Unexpected Phosphoryl Transfer from Asp^{351} to Fluorescein Attached to Lys515 in Sarcoplasmic Reticulum Ca^{2+}-ATPase†

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ABSTRACT: Sarcoplasmic reticulum Ca^{2+}-ATPase is an ion pump whose catalytic cycle includes the transient formation of an acyl phosphate at Asp^{351}, and fluorescein isothiocyanate is a covalent inhibitor of ATP binding to this pump, known to specifically derivatize Lys^{515} in the nucleotide-binding site. It was previously found that an unusually stable, phosphorylated form of fluorescein-ATPase, with low fluorescence, is obtained following Ca^{2+} loading with acetyl phosphate as energy source and then chelation with EGTA of Ca^{2+} on the cytosolic side. Here we show that the phospho-linkage in this low fluorescent species is stable at alkaline pH, unlike the acyl phosphate at Asp^{351}. Moreover, the low fluorescence and stable phosphoryl group track together in primary and secondary tryptic subfragments, separated by SDS−PAGE after denaturation. Finally, normal fluorescence and absorbance are recovered upon treatment with alkaline phosphatase after extensive trypsinolysis. We conclude that the low fluorescent species is the result of the phosphoryl group being transferred from Asp^{351} to the fluorescein moiety during pump reversal, yielding fluorescein monophosphate tethered to Ca^{2+}-ATPase.

Ca^{2+}-ATPase (SERCA1a) from skeletal muscle sarcoplasmic reticulum (SR)[1] is an ATP-dependent Ca^{2+}/H^+ pump belonging to the family of P-type ATPases, so named because an acyl phosphate, formed through transient phosphorylation of an aspartyl group (Asp^{351} in the case of SR Ca^{2+}-ATPase), is an essential part of the catalytic cycle. The cycle (resulting in Ca^{2+}/H^+ counter-transport) comprises several intermediates, which are often separated into E1 and E2 forms and include intermediates with occluded ions (represented below with square brackets):

$$2Ca^{2+}_{cyt} + nH^+_{lum} + ATP \rightarrow 2Ca^{2+}_{lum} + nH^+_{cyt} + ADP + Pi$$

$$Ca_2.E1 \rightarrow Ca_2.E1.ATP \rightarrow [Ca_2].E1 \sim P \rightarrow H_n.E2P \rightarrow [H_n].E2 \rightarrow Ca_2.E1$$

The transitions between some of these intermediates have often been studied using fluorescein as an extrinsic reporter group. Fluorescein isothiocyanate (FITC) specifically derivatizes SR Ca^{2+}-ATPase at Lys^{515} in the nucleotide-binding site, and the fluorescence level of bound fluorescein is sensitive to its specific environment in various conformational states. Fluorescein-ATPase, although unable to use MgATP as a substrate, remains capable of Ca^{2+} pumping with smaller substrates like acetyl phosphate or *p*-nitrophenyl phosphate (*1*–*3*).

Many years ago it was reported that loading FITC-labeled SR vesicles with Ca^{2+} using acetyl phosphate as energy source and then adding EGTA (or adding EGTA and inorganic phosphate to FITC-labeled SR vesicles previously passively loaded with Ca^{2+}) converts fluorescein-ATPase into an extremely low fluorescence species (here dubbed "Lfs") (*4*). We subsequently found that this species also has low absorbance, that it is a phosphorylated species, but that the phospho-species formed has unusual properties: it is stable for several weeks in the absence of free Ca^{2+} (even after dissipation of the Ca^{2+} gradient required for its formation), and yet it is rapidly hydrolyzed upon readdition of Ca^{2+}, through Ca^{2+} binding to high-affinity sites on the cytoplasmic side of the vesicles (i.e., the ATPase can apparently re-enter the catalytic cycle in the forward direction) (*5*). This phospho-species first seemed related to the $[Ca_2].E1 \sim P$ catalytic intermediate, yet without the 2 occluded Ca^{2+} ions at the transport sites. However, this view was subsequently not confirmed by cross-linking experiments with glutaraldehyde within the cytosolic domain, nor by an 8 Å resolution structure of Lfs obtained by electron microscopy (which showed that, in the presence of decavanadate and thapsigargin (TG), Lfs was similar to the classical TG.E2.decavanadate structure); proteolytic digestion patterns with proteinase K and trypsin also failed to reveal any specific property of Lfs,

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[1] Abbreviations: FITC, fluorescein 5′-isothiocyanate; Lfs, low fluorescent species formed after Ca^{2+}-loading of FITC-labeled sarcoplasmic reticulum vesicles with acetyl phosphate followed by addition of EGTA; SR, sarcoplasmic reticulum; TG, thapsigargin.

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compared with control unphosphorylated fluorescein-ATPase (*6*). Questioning the previous idea that Lfs was a fluorescein-ATPase form phosphorylated at the catalytic Asp^{351} but endowed with an unusual conformation, we then considered the possibility that Lfs could result, instead, from chemical modification of the fluorescein itself. Nevertheless, our initial experiments did not succeed in confirming this possibility (*6*).

Here we examine additional properties of Lfs (pH stability, fluorescence level and ^{32}P content of tryptic peptides, as well as sensitivity of the fluorescence intensity to alkaline phosphatase), and show that they are fully consistent with transfer of the phosphoryl group from Asp^{351} to the fluorescein moiety, by reversal of the catalytic cycle. Such transfer results in formation of a tethered phosphofluorescein which, conversely, can be used as substrate for a single enzyme turnover in the forward direction after readdition of Ca^{2+}. Phosphorylation of fluorescein on the 3-O or 6-O of the xanthenone ring is indeed known to be possible and to produce a low absorbance and resultant low fluorescence compound, relatives of which are commonly used as fluorogenic substrates for assaying protein phosphatases (*7*–*9*).

