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A Remarkably Stable Phosphorylated Form of Ca^{2+}-ATPase Prepared from Ca^{2+}-loaded and Fluorescein Isothiocyanate-labeled Sarcoplasmic Reticulum Vesicles*

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After the nucleotide binding domain in sarcoplasmic reticulum Ca^{2+}-ATPase has been derivatized with fluorescein isothiocyanate at Lys-515, ATPase phosphorylation in the presence of a calcium gradient, with Ca^{2+} on the lumenal side but without Ca^{2+} on the cytosolic side, results in the formation of a species that exhibits exceptionally low probe fluorescence (Pick, U. (1981) *FEBS Lett.* 123, 131–136). We show here that, as long as the free calcium concentration on the cytosolic side is kept in the nanomolar range, this low fluorescence species is remarkably stable, even when the calcium gradient is subsequently dissipated by ionophore. This species is a Ca^{2+}-free phosphorylated species. The kinetics of Ca^{2+} binding to it indicates that its transport sites are exposed to the cytosolic side of the membrane and retain a high affinity for Ca^{2+}. Thus, in the ATPase catalytic cycle, an intrinsically transient phosphorylated species with transport sites occupied but not yet occluded must also have been stabilized by fluorescein isothiocyanate (FITC), possibly mimicking ADP. The low fluorescence mainly results from a change in FITC absorption. The Ca^{2+}-free low fluorescence FITC-ATPase species remains stable after addition of thapsigargin in the absence or presence of decavanadate, or after solubilization with dodecylmaltoside. The remarkable stability of this phosphoenzyme species and the changes in FITC spectroscopic properties are discussed in terms of a putative FITC-mediated link between the nucleotide binding domain and the phosphorylation domain in Ca^{2+}-ATPase, and the possible formation of a transition state-like conformation with a compact cytosolic head. These findings might open a path toward structural characterization of a stable phosphorylated form of Ca^{2+}-ATPase for the first time, and thus to further insights into the pump's mechanism.

The SERCA1a[1] Ca^{2+} pump is a P-type membrane ATPase, whose catalytic cycle comprises several intrinsically transient auto-phosphorylated forms, the processing of which is tightly coupled to the binding or dissociation of calcium and hydrogen ions at distant transport sites. Twenty years ago, Pick and Karlish (1) showed that the use of fluorescein isothiocyanate (FITC) as a fluorescent covalent label of Ca^{2+}-ATPase made it possible to monitor conformational changes of the protein. It was subsequently found that FITC specifically labels lysine 515 in the ATPase nucleotide binding domain (2). The fluorescence changes observed upon vanadate or Ca^{2+} binding to FITC-ATPase were generally of relatively small amplitude, but they nevertheless have been widely exploited. Pick also described the formation under specific conditions of an FITC-ATPase species with an exceptionally low fluorescence (3). Surprisingly, however, these latter results were not, to our knowledge, much exploited or mentioned later.

We report here that this FITC-ATPase species of low fluorescence has very unusual functional and energetic characteristics; it is a remarkably stable phosphorylated species, with Ca^{2+} binding sites empty, but oriented toward the cytosolic side and of high affinity. These results are discussed in relation to the putative mechanism for ion transport by Ca^{2+}-ATPase and in relation to the environment of FITC in the ATPase nucleotide binding pocket. The stability of the low fluorescence FITC-ATPase species makes it a good candidate for helping the long-sought structural characterization of a phosphorylated form of Ca^{2+}-ATPase, which would provide significant insight into the conformational flexibility of an ion transport ATPase during its catalytic cycle.

EXPERIMENTAL PROCEDURES

In most experiments, the medium contained 100 mM KCl, 5 mM Mg^{2+}, and 50 mM MOPS-Tris at pH 7 and 20 °C (buffer A). SR vesicles (prepared as in Ref. 4) were labeled with FITC (Sigma F 7250) as described previously (5). In most cases, this incubation was followed by pH neutralization, centrifugation, and resuspension at 20 mg/ml protein in buffer A to which 0.25 M sucrose had been added. Certain aliquots of resuspended labeled vesicles at 20 mg/ml protein were passively loaded by 1–2 h of equilibration with 5 mM Ca^{2+} in buffer A without Mg^{2+}, and frozen without sucrose. Control vesicles were treated similarly but without FITC.

The procedures used for ordinary fluorescence or stopped-flow fluorescence measurements, for $^{45}Ca^{2+}$ binding measurements (either at equilibrium or during rapid filtration with Bio-Logic equipment), and for [^{32}P]*E*P measurements (either without acid quenching or after acid quenching), have already been described (4–8). FITC fluorescence

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[1] The abbreviations used are: SERCA1a, sarcoplasmic or endoplasmic reticulum ATPase, type 1a; SR, sarcoplasmic reticulum; $C_{12}E_8$, octaethylene glycol monododecyl ether; DM, β-D-dodecyl maltoside; MOPS, 4-morpholinepropanesulfonic acid; A23187, calcimycin; P_i, inorganic phosphate; FITC, fluorescein 5′-isothiocyanate; AcP, acetylphosphate; VO_4, vanadate; TG, thapsigargin; *E*1P, *E*2P, names given to the postulated different conformations of phosphorylated ATPase.

(Spex fluorolog or PerkinElmer Life Sciences 650-40 instrument) was generally recorded with excitation and emission wavelengths of 495 and 520 nm, respectively (2- and 5-nm bandwidths), and plotted as percentage of the value in the presence of Ca^{2+} without any correction for dilution effects (dilution was kept below 1.2% for every addition, except for that of 10 mM AcP, which resulted in 4% dilution). The stopped-flow experiments were performed with a Biologic SFM 3 stopped-flow instrument equipped with a short pathlength optical cell (1.5-mm cell, FC15), and data points were collected every 2 or 5 ms. The excitation wavelength was 460 nm, and the emission filter was a broad MTO 531 filter (Massy, France). For the experiment at the final free concentration of about 30 μM, the low fluorescence species was first formed by addition of 6 mM EDTA instead of 2 mM EGTA, and then mixed with 2 mM Ca^{2+} plus 4 mM Mg^{2+}. For phosphorylation experiments performed in the presence of detergent, the phosphorylation reaction was quenched with 15 mM P_i and 0.5 M perchloric acid (*i.e.* 4% v/v) instead of our usual 0.12 M perchloric acid, to precipitate the detergent-solubilized ATPase more easily.

Ca^{2+} uptake into SR vesicles was measured in different ways, among which through changes in absorbance of the calcium-sensitive dye antipyrylazo III (Fluka no. 10795; *e.g.* Ref. 9). Absorbance and turbidity were measured either at a single wavelength or at multiple wavelengths with a diode array HP 8453 spectrophotometer, in a continuously stirred temperature-controlled cuvette. AcP hydrolysis was deduced from proton release, using a pH meter (PHM 62, Radiometer) whose analogic output was amplified (thanks to G. Lecointe in Saclay) to allow acquisition and digitalization.

