Use of Glycerol-Containing Media To Study the Intrinsic Fluorescence Properties of Detergent-Solubilized Native or Expressed SERCA1a[†]

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ABSTRACT: Rapid irreversible inactivation of Ca^{2+}-free states of detergent-solubilized SERCA1a (sarco-endoplasmic reticulum calcium ATPase 1a) has so far prevented the use of Trp fluorescence for functional characterization of this ATPase after its solubilization in various detergents. Here we show that using 20−40% glycerol for protection makes this fluorescence characterization possible. Most of the ligand-induced Trp fluorescence changes previously demonstrated to occur for SERCA1a embedded in native sarcoplasmic reticulum membranes were observed in the combined presence of glycerol and detergent, although the results greatly depended on the detergent used, namely, octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) or dodecyl maltoside (DDM). In particular, at pH 6, we found a $C_{12}E_8$-dependent unexpectedly huge reduction in SERCA1a affinity for Ca^{2+}. We suggest that a major reason for the different effects of the two detergents is that high concentrations of $C_{12}E_8$, but not of DDM, slow down the E2 to E1 transition in solubilized and delipidated SERCA1a. Independently of the characterization of the specific effects of various detergents on SR vesicles, our results open the way to functional characterization by Trp fluorescence of heterologously expressed and purified mutants of SERCA1a in the presence of detergent, without their preliminary reconstitution into liposomes. As an example, we used the E309Q mutant to demonstrate our previous suspicion that Ca^{2+} binding to Site I of SERCA1a in fact slightly *reduces* Trp fluorescence, and consequently that the rise in this fluorescence generally observed when two Ca^{2+} ions bind to WT SERCA1a mainly reflects Ca^{2+} binding at Site II of SERCA1a.

Sarcoplasmic reticulum (SR[1]) Ca^{2+}-ATPase (SERCA1a) is an integral membrane protein of 994 amino acids. It is involved in the transport of Ca^{2+} from the cytosol to the SR lumen during the relaxation of fast twitch muscle, an energy-consuming active transport process which is coupled to ATP hydrolysis by this protein. The catalytic cycle for this process comprises Ca^{2+} binding to SERCA1a, followed by the transient formation of an ATP-derived, energy-rich intermediate, a covalent aspartyl-phosphorylated enzyme (thus, like other cation transporters, SERCA1a belongs to the family of P-type ATPases). After this formation, a spontaneous conformational transition of the phosphorylated enzyme allows the transport sites to change their orientation from one side of the SR membrane to the other, lose their affinity for Ca^{2+}, and therefore release the bound ions toward the SR lumen. Enzyme dephosphorylation then occurs and returns SERCA1a to its initial state. Detailed study of the ATPase catalytic cycle was originally made possible by the high level of expression of SERCA1a in rabbit skeletal muscle (about 70% of the total membrane protein in SR), and later by the heterologous expression of many mutants (e.g., refs (*1*−*5*), see more references in the Supporting Information). In 2000, detergent-solubilized SERCA1a was crystallized and the revelation of its structure at atomic resolution provided invaluable new insights (*6*). The transmembrane region was found to be organized into 10 α-helical transmembrane spans (M1 to M10), two of which (M4 and M6) are interrupted at the level of the Ca^{2+} binding sites. This region (and the corresponding stalk above the membrane) is surmounted by a large cytosolic head, subdivided into three domains: a phosphorylation domain (P), a nucleotide-binding domain (N) and an "actuator" domain (A). This first successful crystallization, obtained in the presence of Ca^{2+}, was followed within a few years by the successful crystallization of other forms of SERCA1a, thought to be reliable analogues of various intermediates in the catalytic cycle (see references in the Supporting Information). Crystals of wild-type and mutated Ca^{2+}-ATPase, purified after heterologous expression in yeast, were also recently obtained

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[1] Abbreviations: SERCA1a, sarco-endoplasmic reticulum Ca^{2+}-ATPase, isoform 1a; SR, sarcoplasmic reticulum; ATPase, adenosine triphosphatase; EGTA[ethylenebis-(oxyethylenenitrilo)]tetraacetic acid; Mops, 4-morpholinepropane sulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Mes, 4-morpholineethane sulfonic acid; FITC, fluorescein 5-isothiocyanate; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; DDM, *n*-dodecyl β-D-maltopyranoside; E2 and E1, nonphosphorylated forms of Ca^{2+}-ATPase; E2P and E1P, phosphorylated forms of Ca^{2+}-ATPase, respectively ADP-insensitive and ADP-sensitive; TG, thapsigargin; AlF_4, aluminum fluoride; BeF_3, beryllium fluoride; VO_4, orthovanadate; DOPC, 1,2-dioleoylphosphatidylcholine; cmc, critical micellar concentration; csc, critical solubilization concentration; BAD, biotin-acceptor domain.

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with satisfactory resolution (*7, 8*). All these results have allowed significant clarification of the structural basis of ATP-driven ion pumping by SERCA1a. It nevertheless remains desirable to broaden the range of techniques available for studying the functional features of the SERCA1a protein and some of its mutants, as well as the specific effects of various detergents on this protein, considered as a model.

One way of following the changes in the conformation of a protein at a particular step of its catalytic cycle is to take advantage of the spectroscopic properties of its tryptophan residues, which may act as intrinsic reporters. SERCA1a contains thirteen tryptophan residues, one in the nucleotide binding domain and twelve in the transmembrane region of the protein, and over many years it has been shown that the addition of various ligands to SERCA1a triggers specific changes in their fluorescence intensity (*9*−*12*). Such experiments, originally performed with native SR SERCA1a, have also been extended to heterologously expressed SERCA1a after its purification to an appropriate degree. However, with one exception (*13*) these Trp fluorescence measurements with expressed and purified SERCA1a were restricted to proteins previously reconstituted into proteoliposomes (*14*−*16*). One good reason for this preliminary reconstitution was that the incubation of native SERCA1a in detergent in the absence of Ca^{2+} was known to lead to relatively fast irreversible inactivation of its activity, much faster than when SERCA was kept embedded in lipids or in the presence of Ca^{2+} (*17*−*22*). Another reason for preliminary protein reconstitution was that, even before denaturation could take place, the presence of detergent was suspected to alter and often reduce the amplitude of the observable fluorescence changes (*23*−*26*). On the other hand, elimination of the detergent around the protein for its reconstitution into proteoliposomes is generally lengthy, and unfortunately often leads to loss of material and significant protein inactivation. It would therefore be desirable to skip the reconstitution step before Trp fluorescence measurements.