EXPERIMENTAL METHODS

[^{32}P]Acetyl phosphate was synthesized by the method of Bodley and Jencks (*10*). SR was isolated from rabbit skeletal muscle by the method of Champeil et al. (*11*). SR derivatization with FITC (Sigma # F-7250, prepared as a 10 mM solution in dimethyl formamide or dimethyl sulfoxide) was performed at room temperature for 20 min at pH 8.0 (as in ref (*12*)), in 50 mM EPPS/TMAH, 0.3 M sucrose, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, with 4 mg of SR protein/mL and a quasi-stoichiometric amount of FITC (25 μM FITC). The pH was then adjusted to pH 7.5 with maleic acid and the preparation placed on ice.

Lfs formation from [^{32}P]acetyl phosphate was performed at room temperature by loading FITC-labeled SR vesicles (0.4 mg of protein/mL) with Ca^{2+} for 2 min in the presence of 50 mM MOPS/TMAH, pH 7.5, 0.3 M sucrose, 100 mM NaCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, and 1 mM [^{32}P]acetyl phosphate (a relatively low concentration for Lfs formation, nevertheless chosen to reduce the total amount of radioactivity handled), and then adding EGTA (5 mM); in some cases, Lfs stability was enhanced by adding EDTA (25 mM) as well.

The pH-dependence of the stability of the phospho-species was investigated by first preparing ^{32}P-labeled Ca^{2+}-ATPase in the Lfs medium (adding [^{32}P]acetyl phosphate, EGTA and EDTA as described above), then denaturing the protein by vortexing aliquots (0.25 mL, i.e. 0.1 mg of protein) with solid urea (150 mg per aliquot, resulting in 7−8 M urea) to which small amounts of concentrated acetic acid, maleic acid, or Tris had been added to adjust pH (to 4, 5−7 or 8−9.5, respectively). After 1 h incubation at 37 °C, samples were quenched with 5 mL of ice-cold 4% (w/v) trichloroacetic acid and 20 mM phosphoric acid, filtered on Whatman GF/F filters, washed thoroughly with the acid mix, and the radioactivity remaining on the filters was measured.

Limited trypsinolysis of ^{32}P-labeled Lfs was carried out by adding trypsin at room temperature in the same medium (0.4 mg of SR protein/mL, trypsin to SR protein ratio = 2% or 4% w/w). The reaction was stopped by vortexing aliquots (75 μL) with solid urea (48 mg) plus solubilization solution (8 μL of 12% sodium dodecyl sulfate, 9 M β-mercaptoethanol, and a trace amount of Bromophenol Blue). The samples were analyzed by SDS−PAGE, according to the method of Laemmli (*13*) (3 h at pH 8.6, performed in a cold room at 4 °C, and yet gel plates warm to touch at the end of electrophoresis, perhaps 30−40 °C) with either 7.5% acrylamide or a gradient of 7−18%. $CaCl_2$ (1 mM) was added to the running and stacking gels, to favor separation of A and B fragments (*14*).

For Lfs formation from nonradioactive acetyl phosphate before the alkaline phosphatase experiments, the conditions were similar but 10 mM acetyl phosphate was used, instead of only 1 mM, because of the poor apparent affinity of this substrate (*5*); the medium also contained 50 mM TES-Tris (at pH 7.5), 0.3 M sucrose, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 100 mM NaCl, and again 0.4 mg of FITC-labeled SR protein/mL. After 5 min loading, 2 mM EGTA was added, followed by 4 μg/mL TG (an inhibitor of Ca^{2+} binding to Ca^{2+}-ATPase) to stabilize Lfs. Control fluorescein-ATPase was prepared similarly, but with a different order of addition of the various ligands: EGTA, then TG, and only then acetyl phosphate. Extensive trypsin digestion of either Lfs or the control fluorescein-ATPase was then carried out, by adding trypsin and Tris-base from concentrated solutions and raising the temperature: thus, trypsinolysis occurred at 37 °C and pH 8, with 12.5% trypsin (w/w) (about 0.4 mg/mL FITC-labeled SR protein/mL and 0.05 mg/mL trypsin). Other aliquots were incubated simultaneously but without trypsin ("untreated samples"). After 3−4 h of incubation, samples were diluted 10-fold into 100 mM NaCl, 0.3 mM sucrose, 5 mM Mg^{2+}, 100 mM Tris-Cl, pH 8 at 37 °C, the fluorescence level and absorbance spectrum measured, and their changes upon addition of alkaline phosphatase (final concentration 1 μg/mL, 7.5 units/mL) recorded.

RESULTS

Some of the characteristics of Lfs have been detailed previously, its main features being an unusually low fluorescence and a different absorption spectrum with lower absorbance at 495 nm and higher absorbance at 430 nm (typical experiments are recalled in Figure 1A and B), as well as an unusually high stability when kept at a very low concentration of free Ca^{2+} (*5*). In Figure 1C, we now report an additional characteristic, i.e. the pH stability of the phospho-species at 37 °C following denaturation of the protein in 7−8 M urea. The control experiment with unmodified ATPase phosphorylated at Asp^{351} (closed circles) reveals the expected lability at alkaline pH of the acyl phosphate bond (*15*). In contrast, in Lfs (triangles), the phospho-species is stable at alkaline pH, suggesting a phosphate ester. This type of bond, known to be formed, for instance, upon kinase-dependent phosphorylation of serine, threonine, or tyrosine, in our case would also be consistent with phosphorylation on the 3-O or 6-O group of the xanthenone ring of the fluorescein attached to Lys^{515}. Note that a second control experiment was performed with FITC-labeled SR vesicles incubated with [^{32}P]acetyl phosphate but to which EGTA had not been added (open circles in Figure 1). In this case the pH-dependence of stability was