Acetylphosphate (AcP) was from Sigma (catalog no. A-0262) and was freshly prepared at 250 mM. Orthovanadate solutions were prepared freshly as 100 mM alkaline (colorless) solutions at pH 12, by simple dissolution in water (Sigma S-6508). Decavanadate (yellow) solutions were prepared by titrating and diluting the former solutions first to pH 2 and then to pH 7 (and 50 mM), or preferably to pH 6 to minimize the presence of other forms of vanadate (10). The nonionic detergents $C_{12}E_8$ and DM were obtained from Nikko and Calbiochem (or Anatrace), respectively. Free Ca^{2+} concentrations were computed as described previously (8, 11).

RESULTS

The Puzzling Properties of an Unusually Stable Low Fluorescence FITC-ATPase Species Formed from AcP in the Presence of a Ca^{2+} Gradient—Pick (3) reported previously that, after AcP-dependent Ca^{2+} uptake by FITC-labeled SR vesicles and an additional EGTA-induced rise in the Ca^{2+} gradient across the membrane of these vesicles, an FITC-ATPase species with an unusually low fluorescence was formed. *Trace A* in Fig. 1 shows that this low fluorescence species can be very stable, much more in fact than initially reported by Pick. The poorer stability of the low fluorescence species in Pick's original report appears to be due to the fact that, in his experiment, EGTA addition did not result in a free Ca^{2+} concentration as low as in the experiment illustrated in our *trace A*; *trace B* in Fig. 1 demonstrates this effect of a higher free Ca^{2+}. The low fluorescence species was back-converted to a species of higher but intermediate fluorescence as soon as subsequent addition of Ca^{2+} raised the external Ca^{2+}, and the addition of ionophore immediately brought the fluorescence level up to about 100%. When the ionophore was added before AcP and EGTA, it completely prevented the appearance of any low fluorescence species (*trace C* in Fig. 1). *Traces A--C* therefore imply that large drops in the fluorescence level depend on the presence of a high calcium concentration on the lumenal side of the vesicles, as concluded previously by Pick. In agreement with this view, we found that the half-time for the AcP-induced initial slow drop in fluorescence (in the absence of any Ca^{2+}-precipitating anion) was similar to that required for the accumulation of $^{45}Ca^{2+}$ in FITC-labeled SR vesicles (20–30 s, data not shown).

However, much to our surprise, we observed that when the ionophore was added to the cuvette *after* the formation of the low fluorescence species, the previous drop in FITC-ATPase fluorescence was not reversed, at least when the free Ca^{2+} concentration on the cytosolic side of the ATPase (*i.e.* outside the vesicles) was very low (*trace D* in Fig. 1). Therefore, the existence of a Ca^{2+} gradient is absolutely necessary for the formation of the low fluorescence species, but once this species is formed, it remains stable even if the gradient collapses. *Trace E* in Fig. 1 illustrates another puzzling feature of this low fluorescence species; although its formation is known (3) to require Mg^{2+}, a cofactor of Ca^{2+}-ATPase phosphorylation, once this species was formed, it no longer required Mg^{2+} for stability. In fact, it was even more stable in the absence of Mg^{2+} than in its presence, presumably partly due to EDTA-induced Ca^{2+} chelation and clamping of the free Ca^{2+} concentration at an extremely low value. The low fluorescence species also remained stable after complete detergent-induced solubilization of the vesicles (*trace F*), eliminating the possibility that the resistance to ionophore in *panel D* could be due to lack of permeabilization, and revealing an unusual stability for this detergent-solubilized ATPase in the presence of EGTA. The low fluorescence species remained stable, too, when thapsigargin was added to it (data not shown,[2] but see Fig. 2 below). Addi-

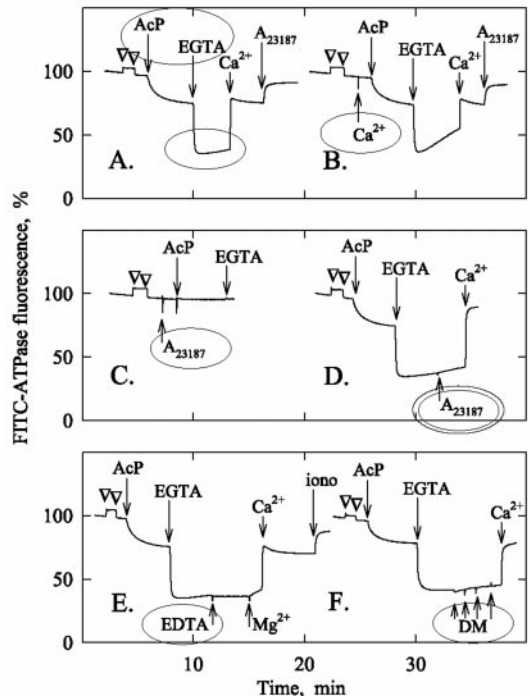

FIG. 1. **The low fluorescence FITC-ATPase species formed from AcP in the presence of a Ca^{2+} gradient has puzzling properties.** FITC-labeled SR vesicles were suspended in 2 ml of buffer A, at 20 μg/ml protein. First, as an internal control for each experiment, 40 μM EGTA followed by 50 μM Ca^{2+} were added (*upside-down triangles*), resulting in the small well known Ca^{2+}-dependent FITC fluorescence changes. In the experiment illustrated by *trace A*, 10 mM AcP was then added, followed by 2 mM EGTA (resulting in a low free Ca^{2+} concentration of about 10 nM), 2 mM Ca^{2+}, and finally 1 μg/ml A23187. In the experiment illustrated by *trace B*, 150 μM Ca^{2+} was added before AcP (EGTA addition now resulted in a slightly higher free Ca^{2+} concentration, about 40 nM). In the experiments illustrated by *traces C* and *D*, similar additions were made, but ionophore was added either before (*C*) or after (*D*) AcP and EGTA. In the experiment illustrated by *trace E*, 6 mM EDTA, followed by 6 mM Mg^{2+}, were added after EGTA; in this case, 1 μg/ml ionomycin was added at the end, instead of A23187 (*iono*). In the experiment illustrated by *trace F*, various aliquots of DM were sequentially added, resulting in final concentrations of 0.1, 0.2, 0.4, and 1 mg/ml; this experiment was performed in a medium containing 250 mM NaCl instead of 100 mM KCl, but qualitatively similar results were obtained in buffer A with $C_{12}E_8$. The first addition of DM was sufficient to reduce sample turbidity to a minimum. The starting time for each trace is arbitrary.

