as an E1P-like form (or perhaps a transition state-like form) (5), and the remarkable resistance of the aspartyl phosphate to hydrolysis by water confirms this classification. Structural analysis of these arrays by electron microscopy (by projection and three-dimensional reconstruction methods) should be of considerable interest. The fact that the phosphorylated FITC-ATPase remained stable after more than 1 week in the presence of a solubilizing concentration of detergent (Fig. 1*b*) might also open the way toward the future three-dimensional crystallization of a phosphorylated form of ATPase and its analysis by x-ray diffraction. This would provide insight into the detailed structure of at least one of the various phosphorylated intermediates of the Ca^{2+} pump and into the conformational flexibility of the pump.

[2] F. Delavoie, *et al.*, manuscript in preparation.

REFERENCES

1. Toyoshima, C., Nakasako, M., Nomura, H. & Ogawa, H. (2000) *Nature* **405,** 645–655
2. MacLennan, D. H. & Green, N. M. (2000) *Nature* **405,** 633–634
3. McIntosh, D. B. (2000) *Nat. Struct. Biol.* **7,** 532–535
4. Pick, U. (1981) *FEBS Lett.* **123,** 131–136
5. Champeil, P., Henao, F., Lacapère, J.-J. & McIntosh, D. B. (2000) *J. Biol. Chem.* **276,** 5795–5803
6. Highsmith, S., Barker, D. & Scales, D. J. (1985) *Biochim. Biophys. Acta* **817,** 123–133
7. Dux, L. & Martonosi, A. (1983) *J. Biol. Chem.* **258,** 2599–2603
8. Sagara, Y., Wade, J. B. & Inesi, G. (1992) *J. Biol. Chem.* **267,** 1286–1292
9. Stokes D. L. & Lacapère, J.-J. (1994) *J. Biol. Chem.* **269,** 11606–11613
10. Zhang, P., Toyoshima, C., Yonekura, K., Green, N. M. & Stokes, D. L. (1998) *Nature* **392,** 835–839
11. Hardwicke, P. M. D. & Bozzola, J. J. (1989) *J. Muscle Res. Cell Motil.* **10,** 245–253
12. Lacapère, J.-J., Delavoie, F., Henao, F., McIntosh, D. B. & Champeil, P. (2001) *Biophys. J.* **80,** 35.a

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