Here, we show that it is possible to solubilize native SR SERCA1a in the presence of excess detergent and still be able to monitor ligand-dependent changes in Trp fluorescence, provided the medium is supplemented with a high concentration of glycerol. In fact, glycerol has often been added to SERCA1a to stabilize it during long-term storage or purification in the presence of detergent (*27*−*29*). In the presence of glycerol and of two of the detergents commonly used to isolate and characterize Ca^{2+}-ATPase, most of the previously documented ligand-dependent fluorescence signals remained of acceptable size, at least for one or the other of these detergents. This enabled us to study here the specific effects of two detergents, DDM and $C_{12}E_8$, on solubilized SERCA1a derived from native SR vesicles, effects which have only rarely been documented in detail beyond the overall alteration of SERCA1a activity and stability (among the few examples, see refs *30, 31*). Similar experiments were also possible for heterologously expressed and purified mutants of SERCA1a, extending the range of functional tests applicable to SERCA1a mutants. As an example (other examples are given in ref *8*, we studied here the E309Q mutant of SERCA1a, modified at one of its two Ca^{2+} sites.

EXPERIMENTAL PROCEDURES

SR Membranes and Chemicals. SR membranes were prepared as previously described (*32, 33*). Octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) was purchased from Nikkol Chemical (Tokyo, Japan), and *n*-dodecyl β-D-maltopyranoside (DDM), from Anatrace (Maumee, OH). Streptavidin Sepharose high performance resin was provided by GE Healthcare (Orsay, France). Thapsigargin (TG stock solution was 1 mg/mL in DMSO, i.e., about 1.5 mM) was from VWR International (#586005, Fontenay-sous-Bois, France). 1,2-dioleoylphosphatidylcholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL). The concentrated stock solutions of detergent:DOPC mixtures (2:1, w/w), prepared at 40 mg/mL:20 mg/mL, were clear (the DDM/DOPC mixture was quite viscous). As glycerol of low quality may contain contaminants contributing undesired UV absorbance and fluorescence, we used the Merck *pro analysi* glycerol (#1.04092). Glycerol concentrations are expressed as w/w or v/v, depending on the particular protocol used for preparation of the buffer in the corresponding experiments. All other chemical products were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). [^{32}P]Pi was from Perkin-Elmer (NEX 054).

Trp Fluorescence Measurements. Fluorescence was measured with a SPEX Fluorolog spectrofluorometer (Horiba/Jobin-Yvon, Longjumeau, France). The intrinsic fluorescence of SR SERCA1a (*34*−*36*) was recorded with SR membranes at either 0.02 mg/mL or 0.1 mg/mL protein in a temperature-regulated and continuously stirred 2 mL cuvette, with excitation and emission wavelengths set at 295 and 320 nm respectively (bandwidths were 5 nm and either 10 or 5 nm, depending on the protein concentration). The increased viscosity provided by glycerol, when present, did not prevent efficient stirring of the corresponding cuvette. The medium used for each experiment is indicated in the corresponding figure legend. Depending on the experiment, this medium was supplemented with various concentrations of Ca^{2+}, EGTA, Mg^{2+}, nucleotides, or Pi. To compute $[Ca^{2+}]_{free}$ concentrations, the dissociation constant of the Ca·EGTA complex was taken to be 0.4 μM at pH 7 in the absence of Mg^{2+} (*37*), and was assumed to be the same in the presence of glycerol. The Maxchelator software was used (http://maxchelator.stanford.edu/ and ref *38*). In some of the experiments performed in the presence of Mg^{2+}, $[Ca^{2+}]_{free}$ was also varied by adding different concentrations of a Mg−EDTA mixture to 100 μM $[Ca^{2+}]_{total}$ (*35, 39*), and the results were similar. In the latter case, the apparent affinity for Ca^{2+} depends on the *ratio* of dissociation constants for Ca·EDTA and Mg·EDTA, and this ratio cancels off any putative effect of glycerol on individual constants.

Steady-State ATPase Activity Measurements. These measurements were performed using a classical enzyme coupled assay, by measuring the rate of NADH oxidation in the presence of 0.1 mg/mL lactate dehydrogenase, 0.1 mg/mL pyruvate kinase, 1 mM phosphoenolpyruvate and 0.1−0.25 mM NADH (*17*).

Phosphorylation from [^{32}P]Pi. SR vesicles, suspended at 0.1 mg/mL in the absence or presence of detergent, were supplemented with [^{32}P]Pi (whose specific activity was set to about 0.025 mCi/μmol). After 30 s at 20 °C, the reaction (in 0.3 mL) was quenched by rapidly adding excess acid

(2 mL of TCA 500 mM + H_3PO_4 30 mM). The quenched sample was loaded onto two superimposed filters (Millipore HA) and thoroughly rinsed with the same acid solution before counting. Using this combination of strong acid and HA filters made it possible to precipitate and recover essentially all protein on the top filter (in the presence of detergent too, as in ref (*54*)), as testified by the absence of protein-associated counts on the second filter.