[2] Further details were submitted with the manuscript, but were

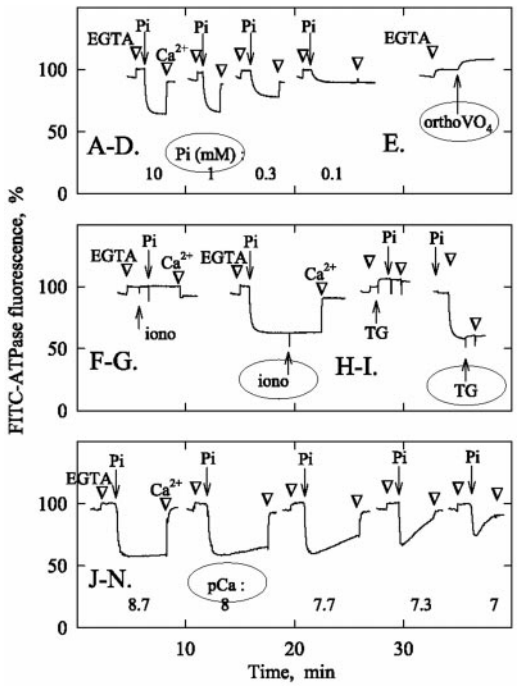

FIG. 2. **The low fluorescence FITC-ATPase species formed from P_i in the presence of a Ca^{2+} gradient has properties similar to those of the species formed from AcP.** FITC-labeled SR vesicles that had been passively loaded with Ca^{2+} were diluted to 20 μg/ml in buffer A. As an internal control for each experiment, EGTA was initially added (*first upside-down triangle*; its concentration was 2 mM for *traces A–I*). In the experiment illustrated by *traces A–D*, P_i was then added at various concentrations (as indicated), followed by 2 mM Ca^{2+} (*second upside-down triangle*). In the experiment illustrated by *trace E*, 0.25 mM orthovanadate (VO_4) was added instead of P_i. In the other experiments, P_i was added at 10 mM; in those illustrated by *traces F* and *G*, additions of EGTA, Ca^{2+}, P_i, and ionophore (2 μM ionomycin) were made in various orders. In the experiment illustrated by *traces H* and *I*, 1 μg/ml TG, was added, either after EGTA and before P_i (*trace H*), or after formation of the low fluorescence species, which here had been formed by adding EGTA after P_i (*trace I*); in the presence of TG, addition of 2 mM Ca^{2+} at the end (*triangle*) was no longer efficient. In the experiment illustrated by *traces J–N*, various concentrations of EGTA were initially added, resulting in calculated final free Ca^{2+} concentrations corresponding to the *p*Ca values indicated.

tion of up to 10 mM ATP or ADP, either in the presence or absence of Mg^{2+}, had no effect either on the low fluorescence species, including after its formation from partially labeled vesicles (data not shown).

The Similar Properties of the Low Fluorescence FITC-ATPase Species Formed from Inorganic Phosphate in the Presence of a Ca^{2+} Gradient—Pick reported that a low fluorescence species could also be formed after adding both EGTA and P_i (irrespective of the order) to FITC-labeled vesicles previously loaded passively with Ca^{2+}, *i.e.* after phosphorylation from P_i in the presence of a calcium gradient (3). We fully confirmed this observation (Fig. 2*A*). The maximal amplitude of the observed signal was smaller than that obtained when experiments were performed with AcP and FITC-labeled vesicles. This smaller amplitude was presumably due to the fact that some of the vesicles had lost their impermeability during passive Ca^{2+} loading (12) and/or freezing. The P_i-dependence of the formation of the low fluorescence species revealed a relatively high apparent affinity for P_i (half-amplitude was obtained for 0.2–0.3 mM P_i, see *traces A–D* in Fig. 2), characteristic of gradient-dependent phosphoenzyme formation (13). Note that orthovanadate (Fig. 2, *trace E*) was not able to replace P_i and induced the same changes with Ca^{2+}-loaded vesicles as those previously observed with nonloaded vesicles (14).

We found that the low fluorescence species formed from P_i and Ca^{2+}-loaded vesicles had puzzling properties, too. Although the formation from P_i of this low fluorescence species was strictly dependent on lumenal Ca^{2+} (as deduced from the fact that preliminary addition of ionophore completely prevented its appearance), once formed, this low fluorescence species was again resistant to the subsequent addition of ionophore (Fig. 2, *traces F* and *G*), or even to the addition of detergent at solubilizing concentrations, as already described for the species formed from AcP in Fig. 1 (data not shown). Similarly, although the formation from P_i of this low fluorescence species was inhibited by thapsigargin, an inhibitor of Ca^{2+}-dependent changes as well as of phosphorylation from P_i (15), once formed, this species was resistant to the addition of thapsigargin (Fig. 2, *traces H* and *I*). Addition of orthovanadate to a preformed low fluorescence species brought the fluorescence back to a high level, at a concentration-dependent rate (data not shown), but decavanadate could bind to this species without destabilizing it; about the same relative quenching was observed when decavanadate was added to control FITC-ATPase (16) or to a low fluorescence TG-stabilized FITC-ATPase species formed from AcP (data not shown).[2]

Traces J–N in Fig. 2 show how formation of the low fluorescence species was influenced by the final free Ca^{2+} concentration in the medium. The low fluorescence species, once formed, was much more stable when the free Ca^{2+} concentration dropped to a very low level than when it was only moderately low, as shown above when the low fluorescence species was formed after AcP-mediated calcium uptake (*cf. traces A* and *B* in Fig. 1). The *p*Ca dependence of the amplitude of the P_i-induced drop revealed a high affinity (*p*Ca½ was about 7, *i.e.* Ca½ was submicromolar), which in fact was slightly higher than the overall affinity for Ca^{2+} binding to SR ATPase under the same conditions (micromolar Ca½; see Ref. 7).

Ca^{2+} Is Not Bound to the Low Fluorescence FITC-ATPase, yet This ATPase Species Remains Phosphorylated and Phosphorylation Is More Stable than without FITC—Since the existence of a Ca^{2+} gradient is required for the initial formation of a low fluorescence FITC-ATPase species, Pick initially concluded that this species would contain 2 or at least 1 bound Ca^{2+} ion(s) (3). If this were the case, as this species, once formed, is resistant to the subsequent addition of ionophore or detergent, the putative bound Ca^{2+} ion(s) should be occluded. We tested this possibility by loading FITC-labeled SR vesicles with $^{45}Ca^{2+}$, creating a low fluorescence species by adding EGTA, P_i, and then ionophore, and measuring the amount of $^{45}Ca^{2+}$ bound to the ATPase: under these conditions, however, this residual amount dropped to values smaller than 0.2 nmol/mg of protein, *i.e.* to values much smaller than the ATPase contents in SR vesicles, which typically is 5–7 nmol/mg (Fig. 3*A*; see also Ref. 6). The results were similar when the Ca^{2+}-loaded vesicles were first diluted in EGTA and P_i was added afterward, or if partially labeled vesicles were used instead of fully labeled vesicles (data not shown). Therefore, Ca^{2+} is not occluded in the low fluorescence FITC-ATPase species; in this ATPase species, the transport sites are *not* occupied by Ca^{2+} at all.