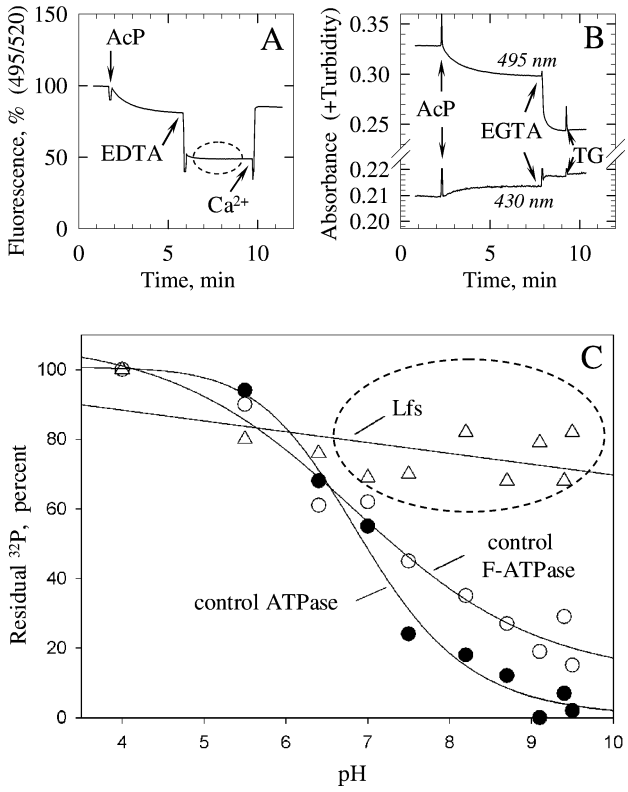

FIGURE 1: Formation of the "low fluorescence species" (Lfs), and pH stability of denatured phospho-species. (A) Typical fluorescence recording (495 nm/520 nm) of the formation of Lfs upon addition of 10 mM acetyl phosphate and then 7 mM EDTA (in the presence of 5 mM Mg^{2+}) to FITC-labeled SR vesicles at 0.02 mg/mL protein in Lfs medium; the subsequent addition of 3 mM Ca^{2+} allows fluorescence to recover, as previously observed (*5*). (B) This formation can also be monitored by absorbance measurements; here, FITC-labeled SR vesicles were at 0.4 mg/mL protein, and addition of acetyl phosphate was followed by addition of EGTA (2 mM) and then TG (4 μg/mL). Traces recorded at 495 nm and at 430 nm are shown, and they evidence a change in the spectrum of the bound chromophore, as previously observed (*5*). (C) pH-dependence of the denatured phospho-species. Control, underivatized SR vesicles ("control ATPase") and control, FITC-labeled SR vesicles ("control F-ATPase") were Ca^{2+} loaded in Lfs medium with 1 mM [^{32}P]acetyl phosphate for 2 min at room temperature. In a third sample, the low fluorescent species ("Lfs") was formed by adding EGTA to control FITC-labeled SR vesicles after this 2 min loading period. In all cases, proteins were then denatured, by vortexing aliquots with solid urea to which small aliquots of acid or Tris had been added, to bring the urea concentration to 7−8 M and the solution pH to that shown on the abscissa. The samples were incubated at 37 °C for 1 h, acid quenched, the protein collected on filters, and the radioactivity on the filters measured. The radioactivity is expressed as a percentage of the value at pH 4.0.

more similar to that for control ATPase, although it included a small pH-independent component. The latter would be consistent with the partial decrease in fluorescence and absorbance observed during Ca^{2+} loading with acetyl phosphate (i.e., formation of a small proportion of phosphofluorescein), even before EGTA addition (*4, 5*) (as recalled in Figure 1A).

Ca^{2+}-ATPase has a particularly sensitive tryptic cleavage site at Arg^{505} (T1 cleavage site), which separates the protein in two fragments of approximately equal size, one containing Asp^{351} (A fragment, comprising residues 1−505) and the other Lys^{515}, with the attached fluorescein if present (B fragment, comprising residues 506−994) (*16*). Figure 2 shows the results of T1 cleavage of Lfs compared to a control fluorescein-ATPase. All samples were applied in duplicates on the same gel, 8 lanes apart, so that after fluorescence imaging (see central part of Figure 2) the gel could be cut down the center and the left part submitted to Coomassie Blue staining while the right duplicate section was kept for radioactivity imaging. Separation of the A and B fragments was achieved in these experiments (thanks to the presence of excess Ca^{2+} during separation, ref *14*), and, as expected, fluorescein attached to Lys^{515} migrated together with the B fragment (see the left part of the figure). Two significant and unexpected facts were observed. First (middle right frame, but this is of course also visible in the other half of the gel, in the middle left frame), and irrespective of cleavage, the fluorescein-ATPase in Lfs (as well as in its B fragment) had distinctly lower fluorescence compared with the control fluorescein-ATPase, i.e. low fluorescence remained associated with the Lfs sample despite its treatment with urea and SDS before SDS−PAGE, and the low fluorescence persisted in the B fragment following T1 cleavage, showing that native association with the A fragment (with Asp^{351}) has nothing to do with this low fluorescence. Such low fluorescence for the denatured Lfs protein and its major fragment tends to rule out the possibility that it could be due to an unusual conformation of the membraneous ATPase chain, phosphorylated at Asp^{351} or any other amino acid residue, and suggests that the fluorescein label itself may well have been modified chemically in Lfs. Second, after radioactive imaging of the right part of the gel (extreme right frame) the phosphoryl group in Lfs was found to associate with the B fragment, not with the A fragment which bears Asp^{351} (horizontal lines inserted to help visual alignments). Note that in the last lane of the right part of the gel (Lfs, 10 min trypsin; fluorescence and radioactivity), slight abnormalities in the running of the gel, as clearly seen by the position and upward tilt of the residual small band corresponding to full length Ca^{2+}-ATPase, artifactually raise the position of the B band; the opposite is true of the last lane of the left part of the gel.