Expression, Purification and Assay of E309Q and E309A Mutants. The yeast expression plasmid pYeDP60-SERCA1a-BAD(WT) was the one described by Jidenko et al. (*16*). To insert the E309 to Q mutation into the expression vector, a previously prepared vector, pYeDP60-SERCA1a[$E^{309}Q$]-6His (*15*), was digested by *Eco*RI and *Bss*HII to extract the DNA fragment containing the mutation. This fragment was used to replace the corresponding wild-type sequence in pYeDP60-SERCA1a-BAD(WT). For the mutation of E309 to A, the initial vector containing the appropriately mutated sequence of SERCA1a was generously given by J. P. Andersen, and treated as described in ref (*8*). All constructions were checked by sequencing. The resulting plasmids for the two mutants were named pYeDP60-SERCA1a[$E^{309}Q$]-BAD and pYeDP60-SERCA1a[$E^{309}A$]-BAD. The plasmids were used to transform the *Saccharomyces cerevisiae* yeast strain W303.1b/Gal4 (*a, leu2, his3, trp1::TRP1-GAL10-GAL4, ura3, ade2-1, can^r, cir$^+$*) using the lithium acetate/single stranded carrier DNA/PEG method (*40*). Growth conditions, clone selection, recombinant protein expression and membrane preparation have all been previously described (*41*). Expression levels for the mutated proteins were similar to those observed for WT, i.e., about 0.1 mg of recombinant protein/g of yeast (about 4 mg/L of culture) was found in the "light membrane" fraction.

This fraction was solubilized by DDM for subsequent purification of recombinant Ca^{2+}-ATPase by Streptavidin affinity chromatography (*8*). After purification, the recombinant proteins were concentrated 15- to 20-fold on Centriprep 30 filtration units (Millipore, Saint Quentin en Yvelines, France). Final samples contained about 0.2−0.6 mg/mL of protein, in a buffer consisting of 40 mM Mops-Tris at pH 7, 80 mM KCl, 40% (w/w) glycerol (the glycerol concentration was increased from 20 to 40% at the end of the concentration step, to ensure SERCA1a stability during storage at −80%) and 5−8 mg/mL DDM. This high final concentration of DDM results from our use of the Centriprep 30 units, which retain and therefore concentrate the DDM micelles together with the purified proteins. Note that, unlike Jidenko et al. (*16*), we did not further purify our samples by HPLC, as this proved unnecessary for the present fluorescence measurements.

RESULTS

Effect of Glycerol on the Critical Micellar Concentration and Critical Solubilization Concentration of DDM and $C_{12}E_8$. As a preliminary step, the effects of glycerol on the critical micellar concentration (cmc) and the critical solubilization concentration (csc) of the DDM and $C_{12}E_8$ detergents were tested. The cmc and csc of DDM and $C_{12}E_8$ only rose by less than a factor of 2 at 40% w/w glycerol, with intermediate values at 20% glycerol (data not shown; see corresponding methods and references in the Supporting Information).

Effect of Ca^{2+} on the Trp Fluorescence of Glycerol-Stabilized Detergent-Solubilized SERCA1a at pH 7. It is well-known that Ca^{2+} binding to the high affinity binding and transport sites in the transmembrane domain of SERCA1a affects the intrinsic (i.e., Trp) fluorescence of this protein by a few percent (*9*). This classical observation is illustrated in the experiment shown as panel A in Figure 1, using native SR membrane vesicles. In this experiment, an initial addition of Ca^{2+} on top of the contaminating Ca^{2+} in the medium left the observed Trp fluorescence of SERCA1a unaltered, because, under these conditions at pH 7, the SERCA1a sites were already saturated with the contaminating Ca^{2+} (3−5 μM). In contrast, Ca^{2+} chelation by EGTA reduced this fluorescence by a few percent, and subsequent addition of Ca^{2+} in several steps allowed the fluorescence gradually to recover its initial high level. The concentration of free Ca^{2+} for half-recovery ($Ca_{1/2}$) was in the submicromolar range, as previously found at pH 7 in the absence of Mg^{2+} (e.g., ref (*42*)).

It is also well-known that similar fluorescence measurements are impossible, or at least extremely problematic, in the presence of solubilizing concentrations of detergents such as the DDM or $C_{12}E_8$ which will be used in this work, because solubilized SERCA1a is prone to fast irreversible inactivation (within minutes) if its Ca^{2+} binding sites remain unoccupied (*17*−*22*). Such inactivation results in a fairly rapid decrease of ATPase fluorescence, on top of the slow downward drift observed usually (and especially at low membrane concentrations), and further addition of Ca^{2+} after a few minutes is then unable to induce any change back to the initial fluorescence level of the Ca^{2+}-saturated ATPase. For example, this is illustrated for $C_{12}E_8$ in panel C of Figure 1. Here, we found that the presence, together with detergent, of high concentrations of glycerol, known to protect solubilized SERCA1a from inactivation (*27*), kept the fluorescence of solubilized SERCA1a stable, even in the absence of Ca^{2+}, and thus made it possible to observe significant rises in fluorescence intensity upon subsequent addition of Ca^{2+}. This effect of glycerol, used at 40% w/w for the experiments illustrated in Figure 1, was observed for both DDM and $C_{12}E_8$ (see panel D of Figure 1 for $C_{12}E_8$; corresponding data for DDM are illustrated in panels E and F of Figure S1 in the Supporting Information). Protection against inactivation was also observed at only 20% glycerol, although for shorter periods (not shown). In the absence of detergent, glycerol *alone* left Ca^{2+}-dependent changes essentially intact (*32*) (panel B in Figure 1), although the affinity for Ca^{2+} observed was slightly altered (see below for analysis).

As a collateral advantage of the simultaneous use of glycerol and detergent, note that the above-mentioned slow downward drift in fluorescence intensity which is generally superimposed on the ligand-induced changes independently of irreversible inactivation became even slower than in the absence of detergent (compare Figure 1D and Figure S1F in the Supporting Information with Figures 1A and 1B), perhaps because detergent was adsorbed onto the cuvette walls and the stirring bar, thereby preventing adsorption of the protein itself. Note also that the ATPase Trp fluorescence level itself changed slightly when detergent was added (e.g., Figure 1, C and D): this is commented upon in the Supporting Information, in relation with Figure S1 in the Supporting Information.