Nevertheless, we found that the low fluorescence FITC-ATPase species was phosphorylated to a high level (Fig. 3*C*), in fact higher than that measured for unmodified ATPase under similar conditions (Fig. 3*D*). This phosphorylation level (close to 4 nmol/mg, *i.e.* a significant proportion of the above-mentioned ATPase contents) remained stable, whereas the level of the phosphoenzyme formed from unmodified vesicles slowly declined with time over minutes, presumably due to the dissi-

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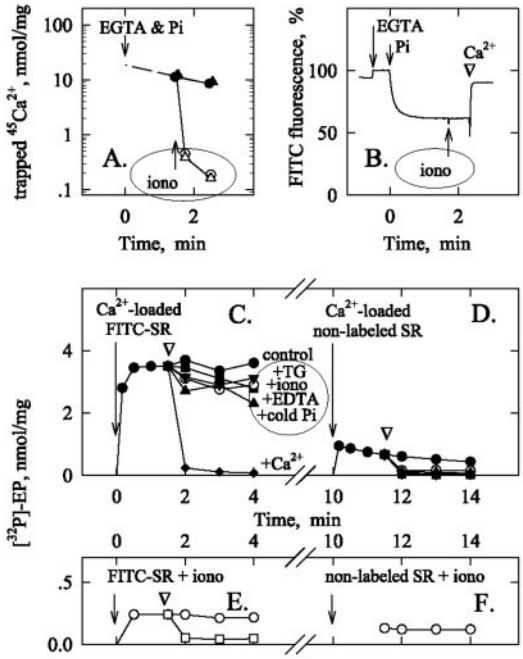


FIG. 3. **The low fluorescence FITC-ATPase species formed from P_i in the presence of a Ca^{2+} gradient does not retain Ca^{2+}; the resulting phosphoenzyme is stabilized by FITC.** *Panel A*, FITC-labeled SR vesicles (20 mg/ml) were passively loaded with 5 mM $^{45}Ca^{2+}$. At time 0, vesicles were diluted 1:20 in buffer A, to which 5 mM EGTA and 10 mM P_i had been added. After various periods, 40-μl aliquots were diluted in 4 ml of buffer A, containing either 0.1 mM Ca^{2+} (*circles*) or EGTA and P_i as in the initial medium (*triangles*), filtered on an HA Millipore filter, washed twice with the same medium, and counted (*closed symbols*). Alternatively (*open symbols*), 0.01 mg/ml ionomycin was added to the vesicles after 1.5 min, and 40-μl aliquots were again processed after various periods. *Panel B* illustrates a control fluorescence experiment (*cf.* Fig. 2*G*). *Panels C* and *D*, at (nominal) time 0 or 10, Ca^{2+}-loaded FITC-labeled vesicles (*panel C*) or unlabeled vesicles (*panel D*) (at 20 mg/ml in a 5 mM Ca^{2+} medium) were diluted to 1 mg/ml in buffer A supplemented with 1 mM $[^{32}P]P_i$ and 10 mM EGTA (free Ca^{2+} was about 10 nM). Aliquots (40 μl) were acid-quenched after various periods and filtered (*closed circles*). For some of the samples, after 1.5 min we added one of the following: 0.01 mg/ml ionomycin (*open circles*), 8 mM EDTA (*squares*), 10 μg/ml TG (*upside-down triangles*), 20 mM cold P_i (*rightside-up triangles*), or 10 mM Ca^{2+} (*diamonds*). *Panels E* and *F*, same experiment as that illustrated in *panels C* and *D*, but with vesicles made leaky with ionomycin before phosphorylation (ionophore/protein was 1% w/w). *Circles*, control experiments. *Squares* in *panel E*, EDTA was added after 1.5 min.

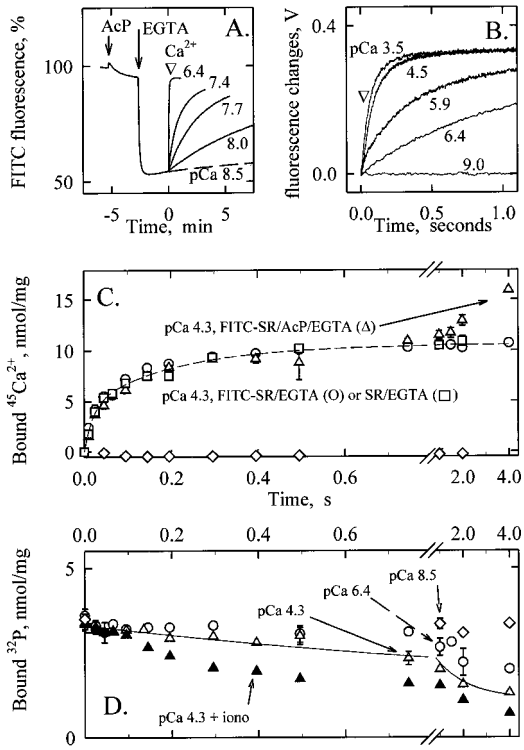


FIG. 4. **Ca^{2+} binding to the low fluorescence species occurs with fast kinetics and returns the ATPase to the normal cycle for Ca^{2+} internalization and dephosphorylation.** *Panel A*, conventional fluorescence experiments. The low fluorescence FITC-ATPase species was formed essentially as in Fig. 1, by sequential addition of 2 mM AcP and 2 mM EGTA (final *p*Ca was about 8.5, because of the presence of 15 μM Ca^{2+}). At (nominal) time 0, Ca^{2+} was then added, leading to the *p*Ca values indicated. *Panel B*, stopped flow fluorescence experiments. The low fluorescence species, initially formed (at 80 μg protein/ml) by 2.5-min incubation with 2 mM AcP, followed by Ca^{2+} chelation, was mixed with various EGTA- or calcium-containing solutions (final *p*Ca values are indicated). *Panel C*, kinetics of $^{45}Ca^{2+}$ binding during rapid perfusion. FITC-ATPase was prepared either in its low fluorescence phosphorylated state (as shown in *panels A* and *B*, but now at 0.3 mg of protein/ml, *triangles*), or in its control nonphosphorylated but Ca^{2+}-deprived state (*circles*); control unmodified ATPase was also prepared (*squares*). In all cases, 0.3 mg of protein was adsorbed onto a Millipore HA filter, manually rinsed for a few seconds with 100 μM EGTA, and perfused with 50 μM $^{45}Ca^{2+}$ for various periods (see *abscissa*). Experiments were repeated in the absence of membranes for control (*diamonds*). *Panel D*, kinetics of Ca^{2+}-induced dephosphorylation. ^{32}P-labeled phosphoenzyme was prepared as in Fig. 3*C*. At t = 1.5 min, aliquots were diluted to 2 ml with a solution containing 1 mM EGTA, filtered, rinsed twice with the same solution and a third time with a solution containing only 0.1 mM EGTA, and at t = 2 min, the sample was finally perfused for various periods (see *abscissa*), with a solution buffered at *p*Ca 4.3 (*triangles*), *p*Ca 6.4 (*circles*), or *p*Ca 8.5 (for control, *diamonds*). To some of the samples (*closed triangles*), 0.01 mg/ml ionomycin had been added at t = 1.2 min.

pation of the Ca^{2+} gradient. Just like the low fluorescence species, the phosphoenzyme formed from FITC-ATPase and $[^{32}P]P_i$ was resistant to ionophore-induced (or detergent-induced) collapse of the Ca^{2+} gradient, the EDTA-induced removal of Mg^{2+}, addition of thapsigargin (with or without decavanadate) and dilution with unlabeled P_i, whereas it was completely abolished by a rise in the Ca^{2+} concentration (Fig. 3*C* and data not shown).[2] These properties are at variance with the conventional ones of gradient-dependent phosphoenzyme formed from unmodified vesicles incubated with $[^{32}P]P_i$ (Fig. 3*D*), as well as with the properties observed with either FITC-modified or unmodified vesicles previously made leaky to Ca^{2+} (Fig. 3, *E* and *F*).