The result of more sustained digestion with trypsin is shown in Figure 3. Among the many subfragments formed (Coomassie Blue-stained, top gel), the ^{32}P-labeled phosphoryl group in Lfs was found to specifically associate (see bottom gel, imaged for radioactivity) with those subfragments (circled bands) which also are fluorescent (center gel, imaged for fluorescence), and even in these smaller fragments the fluorescence was lower than in the corresponding ones in control fluorescein-ATPase ("C1", on the left). This again supports the possibility that fluorescein itself has been modified in Lfs. In these experiments A and B fragments migrated at similar rates, because formation of Lfs had been achieved by adding both EGTA and a high concentration of EDTA for maximal stability, and excess $CaCl_2$ had not been added to the denatured samples.

The second control sample in Figure 3 ("C2", on the right, i.e. FITC-labeled SR vesicles loaded with Ca^{2+} using [^{32}P]acetyl phosphate, but to which EGTA was not added subsequently) is also noteworthy, as prior to electrophoresis it must have been phosphorylated mostly at Asp^{351}: in agreement with Figure 1 (and due to the instability of the acyl phosphate under the alkaline Laemmli conditions), the "C2" lane indeed reveals only a very small amount of residual radioactivity. Again, part of this small amount of

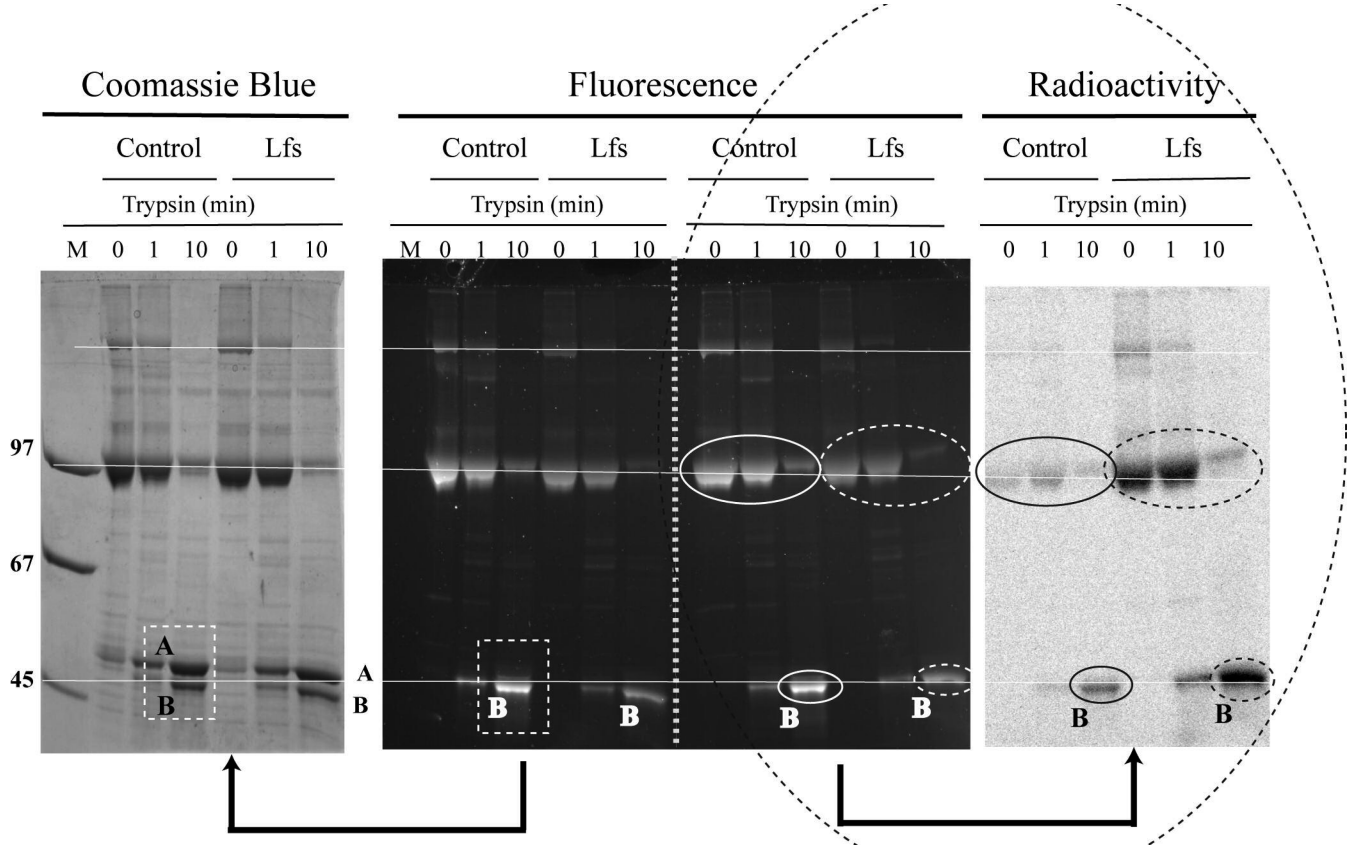

FIGURE 2: Association of phosphoryl group and low fluorescence with primary tryptic fragments. Two samples, both prepared from FITC-labeled SR vesicles, were digested with trypsin for various periods (0.4 mg/mL SR protein and 0.008 mg/mL trypsin, i.e. 2% w/w): either the classically formed Lfs (FITC-labeled SR vesicles loaded with Ca^{2+} for 2 min with [^{32}P]acetyl phosphate, then incubated with excess EGTA for 1 min) or a control sample prepared in the same final medium (here, FITC-labeled SR vesicles incubated with 1 mM [^{32}P]acetyl phosphate in the presence of excess EGTA from the start). At the times indicated, aliquots were vortexed with solubilization solution + urea, excess Ca^{2+} was added (to ensure subsequent separation of A and B fragments), and the samples were subjected to SDS−PAGE. Each sample loaded into a lane in the first half of the gel was duplicated in the second half of the gel. For the zero time point, trypsin was omitted. The gel was visualized for fluorescence, then sliced down the middle, and one-half (the left one) stained with Coomassie Blue (bottom left arrow), and the other (the right one) dried and subjected to radio-imaging (bottom right arrow). The lane marked M shows molecular weight markers. The main band at close to the 97 kDa marker is the uncleaved Ca^{2+}-ATPase, and primary tryptic fragments are labeled A (residues 1−505) and B (residues 506−994). Dotted lines indicate the approximate position of the vertical cut, and horizontal band alignments. An alignment of an unknown protein of high molecular weight (possibly oligomers of denatured ATPase), which was fluorescent and radiolabeled sufficiently, was also performed in order to be more certain of the trypsin fragment alignments.