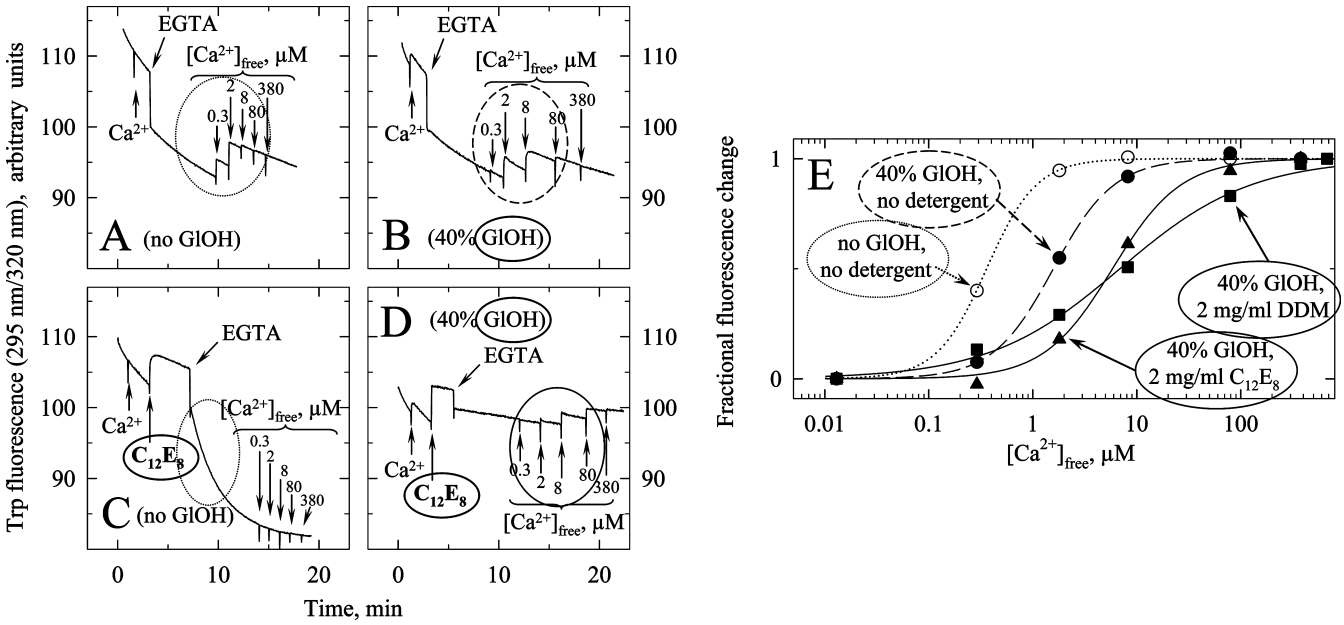

FIGURE 1: Binding of Ca^{2+} to membrane-embedded or detergent-solubilized Ca^{2+}-ATPase in the presence of glycerol (here at pH 7 in the absence of Mg^{2+}), as deduced from Trp fluorescence changes. (A−D) SERCA1a intrinsic fluorescence was measured with SR vesicles suspended at a final protein concentration of 0.02 mg/mL in a 2 mL quartz cuvette, in a buffer containing 100 mM KCl and 50 mM Mops-Tris at pH 7 and 20 °C (panel A), supplemented with 40% (w/w) glycerol as required (panels B and D). The initial step consisted of adding 20 μM Ca^{2+} (4 μL of a 10 mM stock solution) on top of the contaminating Ca^{2+} (3−5 μM) already present in the buffer. This was followed, as required, by the addition of 2 mg/mL $C_{12}E_8$ (panels C and D) or 2 mg/mL DDM (not shown here, but see Figure S1 in the Supporting Information, panels E and F). Subsequent addition of 0.75 mM EGTA (5 μL of a 300 mM stock solution) then reduced $[Ca^{2+}]_{free}$ to about 0.013 μM, and the fluorescence intensity at this step was arbitrarily taken as 100%. Several amounts of extra total Ca^{2+} were then added, in the order and at the concentrations stated below together with the resulting final concentrations of total and free $[Ca^{2+}]$: first, 0.3 mM (as 2 μL of a 300 mM stock solution), resulting in $[Ca^{2+}]_{total} = 0.325$ mM and $[Ca^{2+}]_{free} = 0.29$ μM; 0.3 mM again (2 μL at 300 mM), resulting in $[Ca^{2+}]_{total} = 0.625$ mM and $[Ca^{2+}]_{free} = 1.8$ μM; 0.1 mM (2 μL at 100 mM), resulting in $[Ca^{2+}]_{total} = 0.725$ mM and $[Ca^{2+}]_{free} = 8.2$ μM; 0.1 mM again (2 μL at 100 mM), resulting in $[Ca^{2+}]_{total} = 0.825$ mM and $[Ca^{2+}]_{free} = 79$ μM; and last, 0.3 mM (2 μL at 300 mM), resulting in $[Ca^{2+}]_{total} = 1.125$ mM and $[Ca^{2+}]_{free} = 376$ μM. To estimate $[Ca^{2+}]_{free}$ according to MaxChelator (a rough value is indicated on the figure itself), the dissociation constant of the Ca^{2+}•chelator complex was assumed to be the same, irrespective of the presence or absence of glycerol (see text). The relative error in estimating these free Ca^{2+} concentrations is largest around 10 μM free Ca^{2+}. The traces shown illustrate a representative experiment of several which gave similar results. Fluorescence intensities were corrected for the small changes due to sample dilution on the addition of ligands. (E) Changes in ATPase fluorescence as a function of free Ca^{2+} were deduced from the traces in panels A, B and D, and from the trace in panel F of Figure S1 in the Supporting Information, after correction for the intensity drift, and plotted as fractional values after normalization to 1 of the maximal change in fluorescence in each series. These various curves were fitted to Hill equations with Hill coefficients of 1.8 (no glycerol, no detergent, open circles), 1.5 (40% glycerol, no detergent, closed circles), 1.3 (40% glycerol, 2 mg/mL $C_{12}E_8$, closed triangles) and 0.7 (40% glycerol, 2 mg/mL DDM, closed squares), but exact values for these Hill coefficients are subject to strong uncertainties because of the small number of data points; the only clear fact is the reduced cooperativity in the last curve. Before normalization, the maximal Ca^{2+}-dependent relative changes in fluorescence were about 6.2%, 6.1%, 4.6% and 3.2% for the traces in panels A, B, D, and panel F of Figure S1 in the Supporting Information.