Ca^{2+} Binds to the Phosphorylated Low Fluorescence FITC-ATPase Species from the Cytosolic Side, and Then Gets Transported into the SR Lumen—The fact that, in the experiment illustrated in Fig. 3*C*, the addition of cold P_i had no effect on the $[^{32}P]EP$ level of FITC-ATPase implies that the phosphorylated low fluorescence species is *not* in rapid equilibrium with unphosphorylated ATPase and P_i in the medium. However, when the external Ca^{2+} concentration was raised, the low fluorescence FITC-ATPase species was back-converted to a species with higher fluorescence (Figs. 1 and 2). This implies that Ca^{2+} interacts directly with the phosphorylated low fluorescence species. In view of the fast effect of Ca^{2+} (Figs. 1, 2, and 3*C*) compared with its relatively slow permeation through the membrane (Fig. 3*A*), this also suggests that Ca^{2+} binds to the phosphorylated low fluorescence species from the external, cytosolic side. This would be an unconventional conclusion, because phosphoenzyme formation is generally thought to be associated with the reorientation of Ca^{2+} sites toward the lumen, or at least toward the interior of the membrane for ion occlusion (17, 18). In the next experiments, we therefore studied in some detail the kinetics of the events associated with Ca^{2+} binding to the low fluorescence species.

We initially investigated them by forming a low fluorescence

species of FITC-ATPase (by adding EGTA after AcP-supported Ca^{2+} uptake) and then monitoring the rate at which subsequent addition of Ca^{2+} reversed the previous drop in FITC fluorescence. In the range of submicromolar Ca^{2+} concentrations, this reversal was slow enough to be monitored with a regular fluorometer, but it accelerated when the free Ca^{2+} concentration was raised (Fig. 4*A*). For micromolar Ca^{2+} concentrations, we had to use stopped-flow detection (Fig. 4*B*). We found that the rate constant of the fluorescence rise was about 10 s^{-1} at 30 μM free Ca^{2+} (*p*Ca 4.5), and this was almost the maximal value (the rates measured at 300 μM and 2 mM free Ca^{2+} were 13 and 15 s^{-1}, respectively). This rate was of the same order of magnitude as that for Ca^{2+} binding to dephosphorylated native ATPase in the absence of nucleotides under similar conditions (19, 20). The Ca^{2+} dependence of the stability of the low fluorescence species was qualitatively similar after collapsing the calcium gradient by either ionophore or detergent (data not shown).

We also directly measured, with rapid filtration equipment at a free Ca^{2+} concentration of 50 μM, the kinetics of $^{45}Ca^{2+}$ binding to the low fluorescence phosphorylated species, as well as, in control experiments, the kinetics of $^{45}Ca^{2+}$ binding to either nonphosphorylated FITC-labeled ATPase or unlabeled SR-ATPase. For short perfusion periods of up to 1 s, the binding patterns were essentially similar (Fig. 4*C*), and biphasic as found previously (21, 22); together with the above stopped-flow fluorescence data, this confirms that the Ca^{2+} binding sites on the low fluorescence species are as accessible from the external compartment of the vesicles as the binding sites on the control nonphosphorylated ATPase with or without FITC. However, for longer periods of $^{45}Ca^{2+}$ perfusion onto the FITC-ATPase species of initially low fluorescence, the amount of $^{45}Ca^{2+}$ associated with the vesicles appeared to slowly rise to levels higher than those required for saturation of the two high affinity binding sites (*triangles* in Fig. 4*C*; see below for interpretation).

In the final experiment of this series, we then measured the rate of Ca^{2+}-induced dephosphorylation of the low fluorescence species formed from [^{32}P]P_i and Ca^{2+}-loaded FITC-labeled SR. This rate turned out to be slower than the rate at which the fluorescence rose and the rate at which Ca^{2+} binding took place (*open triangles* in Fig. 4*D*, compare with panels *B* and *C*). The slow dephosphorylation was accelerated (nevertheless remaining slower than Ca^{2+} binding) when the low fluorescence species was treated with ionophore before Ca^{2+} was added (*closed triangles* in Fig. 4*D*), while the phosphoenzyme remained stable when no Ca^{2+} was added, as expected (*diamonds* in Fig. 4*D*). Our interpretation is that addition of Ca^{2+} to the Ca^{2+}-free low fluorescence phosphorylated species allows the ATPase to re-enter the catalytic cycle and permits ATPase dephosphorylation through the normal forward pathway, in which dephosphorylation after Ca^{2+} internalization is slow compared with Ca^{2+} binding, especially in the presence of lumenal Ca^{2+}. In Ca^{2+}-tight vesicles, the internalization of those $^{45}Ca^{2+}$ ions that have initially interacted with the low fluorescence species combines with the passive rebinding of $^{45}Ca^{2+}$ (resulting from continuous perfusion) to newly available dephosphorylated ATPase to explain why bound $^{45}Ca^{2+}$ slowly rises to a final level higher than that required for saturation of the ATPase high affinity binding sites (as shown by the *open triangles* in Fig. 4*C*).

When the Rapid AcP-dependent Turnover of FITC-ATPase Results in Ca^{2+} Depletion, a Low Fluorescence Species Forms Spontaneously; FITC Absorbance Also Changes—In addition, we found that the low fluorescence FITC-ATPase species can form spontaneously after AcP-dependent Ca^{2+} pumping, before

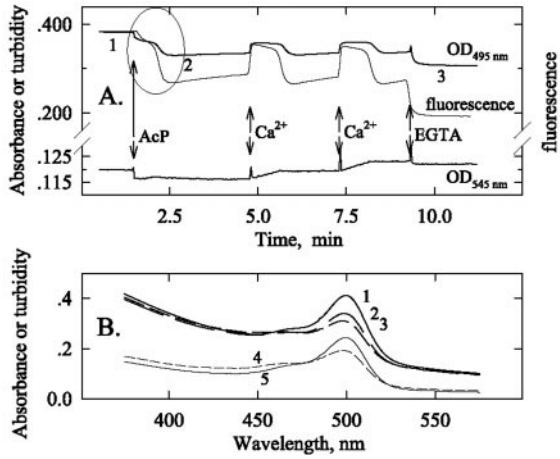


FIG. 5. **Spontaneous conversion of FITC-ATPase into a low fluorescence species, with a different absorption spectrum, after Ca^{2+} depletion in the presence of oxalate.** *Panel A*, buffer A was supplemented with 8 mM oxalate, 40 μM Ca^{2+}, and 0.4 mg/ml SR vesicles that had just been incubated with FITC (2 mg/ml SR and 16 μM FITC for 60 min). 10 mM AcP was added to trigger Ca^{2+} uptake. 100 μM Ca^{2+} was subsequently added twice, followed by 1 mM EGTA. FITC fluorescence at this high protein concentration was recorded (*thin bottom line*, plotted after appropriate normalization). In a parallel measurement, optical densities at 495 nm (*top thick trace*) and 545 nm (*bottom trace*) were recorded. The 545-nm trace reflects changes in turbidity mainly due to (initially delayed) precipitation of Ca^{2+}-oxalate. *Panel B*, absorption spectra recorded at various times during such an experiment. As indicated in *panel A*, spectra were recorded: *1*, in the initial state; *2*, after AcP-dependent withdrawal of Ca^{2+} from the medium, and *3*, after EGTA addition. At this point, 0.5 mg/ml $C_{12}E_8$ was added, resulting in *spectrum 4*. Readdition of Ca^{2+} to the now solubilized sample resulted in *spectrum 5* (see Fig. 1*F* for the related fluorescence recovery).