residual radioactivity could be due to a small proportion of phosphate ester on fluorescein, as the latter is expected to be in equilibrium with the phosphorylated Asp^{351} during Ca^{2+} loading.

As mentioned, phosphorylation of fluorescein on the 3-O or 6-O of the xanthenone ring is known to result in a low absorbance and low fluorescence compound (*8*), and related phosphorylated fluorescein molecules are commonly used as fluorogenic substrates for assaying protein phosphatases, which release fluorescein from the fluorescein phosphate (*7*–*9*). We asked whether normal fluorescence could be recovered from Lfs by adding such a phosphatase (in our case alkaline phosphatase): in our first attempts, the answer was no, even at 37 °C and pH 8 (Figure 4A, left traces), including (data not shown) after heat denaturation or after denaturation with 1% SDS followed by 10-fold dilution. However, extensive trypsinolysis of Lfs prior to alkaline phosphatase treatment did allow Lfs to slowly recover normal fluorescence upon subsequent incubation with alkaline phosphatase (center trace in Figure 4A), whereas alkaline phosphatase was without effect on similarly trypsinized control fluorescein-ATPase. This is critical evidence that the fluorophore itself in Lfs has been chemically modified, to form fluorescein monophosphate and become sensitive to alkaline phosphatase (provided alkaline phosphatase has access to it, thanks to trypsinolysis). Note, in this respect, that adding alkaline phosphatase to trypsinized Lfs allowed the fluorescence to rise much more slowly than when the same concentration of alkaline phosphatase was added to fluorescein diphosphate (Figure 4A, extreme right trace), which is consistent with fluorescein monophosphate bound to tryptic peptides having hindered access to the active site of alkaline phosphatase, compared with free fluorescein monophosphate.

We previously noted that the low fluorescence species Lfs had low absorbance too, around 500 nm (*5*). Experiments identical to the fluorescence experiments described above, but in which absorbance properties were monitored after addition of alkaline phosphatase, also revealed, for trypsinized Lfs, alkaline-phosphatase dependent recovery of absorbance in this region (Figure 4, B−E). Moreover, upon addition of alkaline phosphatase, the time-dependent rise in Lfs absorbance around 500 nm was accompanied by a clear absorbance drop over the 330−460 nm range: judging from the absorbance spectrum of fluorescein monophosphate, this is exactly the range of wavelengths where absorbance of (free) fluorescein monophosphate exceeds that of fluorescein (*8*).