Traces like those illustrated in Figure 1 A, B, D (and in Figure S1F in the Supporting Information) made it possible to deduce, from the progressive recovery of fluorescence upon Ca^{2+} supplementation, the apparent affinity for Ca^{2+} of membrane-embedded or detergent-solubilized Ca^{2+}-ATPase (Figure 1E). Glycerol alone reduced this affinity moderately, as $Ca_{1/2}$ rose from 0.3−0.4 μM to 1.5−2 μM. This reduction in affinity, here deduced from the assumption that Ca^{2+} chelation by EGTA does not change much in the presence of glycerol, is consistent with the fact that in the presence of glycerol and absence of EGTA, the initial addition of 20 μM Ca^{2+} on top of the contaminating Ca^{2+} raised the Trp fluorescence level up to its maximal level, whereas in the absence of glycerol (as shown in Figure 1A), the same addition of 20 μM Ca^{2+} had absolutely no effect. The additional presence of detergent, either DDM or $C_{12}E_8$, reduced this affinity further, as $Ca_{1/2}$ rose to still higher values, 7−9 μM, in the presence of 2 mg/mL of either detergent. However, the effects of DDM and $C_{12}E_8$ did not appear to be identical, as in addition to raising $Ca_{1/2}$, DDM reduced the apparent cooperativity for Ca^{2+} binding quite significantly, whereas this was much less the case for $C_{12}E_8$ (closed triangles versus closed squares in Figure 1E). Similar results were obtained when experiments were repeated in the presence of 5 mM Mg^{2+}, as illustrated in Figure S2 in the Supporting Information: in the presence of Mg^{2+}, while the apparent affinity of solubilized ATPase for Ca^{2+} was poorer than in its absence (a well-known fact for membrane-embedded SERCA1a, presumably due to competition between Mg^{2+} and Ca^{2+}, see, for instance, refs *11, 42*), the additional presence of detergent together with glycerol again shifted $Ca_{1/2}$ to still higher values, and DDM again specifically reduced the apparent cooperativity for Ca^{2+} binding. The poorer affinity and/or possibly poorer cooperativity of Ca^{2+} binding to detergent-solubilized SERCA1a compared with membrane-embedded ATPase have often been alluded to and discussed (or denied) in previous investigations with solubilized ATPase (e.g., refs (*17, 18, 30, 43, 44*), additional references are provided together with the legend to Figure