re-addition of Ca^{2+} leads to another round of pumping. This was shown by the fact that in the presence of oxalate and a high enough concentration of vesicles, *i.e.* when these vesicles are capable of depleting the free Ca^{2+} concentration in the medium down to submicromolar values, FITC-ATPase was to a large extent converted spontaneously into a low fluorescence species, after a lag corresponding to the completion of Ca^{2+} withdrawal from the medium (*thin top trace* in Fig. 5*A*). Subsequent sequential Ca^{2+} additions, which triggered renewed Ca^{2+} uptake, drove the fluorescence level back toward a higher value until the added Ca^{2+} was pumped into the vesicles again. Note that, in such experiments, the slight increase in turbidity concomitant with the lumenal precipitation of calcium oxalate (23, 24) may serve as a marker of the completion of Ca^{2+} uptake, even in the absence of any Ca^{2+}-sensitive dye (*bottom thick trace* in Fig. 5*A*). In parallel experiments, we confirmed that the rates of AcP hydrolysis and AcP-dependent Ca^{2+} uptake after formation of the first microcrystals of calcium oxalate were only marginally slower for FITC-modified ATPase than for unmodified ATPase (in agreement with Ref. 5); coupling ratios were also unaltered by FITC modification. However, FITC-modified ATPase had become completely unable to hydrolyze ATP, as expected (data not shown).[2]

Optical density recordings under the exact same conditions allowed us to conclude that changes in FITC fluorescence were in fact due, at least in part, to changes in FITC absorbance (*thick top trace* in Fig. 5*A*). Using a diode-array spectrophotometer, it was possible to reveal the changes in the entire FITC absorption spectrum, as they were large enough to show up on top of the light scattering by the vesicles (Fig. 5*B*, *spectra 1–3*). These changes were visualized even more easily after detergent-induced solubilization of the vesicles (*spectra 4* and *5*), which was shown previously (Fig. 1*F*) to leave the low fluorescence species stable until Ca^{2+} was re-added. Similar changes

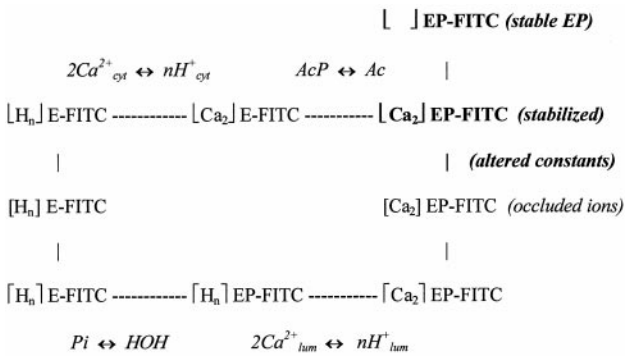
in FITC absorbance were also seen after Ca^{2+} depletion in the presence of 25 mM P_i instead of oxalate, or in the absence of Ca^{2+}-precipitating anions (data not shown). FITC is known to have an absorption spectrum (and not only a fluorescence spectrum) highly sensitive to protonation, polarity, or interactions (25, 26). FITC spectral absorbance changes for phosphorylated FITC-ATPase after Ca^{2+} depletion are qualitatively similar to those occurring for FITC in either an acidic or an apolar medium.

Absence of Special Interactions between Chains in the Low Fluorescence FITC-ATPase—We asked whether the unusual properties of the phosphorylated FITC-ATPase species were due to the appearance of unrecognized ATPase-ATPase interactions. The answer, however, was no: first, because similar spectroscopic properties were observed with ATPases that had been labeled with FITC only partially (1 nmol of FITC molecule bound/mg of protein instead of 5–7 nmol/mg) (data not shown); second, because measurements of the fluorescence polarization of bound FITC (an index of ATPase-ATPase proximity) showed that most of the homotransfer-induced depolarization of the bound FITC in FITC-labeled vesicles was lost upon solubilization at low Ca^{2+} concentrations, whereas the low fluorescence species remained stable (data not shown); and third, because size exclusion chromatography experiments indicated that this detergent-solubilized low fluorescence FITC-ATPase species was still essentially monomeric (data not shown).

DISCUSSION

AcP-dependent Ca^{2+} uptake followed by chelation of external Ca^{2+}, as well as phosphorylation from P_i in the presence of a Ca^{2+} gradient, both permit accumulation of a phosphorylated FITC-ATPase species with unusual properties. The species has an extremely low fluorescein fluorescence, much lower than any other catalytic intermediate of the ATPase cycle. Once it has been formed, it no longer depends on the persistence of a Ca^{2+} gradient or a membranous state. The phosphorylated and low fluorescence species has vacant, outwardly oriented, high affinity Ca^{2+} binding sites. It is very stable, as long as the free Ca^{2+} concentration is kept close to zero, but re-addition of Ca^{2+} causes inward Ca^{2+} transport followed by dephosphorylation. All these properties point to a novel species of Ca^{2+}-ATPase, which appears to be of special interest both for the present functional description of the mechanism of ion transport and for the future structural studies of phosphorylated forms of the pump.

Relationship of the Low Fluorescence Species to the Usual Catalytic Intermediates—It was suggested that the Ca^{2+}-ATPase catalytic scheme comprises four major enzyme intermediate species, namely phosphorylated or nonphosphorylated ATPase with or without bound Ca^{2+} (Ref. 27; reviewed in Ref. 28). In terms of a simple four-species scheme, the Ca^{2+}-free nonphosphorylated form of ATPase must expose its Ca^{2+}-binding sites toward the cytosolic side of the SR, whereas the Ca^{2+}-bound phosphorylated ATPase must expose its Ca^{2+} binding sites toward the lumenal side (29). The same rationale is valid for ATP-supported or AcP-supported activity, since both catalytic cycles appear to be similar (30–32). In Ca^{2+}-accumulating vesicles, phosphoenzyme hydrolysis slowing down by the high lumenal concentration Ca^{2+} should thus lead to steady-state accumulation of the Ca^{2+}-bound phosphoenzyme; if excess EGTA is then added, even more of this phosphoenzyme should form as a result of the reaction of P_i (derived from AcP hydrolysis) with residual nonphosphorylated enzyme, by the reverse reaction. The same Ca^{2+}-bound phosphoenzyme should also be formed from P_i (again by the reverse reaction) in experiments performed with passively Ca^{2+}-loaded vesicles like those illustrated in Fig. 2. However, in the context of such a four-species scheme, a phosphoenzyme species with outwardly oriented Ca^{2+} binding sites cannot be generated upon addition of EGTA to Ca^{2+}-loaded SR vesicles, and this phosphoenzyme cannot remain stable after disruption of the initial Ca^{2+} gradient. Consideration of the more recent hypothesis that during transport, Ca^{2+} ions move from a first, cytosolically oriented pair of sites to a second, lumenally oriented pair of sites (*e.g.* Ref. 33) does not help much, since in that alternative view the cytosolically oriented pair of sites is no longer accessible after ATPase phosphorylation (see Fig. 1 in Ref. 33). In addition, no evidence for Ca^{2+} binding to any lumenal site was found in the atomic structure of Ca^{2+}-ATPase derived from protein crystallized in the presence of 10 mM Ca^{2+} (34).