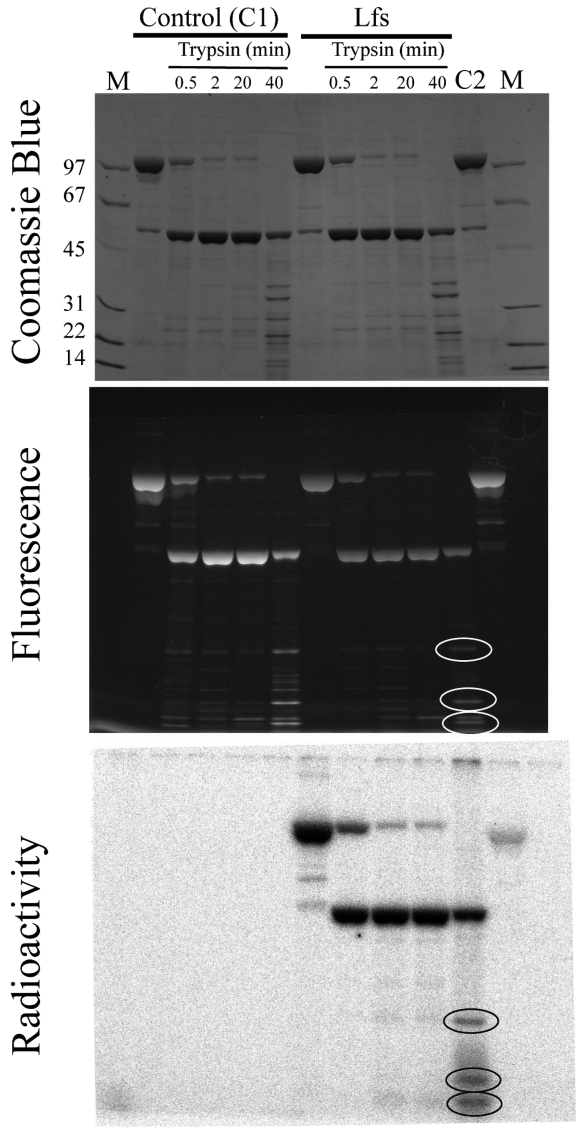

FIGURE 3: Association of phosphoryl group and low fluorescence with secondary tryptic subfragments. Initial samples, similar although not identical to those described for Figure 2, consisted of Lfs (FITC-labeled SR vesicles loaded with Ca^{2+} for 2 min with [^{32}P]acetyl phosphate, then supplemented with EGTA to form Lfs, and then with EDTA to stabilize Lfs further) and a control sample, "C1" (FITC-labeled SR vesicles supplemented with EGTA and EDTA first, and only then [^{32}P]acetyl phosphate). They were here subjected to more sustained tryptic digestion, using 4% (mg of trypsin/mg of SR protein) trypsin and longer digestion periods (for the time labeled 40 min, the digestion was in fact done for 20 min, SDS was then added to 1% (w/v), and the digestion continued for a further 20 min in the presence of SDS; trypsin retains significant activity in the presence of 1% SDS). The digestion was stopped by vortexing aliquots with solubilization solution + urea, and then the samples were subjected to SDS−PAGE with an acrylamide gradient of 7−18%. "C2" is another control where the FITC-labeled vesicles were incubated with [^{32}P]acetyl phosphate for 2 min, the reaction stopped with SDS + β-mercaptoethanol, and then EGTA and EDTA added. The top gel is Coomassie Blue-stained, the middle one shows fluorescence, and the bottom one radioactivity. Small bands in trypsin-treated Lfs which are both radiolabeled and of low fluorescence are circled. The gel used for Coomassie Blue staining was different from that used for fluorescence and radioactivity visualization, although the same samples were used and electrophoresis was performed in parallel. Lanes marked M show molecular weight markers.

Incidentally, note that for both Lfs and control fluorescein-ATPase, the prior trypsinolysis was accompanied by a marked reduction in sample turbidity, as expected. In the case of control fluorescein-ATPase, it was also accompanied by a slight shift of the λ_{max} for bound fluorescence (from 501 to 495 nm), probably due to partial disruption of the protected environment around the bound fluorescein (e.g., Figure 9B in ref *17*.).

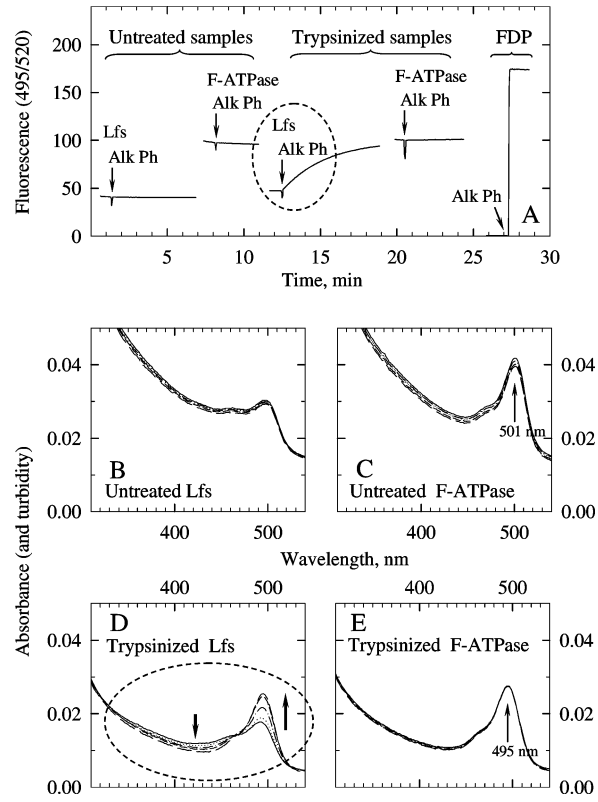

FIGURE 4: The low fluorescence and low absorbance of Lfs revert to normal fluorescence and normal absorbance upon addition of alkaline phosphatase to trypsinized samples. After Lfs formation from nonradioactive AcP (10 mM acetyl phosphate was used, to maximize the amount of Lfs) and preparation of a control fluorescein-ATPase (incubated with the same ligands added in a different order, see Experimental Methods), samples were supplemented with trypsin (12.5% w/w) when desired and slightly alkalinized, and brought to 37 °C for incubation (the final pH was 8). After 3 or 4 h incubation, samples were diluted 10-fold (to 0.04 mg/mL FITC-SR protein) for recording their fluorescence or absorbance characteristics at various times after addition of alkaline phosphatase (final concentration 1 μg/mL). (A) Fluorescence continuous measurements (495 nm/520 nm). In this series of measurements, the effect of alkaline phosphatase addition to fluorescein diphosphate (at about 0.25 μM, roughly equivalent to the concentration of fluorescein in derivatized SR vesicles) was also included. (B−E) Absorbance spectra for the various samples (as indicated) at various times after addition of alkaline phosphatase (0, 0.5, 2, 5 or 10 min). In parallel absorbance experiments with fluorescein diphosphate, the much faster kinetics previously illustrated for fluorescence recovery in panel A was also observed (not shown).