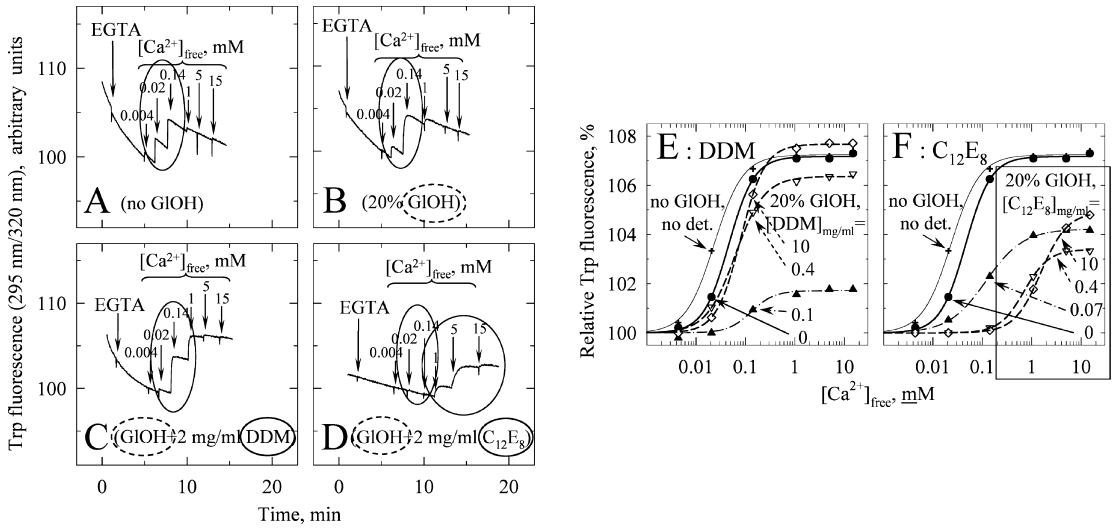

FIGURE 2: Ca^{2+} binding to membrane-embedded, DDM-solubilized or $C_{12}E_8$-solubilized Ca^{2+}-ATPase at pH 6 and absence of KCl, as deduced from Trp fluorescence changes. (A−D) SERCA1a intrinsic fluorescence was measured with SR vesicles suspended at a final protein concentration of 0.1 mg/mL in a 2 mL quartz cuvette, in a buffer containing 150 mM Mes-Tris, 100 mM LiCl and 20 mM $MgCl_2$, at pH 6 and 20 °C (panel A), in the absence of glycerol (panel A) or in the presence of 20% (v/v) glycerol (panels B−D); 2 mg/mL detergent was then added as required (DDM for panel C, $C_{12}E_8$ for panel D). Next (the illustrated traces start here) 2 mM EGTA was added, resulting in a free Ca^{2+} concentration of about 0.1 μM. The fluorescence intensity at this step was taken as 100%. Various amounts of Ca^{2+}, on top of the contaminating 3−5 μM total Ca^{2+}, were then added, resulting in different final concentrations of free [Ca^{2+}]. These amounts are listed below in the order of their addition, together with the resulting concentrations of free [Ca^{2+}]: 0.2 mM, resulting in $[Ca^{2+}]_{total}$ = 0.205 mM and $[Ca^{2+}]_{free}$ = 4 μM ($[Ca^{2+}]_{free}$ was estimated according to MaxChelator); 0.5 mM, resulting in $[Ca^{2+}]_{total}$ = 0.705 mM and $[Ca^{2+}]_{free}$ = 20 μM; 1 mM, resulting in $[Ca^{2+}]_{total}$ = 1.7 mM and $[Ca^{2+}]_{free}$ = 140 μM; 1.3 mM, resulting in $[Ca^{2+}]_{total}$ = 3 mM and $[Ca^{2+}]_{free}$ = 1 mM; 4 mM, resulting in $[Ca^{2+}]_{free}$ = 5 mM; and 10 mM, resulting in $[Ca^{2+}]_{free}$ = 15 mM. Values were corrected for the small changes in intensity due to sample dilution on the addition of ligands. (E, F) After correction for the intensity drift, fluorescence intensities as a function of free Ca^{2+} for a given concentration of detergent (DDM in panel E, $C_{12}E_8$ in panel F) were deduced from the traces in panels A−D and from similar traces at other detergent concentrations, and plotted as %, relative to the fluorescence intensity at the lowest free Ca^{2+} concentration, under the following conditions: crosses and continuous thin line, no detergent and no glycerol; closed circles and continuous thick line, 20% glycerol and no detergent; closed right-side-up triangles and dash−dot lines, glycerol and nonsolubilizing concentrations of detergent (0.1 mg/mL DDM for panel E, 0.07 mg/mL $C_{12}E_8$ for panel F); dashed lines and open symbols, solubilizing concentrations of detergent, either 0.4 mg/mL (upside-down triangles), or 10 mg/mL (diamonds). As the results obtained with 2 mg/mL detergent (deduced from the traces in panels C and D) were close to those obtained with 10 mg/mL (diamonds in panels E and F), they were omitted, for clarity. These various curves were fitted to Hill equations with Hill coefficients between 1.5 and 2 for both intact and solubilized membranes.

S2 in the Supporting Information). This question will be addressed again in the Discussion.

The Dramatic Effect of Solubilizing Concentrations of $C_{12}E_8$ on SERCA1a Affinity for Ca^{2+} at pH 6. As pH *per se*, in the absence of Ca^{2+}, is thought to affect the conformational state of SERCA1a (*55, 67*), we extended to more acidic conditions our characterization by Trp fluorescence of the binding of Ca^{2+} to solubilized SERCA1a, and found that, under these conditions, $C_{12}E_8$-solubilized SERCA1a displayed an unexpected behavior. At pH 6 in the presence of glycerol (here in the absence of potassium but presence of lithium), solubilization of SERCA1a (0.1 mg/mL SR) with excess $C_{12}E_8$ (from 0.4 up to 10 mg/mL $C_{12}E_8$) indeed raised the $Ca_{1/2}$ values for Ca^{2+}-induced changes in Trp fluorescence to extremely high, *millimolar* values (see Figure 2D versus Figure 2B), i.e., the apparent affinity of SERCA1a for Ca^{2+} dropped to extremely poor values. Nevertheless, this was *not* the case for DDM, which, at solubilizing concentrations, only led to a moderate increase in the $Ca_{1/2}$ (Figure 2C versus Figure 2B). The data for different concentrations of solubilizing detergent are given for DDM and $C_{12}E_8$ in Figures 2E and 2F respectively. In the same series of experiments, the apparent affinity of SERCA1a for Ca^{2+} in the presence of low, nonsolubilizing concentrations of detergent was also tested, and the results, also illustrated in Figures 2E and 2F, confirmed the moderate increase in $Ca_{1/2}$ previously observed when SR membranes were "perturbed" by various detergents, either DDM (*39*), $C_{12}E_8$ (data not shown), Triton X-100 (*44*) or SDS (*45*). For large solubilizing concentrations of DDM, the $Ca_{1/2}$ was in fact smaller than for small nonsolubilizing concentrations (Figure 2E) (as previously deduced from ATPase activity measurements under standard ionic conditions, see Figure 5 in ref (*39*)). For $C_{12}E_8$, the opposite was true, because in the presence of large solubilizing concentrations of $C_{12}E_8$, the $Ca_{1/2}$ became much higher than in the presence of nonsolubilizing concentrations (Figure 2F). Note, incidentally, that the presence of 1 mg/mL DOPC together with 2 mg/mL $C_{12}E_8$ left the apparent Ca^{2+} affinity of SERCA1a at an intermediate value, corresponding to the affinity displayed by $C_{12}E_8$-saturated or minimally solubilized SR membranes (data not shown).

The unexpectedly huge shift of $Ca_{1/2}$ to millimolar values in the presence of solubilizing concentrations of $C_{12}E_8$ at pH 6 was not restricted to assay conditions in the absence of KCl and presence of LiCl, but was also observed in the presence of KCl. This was shown not only by Trp fluorescence experiments similar to those reported above but now performed in the presence of KCl (Figure 3A,B), but also by simple ATPase activity measurements, at 10 μM Mg·ATP (Figure 3C), performed with a standard coupled enzyme assay (the presence of KCl in fact was a prerequisite for such ATPase activity measurements with this technique,