SCHEME 1. **Post-Albers-type model with occluded states and transported protons.**

Additional intermediate forms of ATPase have been suggested to exist within the ATPase catalytic cycle (35–40). As a result, more elaborate schemes now explicitly include different forms for Ca^{2+}-bound phosphorylated ATPase (permitting interconversion between the outside and inside orientations of occupied Ca^{2+} sites), different forms for Ca^{2+}-free nonphosphorylated ATPase (permitting interconversion between the outside and inside orientations of free Ca^{2+} sites), and distinct enzyme forms permitting ion "occlusion" (either for Ca^{2+} or for the counter-transported protons) (40, 41); this is illustrated by the main cycle in Scheme 1. Of course, some of the various intermediate forms postulated by such schemes may be very transient during turnover. For instance, with unmodified vesicles, since dissociation of Ca^{2+} ions to the outside of the vesicles was found to become impossible almost concomitantly with ATPase phosphorylation (17, 18), the early phosphoenzyme form with outwardly oriented sites in Scheme 1, $\lfloor Ca_2 \rfloor E$P, must be present in only small amounts. A transient species, however, can conceivably be made more stable by manipulating experimental conditions or modifying the enzyme.

We have shown here that when FITC-modified vesicles are suddenly depleted of external calcium after AcP-dependent loading (Fig. 1), or when Ca^{2+}-loaded FITC-labeled vesicles are phosphorylated from P_i in the absence of cytosolic Ca^{2+} (Fig. 2), a Ca^{2+}-free phosphorylated form accumulates (Fig. 3), with outwardly oriented (cytosolically oriented) high affinity sites (Fig. 4). In the context of the above discussion about the ATPase catalytic scheme, the simplest explanation for these results is that, in FITC-labeled Ca^{2+}-loaded vesicles, the early phosphorylated ATPase with outwardly oriented Ca^{2+} sites, $\lfloor Ca_2 \rfloor E$P-FITC in Scheme 1, is stabilized and now constitutes a very significant fraction of total phosphoenzyme, from which Ca^{2+} can dissociate toward the cytosolic side. Note that stabilization by FITC of the total level of *E*P formed from P_i in the presence of lumenal Ca^{2+} is not an interpretation, but a fact (see Fig. 3, *C* and *D*).

Stabilization of phosphorylated ATPase with Ca^{2+} sites not

yet occluded might be derived from changes in either the forward or the reverse rate of occlusion in Scheme 1. Since it is known that this occlusion reaction is not rate-limiting in the normal cycle, such FITC-dependent alterations would not greatly reduce the overall AcP-dependent turnover in leaky vesicles or in the presence of oxalate, as actually observed. However, in tight vesicles with high lumenal Ca^{2+}, the $\lfloor Ca_2 \rfloor$*E*P-FITC species could accumulate, and at a low enough external free Ca^{2+} concentration a phosphorylated species with unoccupied Ca^{2+} sites facing the cytosol, $\lfloor\ \rfloor$*E*P-FITC in Scheme 1, could finally be formed from the previous one. A scheme similar to Scheme 1 (except for omission of the occluded states) was in fact proposed previously as an alternative to an overly simple four-species scheme, to explain AcP-dependent $^{45}Ca^{2+}$-$^{40}Ca^{2+}$ exchange, an exchange that is slow but nevertheless measurable in normal SR (42). Note that, even though the early phosphoenzyme form might be very transient in the absence of FITC, it implies that phosphorylation actually precedes ion occlusion itself and does not occur simultaneously with it. Note also that the absence of Mg^{2+} at the catalytic site of the "*E*1P" phosphoenzyme has also been shown to stabilize a phosphoenzyme form with open Ca^{2+} sites, thereby permitting Ca^{2+} release toward the cytosolic side (43).

Implications for the Catalytic Cycle of Both FITC-Modified and Unmodified ATPase: A Role for ADP Dissociation in the Occlusion Process, and Long Distance Coupling?—To understand the role of FITC in the stabilization of the early and open $\lfloor Ca_2 \rfloor$*E*P-FITC form (with intermediate fluorescence) that precedes ion occlusion, it is worth mentioning that previous experiments with a photoactivatable analog of ATP (TNP-8-azido-ATP) revealed that, when this analog was covalently tethered to the ATPase active site at Lys-492, most of its slow hydrolysis was uncoupled from Ca^{2+} transport, again as if the transport sites had remained open to the cytosolic medium (44). This did not occur with the free TNP nucleotide, which exhibited tight coupling of calcium transport and phosphoenzyme hydrolysis. In the phosphorylated FITC-labeled ATPase, as in the phosphorylated ATPase with a tethered nucleotide analog, the nucleotide site remains permanently occupied, perhaps mimicking partly a state in which ADP has not yet dissociated itself from the unmodified ATPase (45). It might thus be speculated that, following phosphorylation, ADP dissociation from the phosphoenzyme plays a significant role for fast closure of the cytosolic gate of the ion transport site. With unmodified ATPase, from which ADP dissociates rapidly, the early phosphoenzyme form with transport sites still open toward the external side of the SR vesicles would be very transient, while bound FITC or the tethered analog might stabilize this form by mimicking to some extent bound ADP. To our knowledge, the impact of ADP dissociation on ion dissociation from other P-type phosphorylated ATPases has not much been studied. The possibility we suggest should perhaps be kept in mind as an appealing speculation, although it is fair to say that it is not immediately reconciled with the fact that the tight binding of Cr.ATP to *un*phosphorylated Ca^{2+}-ATPase favors Ca^{2+} occlusion (39, 46).

At this point, it is also worth saying a word about the relative fluorescence level of the two phosphoenzyme forms with outwardly oriented Ca^{2+} sites, $\lfloor Ca_2 \rfloor$*E*P-FITC and $\lfloor\ \rfloor$*E*P-FITC. The latter obviously has a low fluorescence level. The former, if it indeed accumulates at steady state in the presence of external Ca^{2+}, must have intermediate fluorescence (Fig. 1). The difference between the low and intermediate levels of fluorescence (and absorbance, see Fig. 5) reflects rearrangement of the FITC environment upon Ca^{2+} dissociation or binding, and illustrates the long distance coupling between the transport sites and the catalytic domain. Differences in fluorescence level have functional counterparts, since the Ca^{2+}-free $\lfloor\ \rfloor$*E*P-FITC species is unusually stable and hardly reacts with water, while rapid re-binding of Ca^{2+} allows the enzyme to re-enter the cycle for normal handling of the phosphoenzyme (Fig. 4). The absolute requirement for Ca^{2+} at the transport site for normal phosphoenzyme processing highlights the extraordinary degree to which partial reactions at the catalytic site can be coupled to changes at distant transport sites by long range transmission of information (47).