DISCUSSION

The results presented here provide compelling evidence that in the ATPase form (Lfs) generated by acetyl phosphate-supported Ca^{2+} loading of FITC-derivatized vesicles followed by addition of EGTA, the remarkably stable phospho-species formed is not an ATPase with an acyl phosphate bond at Asp^{351} (as in the phosphoenzyme normally found during the catalytic cycle), but instead, a species with a phosphate ester

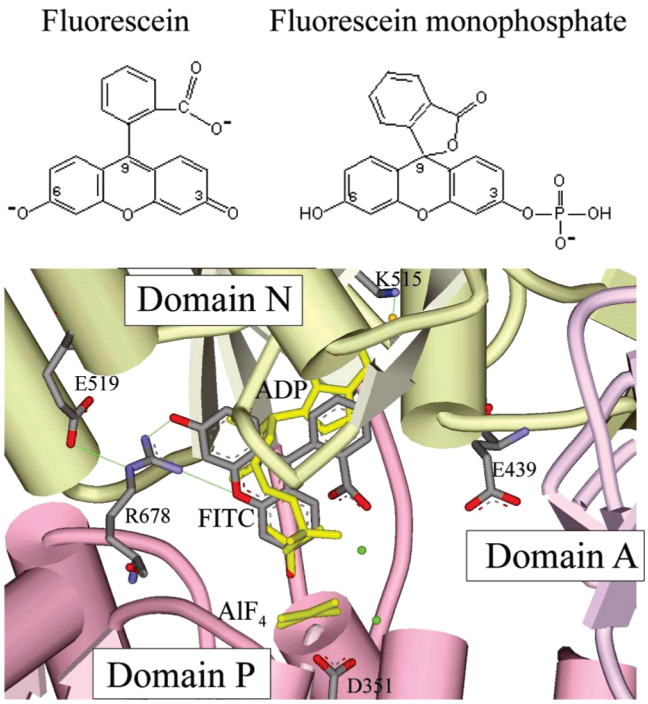

FIGURE 5: Model for phosphorylation of fluorescein: docking of FITC in the active site of Ca^{2+}-ATPase in an E1P-like structure. Top: Chemical formulas for fluorescein (left), here shown as the dianionic fluorescent form, and fluorescein monophosphate (right), here shown as isomer "A" (*8, 9*). Bottom: The structure of FITC, built up manually with software in Accelrys DS ViewerPro, was manually introduced into the structure of Ca^{2+}-ATPase (Ca_2.E1.ADP.AlF_4; PDB accession code 1T5T), in the cavity of the ATPase accommodating ADP. The distance of the nitrogen atom of Lys^{515} to the sulfur atom of the isothiocyanate group is 1.2 Å. The fitted FITC (atom specific coloring) and the ADP experimentally determined (yellow) are shown simultaneously; the adenine ring of ADP and the benzoyl ring of FITC are superimposed and coplanar. AlF_4, close to Asp^{351}, is also shown in yellow. The green spheres indicate Mg^{2+} ions. Favorable contacts (green lines) are made with Arg^{678} that normally hydrogen bonds with 3-O ribose of ADP.

on the fluorescein moiety attached to Lys^{515}. The chemical structure of one of the possible isomers of fluorescein monophosphate (*8, 9*) is shown on top of Figure 5.

This phosphorylation of fluorescein itself, not Asp^{351}, accounts nicely for the stability of the phosphoryl group, for the low fluorescence and the low absorbance (at 500 nm) of Lfs (including after denaturation of the protein), for the coincidence of the ^{32}P radioactivity with fluorescence in the primary tryptic fragment containing Lys^{515} (and not Asp^{351}) as well as in smaller secondary subfragments, and for the ability of alkaline phosphatase to slowly but definitely allow small tryptic fragments of Lfs to recover normal fluorescence and absorbance properties for the fluorescein moiety. It also accounts for most of the puzzling electron microscopy, cross-linking and proteolysis observations reported in ref *6*. One feature still requiring an explanation is that in the 8 Å resolution structure of Lfs in the additional presence of decavanadate and TG also reported in ref *6*, Lfs, rather oddly, accommodated decavanadate at the interface of the three head domains, whereas the unphosphorylated fluorescein ATPase did not: presumably, phosphorylation of the fluorescein moiety forces the fluorescein rings to relocate elsewhere within the binding pocket (see below), away from the decavanadate binding site. A crystal structure of E2.TG.AMPPCP (PDB code 2C88, ref *18*, or 2DQS) shows that the α-and *β*-phosphates of nucleotide, together with a Mg^{2+} ion, focus toward Glu^{439} in domain N, and interact with the protein matrix in a manner significantly different from that in the [Ca_2].E1.AlF_4.ADP species (PDB code 1T5T, ref *19*); if, in the combined presence of EGTA, decavanadate, TG and Mg^{2+}, the phosphorylation of fluorescein in Lfs causes a similar interaction of its phosphoryl group with Glu^{439}, this could well explain a shift in position of the xanthenone structure, compared to control fluorescein-ATPase. Based on handmade modeling, such a pendulum-like movement of the rings is indeed compatible with maintaining the link to Lys515; for the 3O, it would require a move of ∼4 Å from the position in the model in Figure 5 (see below).