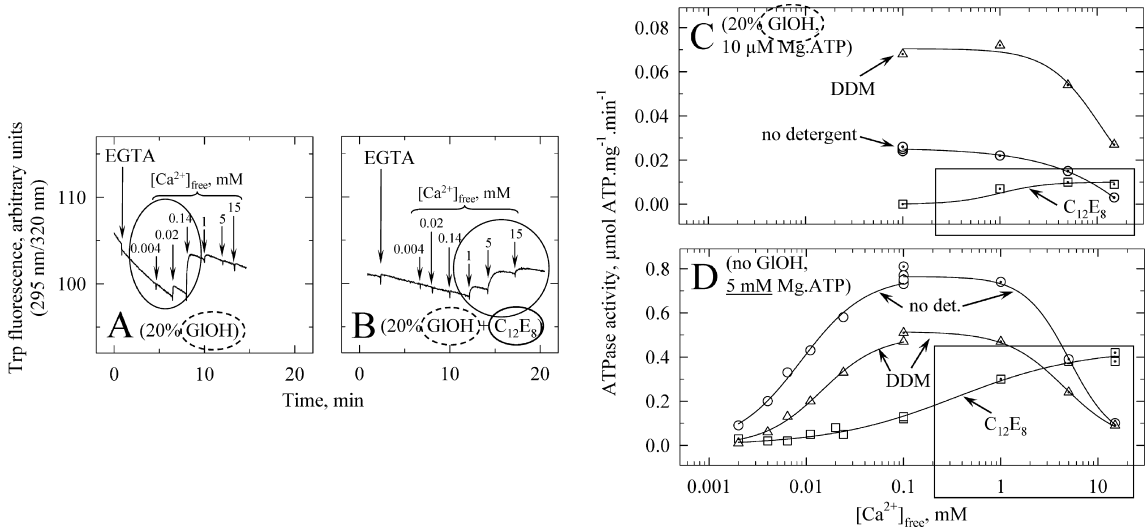

FIGURE 3: In the presence of KCl too, the affinity of SERCA1a for Ca^{2+} at pH 6 drops dramatically after solubilization with $C_{12}E_8$. This affects Ca^{2+}-dependent ATPase activities, both in the presence of glycerol at low Mg·ATP and in its absence at high Mg·ATP. (A, B) Trp fluorescence was measured with 0.1 mg/mL SR vesicles in a buffer containing 20% glycerol, 150 mM Mes-Tris, 100 mM KCl and 20 mM $MgCl_2$ at pH 6 and 20 °C, in the presence of 2 mg/mL $C_{12}E_8$ (panel B) or in the absence of $C_{12}E_8$ (panel A). EGTA (2 mM) was added, resulting in a free Ca^{2+} concentration of about 0.1 μM (at this stage, the fluorescence intensity was taken as 100%); this was followed by additions of Ca^{2+}, on top of the contaminating 3−5 μM total Ca^{2+}, in the order and at the concentrations given below together with the resulting final concentrations of free $[Ca^{2+}]$: 0.2 mM resulting in $[Ca^{2+}]_{total} = 0.205$ mM and $[Ca^{2+}]_{free} = 4$ μM ($[Ca^{2+}]_{free}$ was estimated according to MaxChelator); 0.5 mM, resulting in $[Ca^{2+}]_{total} = 0.705$ mM and $[Ca^{2+}]_{free} = 20$ μM; 1 mM, resulting in $[Ca^{2+}]_{total} = 1.7$ mM and $[Ca^{2+}]_{free} = 140$ μM; 1.3 mM, resulting in $[Ca^{2+}]_{total} = 3$ mM and $[Ca^{2+}]_{free} = 1$ mM; 4 mM, resulting in $[Ca^{2+}]_{free} = 5$ mM; and last, 10 mM, resulting in $[Ca^{2+}]_{free} = 15$ mM. Fluorescence intensities were corrected for the small changes due to sample dilution on the addition of ligands. (C) ATPase activity was measured by a coupled enzyme assay, here in the presence of 10 μM Mg·ATP, under each of three conditions: the absence of detergent (circles), the presence of 2 mg/mL DDM (triangles), or the presence of 2 mg/mL $C_{12}E_8$ (squares). The medium, which also contained 0.1 mg/mL SR vesicles, 20% glycerol, 150 mM Mes-Tris, 100 mM KCl and 20 mM $MgCl_2$ at pH 6 and 20 °C as for the experiments shown in panel A, was first supplemented with 0.1 mM Ca^{2+} and subsequently with final Ca^{2+} concentrations of 1, 5 and 15 mM for the ATPase activity measurements. (D) This panel shows similar ATPase activity measurements, but in the presence of 5 mM Mg·ATP, without glycerol and with 0.01 mg/mL SR (see the different *Y* scale) in otherwise the same media, again in the absence of detergent (circles), in the presence of 2 mg/mL DDM (triangles), or in the presence of 2 mg/mL $C_{12}E_8$ (squares). Here too, the medium was initially supplemented with 0.1 mM Ca^{2+}. Then, either various concentrations of EGTA were added sequentially, to explore the effect of lower free Ca^{2+} concentrations (open symbols), or extra Ca^{2+} was added, to explore the effect of higher free Ca^{2+} concentrations (dotted symbols).

because of the potassium-dependence of the pyruvate kinase activity in this assay). Millimolar concentrations of Ca^{2+} *stimulated* ATPase activity in the presence of glycerol after solubilization by $C_{12}E_8$ at pH 6, contrasting with the more classical *inhibitory* effect of the same high concentrations of Ca^{2+} on the activity of membrane-embedded or DDM-solubilized SERCA1a (Figure 3C). To address the question of whether the $C_{12}E_8$-induced large increase in the $Ca_{1/2}$ for ATPase activation only occurred in the presence of glycerol or, instead, was a more general feature, we performed additional ATPase measurements in the absence of glycerol. Using 5 mM Mg·ATP instead of only 10 μM, we were able to perform these additional experiments in the absence of glycerol reliably, because high Mg·ATP concentrations have been reported to efficiently reverse the instability of solubilized SERCA1a (*17, 46*). Under these conditions, *i.e.* in the absence of glycerol, a very large increase in the $Ca_{1/2}$ for ATPase activation at pH 6 was again apparent for SERCA1a solubilized with $C_{12}E_8$, whereas with DDM, the increase was again much smaller (Figure 3D).