By Which Molecular Mechanism Does Fluorescein Stabilize the Phosphorylated Form of FITC-ATPase?—FITC reacts with lysine 515 at the high affinity nucleotide binding site (2, 5, 48), probably as an affinity label, mimicking nucleotides. Although under our conditions FITC derivatization increased 3–4-fold the amount of phosphoenzyme formed from 1 mM P_i in the presence of a calcium gradient, and made it unusually stable (Fig. 3, *C* and *D*), FITC seems to have only slight effects on most individual steps of the cycle, including dephosphorylation and Ca^{2+} binding (Refs. 5 and 7; see also Fig. 4*B*); Ca^{2+} occlusion can also occur (although with a modified rate, as discussed above), since Ca^{2+} is taken up efficiently in the presence of AcP. Thus, FITC does not sterically block any essential conformational change. For understanding the effect of FITC on the stability of the intermediate and low fluorescence species, $\lfloor Ca_2 \rfloor$*E*P-FITC and $\lfloor\ \rfloor$*E*P-FITC, respectively, a clue might come from the spectroscopic changes experienced by the fluorescein moiety under these conditions.

The absorbance (and therefore fluorescence) of fluorescein is critically dependent on its protonation state and/or the hydrogen bonding power of the environment (25, 26). Fluorescein has two ionizing groups, the xanthene phenolic group, 3-OH, with a pK_a of about 6.7, and the benzoate carboxyl group, with a pK_a of about 4.5. The dianionic species is strongly absorbant at 495 nm, whereas the monoanion is much less so. Therefore, one explanation for the unusually low fluorescence and absorption of the $\lfloor\ \rfloor$*E*P-FITC species is an increase in the pK_a of the phenolic 3-OH, which would stabilize the protonated form. This could arise either from an increase in hydrophobicity around this group or from the close approach of a negatively charged residue. Alternatively the fluorescence may be quenched by a salt linkage of the negatively charged 3-O^- to a positively charged residue. Lys-515, to which the fluorescein moiety is covalently attached in FITC-modified Ca^{2+}-ATPase, is located deep in the nucleotide binding pocket, while the rest of this pocket as well as the region surrounding the phosphorylatable aspartate contains a large number of charged residues (34). Since the ATPase turnover probably involves large relative movements of the various subdomains in the ATPase cytosolic head (34), we suggest that the large drop in FITC fluorescence and absorbance associated with formation of an early phosphoenzyme form might reflect the fact that, at this step, the FITC moiety is brought toward the walls of the cavity, which could easily result in a charged residue being very close to the 3-O^- of the fluorescein. This could result in salt type bonding if a positive charge approaches. It is also possible that if a negative charge approaches and results in protonation (change in pK_a) for the fluorescein 3-OH, the hydroxyl group could now hydrogen-bond to neighboring residues. In both cases this could be viewed as a form of cross-linkage between subdomains. Of course, the carboxyl group of the fluorescein may also contribute interaction energy. The link resulting from these interactions could be of moderate strength in the $\lfloor Ca_2 \rfloor$*E*P-FITC intermediate, but reorganization of the cytosolic head after the dissociation of Ca^{2+} could stabilize it further, result-

ing in the unusually stable, low fluorescence, and Ca^{2+}-free $\lfloor\ \rfloor E$P-FITC species.

The Low Fluorescence Species: A Supercompact Form of the ATPase Cytosolic Head, Almost a Transition-like State? Potential Value for Future Structural Studies—Along this line, it is particularly appealing to further speculate that the above-mentioned "cross-link" could occur between the nucleotide binding domain and the phosphorylation domain. We know that, at some stage during the catalytic cycle, the nucleotide domain and the phosphorylation domain must be able to come close together, to make phosphoryl transfer possible. The early phosphoenzyme form that is stabilized by FITC is as close as possible to that state. A further possibility that we may consider is that the low fluorescence is in fact caused by the interaction of the FITC moiety with the phosphoryl group itself. It could help to fix domain N and domain P (34) together and simultaneously alter the reactivity of the phosphoryl group. The low fluorescence species might therefore be a compact phosphoenzyme form, with the cytosolic head domains tightly associated.

Independently of the precise assignment of the residues interacting with FITC in the low fluorescence species, the structure of the low fluorescence species might mimic not only that of an early phosphoenzyme form, but also that of the transition state for phosphoryl transfer. Indeed, the phosphorylation event for unmodified ATPase can be broken into several substeps, as illustrated in Scheme 2.

$$\lfloor Ca_2 \rfloor E \cdot ATP — \lfloor Ca_2 \rfloor^{a} E \cdot ATP — [\lfloor Ca_2 \rfloor^{*} E \ldots P \ldots ADP] — \lfloor Ca_2 \rfloor EP \cdot ADP$$

$$\quad | $$

$$[Ca_2]EP$$

SCHEME 2

Phosphorylation is thought to be preceded by a rate-limiting nucleotide-induced conformational change to a species, $\lfloor Ca_2 \rfloor^{a} E$·ATP, from which phosphorylation is very rapid (49–51). In Scheme 2, the transition state for phosphoryl tranfer is placed in square brackets to indicate its transient existence. Jencks and co-workers (50, 52) have found that Ca^{2+} dissociates toward the cytosolic side of the membrane from both $\lfloor Ca_2 \rfloor E$·ATP and $\lfloor Ca_2 \rfloor^{a} E$·ATP. The low fluorescence FITC-ATPase species, or more likely the Ca^{2+}-bound species with intermediate fluorescence from which it is immediately derived, might to some extent mimic this transition state for phosphoryl transfer. If this idea is correct, the ATPase cytosolic head can again be expected to be in a very compact state, with restricted access of water or other phosphoryl acceptors to the active site.

At any rate, the low fluorescence phosphorylated form of FITC-ATPase can probably be safely classified as an "*E*1P-like" form, on the basis of its being derived from an early phosphoenzyme in the cycle and of its poor reactivity to water. The structure of its head region is likely to be substantially different from that of either the Ca_2·*E*1 or the *E*2·VO_4 states; the structure of the former state, deduced from Ca^{2+}-ATPase three-dimensional crystals grown in the presence of Ca^{2+}, shows a widely open head region, with the nucleotide binding domain and the phosphorylation domain separated by ~25 Å (34), whereas two-dimensional crystals of the *E*2·VO_4 species show a more closed head region in which full interaction of the nucleotide binding and phosphorylation domains is nevertheless hindered by decavanadate binding at the interface between domains (34, 53). The structure of the low fluorescence species could provide critical new information on head domain interactions in a closed state and, by comparison with the other structures, on the extent and nature of domain movements.

In this direction, the fact that the low fluorescence species remained stable in a detergent-solubilized state (Fig. 1) might be of future value for three-dimensional crystallization attempts (*e.g.* as in Ref. 34 or 54). In addition, we have now found that it is possible to grow two-dimensional crystals from the low fluorescence and phosphorylated ATPase in the presence of thapsigargin and decavanadate (work in progress),[2] *i.e.* under conditions previously shown to induce the formation of two-dimensional arrays of *un*phosphorylated ATPase (53, 55–57). The present demonstration of a stable, phosphorylated form of FITC-ATPase might thus provide a starting point toward the future crystallization and structural analysis for the first time of a phosphorylated form of the pump.

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A Remarkably Stable Phosphorylated Form of Ca^{2+}-ATPase Prepared from Ca^{2+}-loaded and Fluorescein Isothiocyanate-labeled Sarcoplasmic Reticulum Vesicles

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