Thus, unexpectedly, it appears that after acetyl phosphate-supported Ca^{2+}-loading of vesicles containing FITC-derivatized Ca^{2+}-ATPase and pump cycle reversion on the addition of EGTA, the phosphoryl group can be transferred to the fluorescein moiety. This transfer implies proximity of Asp^{351} (in domain P) and the fluorescein moiety (in domain N) in the initial Asp-phosphorylated state (presumably the [Ca_2].E1∼P phosphoenzyme), and also implies that after such transfer Ca^{2+} ions originally bound to this phosphorylated state are no longer occluded and can dissociate from the ATPase (see also below) and thereby trigger disruption of the N/P domain proximity, making the phospho-transfer irreversible at low free [Ca^{2+}] and resulting in a Ca^{2+}-free state with an ordinary conformation (E2) but a phosphorylated fluorescein instead of the normal fluorescein. Conversely, the extreme sensitivity of Lfs to Ca^{2+} (low fluorescence, low absorption and stable phosphorylation are lost immediately upon readdition of Ca^{2+} to nondenatured Lfs, see ref *5*) can now be readily explained by the fact that, upon Ca^{2+} rebinding to the ATPase high affinity transport sites (oriented to the cytoplasmic side), the covalently attached fluorescein monophosphate will come close to Asp^{351} again and will be used as a substrate in the forward direction of catalysis, for a single turnover. Indeed, the kinetics of Ca^{2+} transport during this process is consistent with the phosphoryl group re-entering the cycle after the binding of Ca^{2+} to normal outwardly exposed sites (*5*). As the rate of this binding was found similar to the rate of Ca^{2+} binding to normal fluorescein-ATPase or unlabeled ATPase (*5*), this, incidentally, implies that the tethered fluorescein monophosphate does not accelerate the "E2 to E1" transition, unlike the genuine substrate ATP.

We have suggested previously that FITC may be modeled into the catalytic site of Ca^{2+}-ATPase crystallized with ADP + AlF_4, such that the isothiocyanate moiety is proximal to the amino group of Lys^{515} and the 3-O xanthenone oxygen superimposes with the attacking/departing oxygen of the *β*-phosphate of ADP (*6*). Such a model is presented in Figure 5, showing the 3-O oxygen appropriately positioned for acceptance of the phosphoryl group (mimicked by an AlF_4 group in 1T5T) from Asp^{351}. This supports the possibility that the formation of a tethered fluorescein monophosphate is "simply" the result of the cycle going in the reverse, with fluorescein replacing ADP and the E1-fluorescein monophosphate species being an analogue of the E1.ATP noncovalent species formed before phosphorylation; and the tethered fluorescein monophosphate could well readopt a similar position during re-entry into the cycle in the presence

of Ca^{2+}. Note, however, that the bound fluorescein can probably not adopt the same position during loading of the vesicles with acetyl phosphate, because of likely steric clashes of the edge of the xanthenone ring with the acetyl group: thus the covalently attached (nonphospho) fluorescein, at some stage, possibly during the $[Ca_2]$.E1∼P to E2P transition to account for the strict dependence of Lfs on a Ca^{2+} gradient, must be free enough to adopt alternative positions in the nucleotide-binding site, or alternatively the apposition of domains N and P is variable. The latter would be consistent with the rather weak interactions between them seen in some of the atomic structures, especially in the open Ca_2.E1 structure, coupled with flexing of the linking hinge. The ability of bound fluorescein to adopt different positions was in fact previously suggested on the basis of the ability of ATP (if not MgATP) to affect FITC fluorescence significantly, by binding to a low affinity site (*12*).

Our finding that the fluorescein in derivatized $[Ca_2]$.E1 ∼ P behaves very much like ADP in unlabeled Ca^{2+}-ATPase might have a bearing on the proposal that departure of ADP from the catalytic site is the critical trigger for domains N and P separation, domain A rotation and E2-P state formation (*20, 21*). Obviously, the tethered fluorescein does not depart the catalytic site, and yet the $[Ca_2]$.E1 ∼ P transition to E2P does not seem to be much inhibited when the covalently attached fluorescein monophosphate is used as substrate on the addition of Ca^{2+} or when acetyl phosphate is used as substrate with the fluorescein-ATPase. Domains N and P are mainly held together by Glu^{519}-Arg^{678} and Arg^{560}-Asp^{627} links, where both arginines are also interacting with the ADP (ribose hydroxyls and β-phosphate oxygens, respectively). There is an additional hydrogen bond between the attacking/departing oxygen atom of the β-phosphate and Thr^{353} of domain P. When fluorescein replaces ADP, the interdomain interactions could remain (the Glu^{519}-Arg^{678} interaction is shown in Figure 5; the other interactions are omitted in order not to complicate the view). As shown in Figure 5, Arg^{678} could also interact very favorably with the xanthenone ring. The side chain of Arg^{560} could salt-link favorably with the carboxyl of the smaller phenyl ring of the fluorescein and the 3O-Thr^{353} interaction could also survive, as positioning this attacking/departing oxygen must be critical for catalysis. Thus, it is not clear that replacing the ADP with fluorescein necessarily weakens head domain interactions, and one then may query whether release of ADP really is the critical trigger for the $[Ca_2]$.E1∼P to E2P transition. Perhaps, dissociation of ADP may be rather seen as a means of driving the reaction forward by preventing the backward reaction to ATP, rather than a trigger to E2P. The only requirement for the transition from $[Ca_2]$.E1∼P to E2P probably is that the phosphate chain in ADP reorients (while the base moiety could remain bound to Phe^{487}) and leaves the vicinity of the now phosphorylated Asp^{351}.

Finally, beyond accounting for the mystery of the low fluorescent intermediate reported by U. Pick so many years ago, the phosphorylation of fluorescein by reversal of the pump, and its dependence on lumenal Ca^{2+}, might turn out to be useful in the development of an assay for measuring Ca^{2+} binding to lumenal sites of mutant ATPase, or for detecting mutants with stabilized phosphorylated states. Such an assay would shed light on the pathway for Ca^{2+} release from the transport sites located midway in the membrane to the lumen.

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