The different Effects of DDM and $C_{12}E_8$ at Solubilizing Concentrations on the Kinetics of Ca^{2+}-Induced Conformational Rearrangement. In addition to making it possible to monitor Ca^{2+} binding to solubilized SERCA1a at *equilibrium*, the presence of glycerol in the above-mentioned fluorescence experiments at pH 6, by slowing down the Ca^{2+}-induced conformational rearrangement of SERCA1a sufficiently (*32*), made it possible, at least at pH 6, to resolve the *kinetics* of the Ca^{2+}-induced fluorescence rises, without resorting to more complicated stopped-flow experiments. Figure 2B−D shows that the rate of this Ca^{2+}-induced rearrangement was slightly accelerated by solubilizing concentrations of DDM, whereas solubilizing concentrations of $C_{12}E_8$ did not result in such acceleration, but in *significant slowing down* instead; as concerns $C_{12}E_8$, the same conclusion can be derived from Figure 3A,B. This unexpected slowing down of the Ca^{2+}-induced rearrangement by high concentrations of $C_{12}E_8$, but not DDM, contrasts with the fact that this rearrangement is accelerated in the presence of either $C_{12}E_8$ or DDM at low, nonsolubilizing concentrations (*25, 26, 47*), or in the simultaneous presence of $C_{12}E_8$ and lipid at a $C_{12}E_8$ to DOPC ratio of about 2 to 1 (data not shown; see also panels B and F versus panel A in the subsequent Figure 5).

A possible consequence of this slowing down of the Ca^{2+}-induced transition by high concentrations of $C_{12}E_8$ (as opposed to DDM) in the absence of extra lipid is that the E2/E1 equilibrium of SERCA1a might shift toward E2 in the presence of solubilizing and delipidating concentrations of $C_{12}E_8$, rendering Ca^{2+} binding strongly unfavorable, especially at pH 6. This will be a key point when the effects of $C_{12}E_8$ versus those of DDM are discussed below.

Consequences of the Ca^{2+} Affinity Changes for Trp Fluorescence Detection of the Turnover of SERCA1a Solu-

bilized in Different Detergents at pH 6. Previous studies with membrane-embedded SERCA1a have suggested that the ability of Trp fluorescence to monitor SERCA1a enzymatic turnover depends on whether the E2P and E2 species, often considered to display lower fluorescence than the E1 and E1P species, accumulate to a significant or insignificant extent at steady-state during turnover (see, for instance, refs *48, 49*). In previously published reports, different media have been shown to favor accumulation of E2P or E2 species at steady state (and therefore observation of clear turnover-dependent drops in fluorescence), and a pH 6 medium, devoid of potassium and supplemented by lithium if desired (*50*), fulfills these requirements (*47, 49*). With such a medium, in the presence of ionophore to make SR vesicles permeable to Ca^{2+} (note that as a side effect, the ionophore we used, calcimycin [A23187], quenched a large fraction of SERCA1a fluorescence (*49*), accounting for the low initial fluorescence level illustrated in Figure 4), and first in the absence of glycerol (panel A in Figure 4), a clear turnover-dependent drop in fluorescence was found to result from ATP addition to the permeabilized vesicles at both 100 μM and 1 mM Ca^{2+}, as illustrated by the beginning of the fluorescence trace in Figure 4A (the Ca^{2+} concentration was raised from 100 μM to 1 mM Ca^{2+} to allow full saturation of the Ca^{2+} binding sites of membrane-embedded SERCA1a, despite the acidic pH). Increasing concentrations of detergent were then added, and the result for DDM, here in the absence of glycerol, is also shown in Figure 4A. Small, nonsolubilizing (or minimally solubilizing) concentrations of DDM (0.1 mg/mL) reduced the size of the turnover-dependent fluorescence drop but not its rate, as previously reported (*26*). When higher concentrations of detergent were added, from 0.5 to 10 mg/mL, solubilization of the membranes occurred, as confirmed by the recovery of Trp fluorescence from its previous quenching by the nearby calcimycin ionophore (*49*). However, when ATP-dependent signals were then recorded, signs of turnover-dependent irreversible inactivation were detectable at the highest concentrations of DDM (Figure 4A, see dotted zone for 10 mg/mL), as expected on the basis of a previous report with $C_{12}E_8$ at pH 8.5 (*48*). Similar signs of inactivation were also observed with $C_{12}E_8$ at pH 6 in the absence of glycerol (not shown).

In the *presence* of 20% glycerol (here, v/v) (Figure 4B), the initial turnover rate for membrane-embedded SERCA1a was slower than in its absence, and raising the Ca^{2+} concentration from 100 μM to 1 mM again accelerated this rate slightly, because of the more complete saturation of the Ca^{2+} sites (see the beginning of the trace in Figure 4B). After solubilization, the presence of glycerol apparently eliminated turnover-dependent irreversible inactivation (Figure 4B). Highly reproducible ATP turnover-dependent fluorescence drops were therefore observable for all concentrations of DDM up to 10 mg/mL (higher concentrations were not tested). The glycerol-induced slowing down of the ATPase activity of membrane-embedded SERCA1a was reversed by both low and very high concentrations of DDM, although not exactly to the same extent. Similar experiments performed at pCa 4 only (instead of pCa 3) revealed the same qualitative features. Thus, assay conditions at pH 6 in the presence of glycerol are appropriate for revealing, by intrinsic fluorescence, the ATPase activity of DDM-solubilized ATPase.

When the same experiments (at pH 6 in the presence of glycerol) were repeated in the presence of $C_{12}E_8$, there was again no sign of any irreversible inactivation of the solubilized SERCA1a, and ATP turnover was again accelerated by the nonsolubilizing or only minimally solubilizing concentration of 0.07 mg of $C_{12}E_8$/mL (as with 0.1 mg/mL DDM). In this case, however, higher, fully solubilizing concentrations of $C_{12}E_8$ inhibited turnover dramatically: Figure 4C shows the results for 0.5 and 2 mg/mL $C_{12}E_8$, and the same was true at 10 mg/mL (not shown). In accordance with the results in Figures 2 and 3, this dramatic inhibition of turnover proved to be due, at least in part, to the fact that SERCA1a had acquired a very poor affinity for Ca^{2+} under these new conditions, because when Ca^{2+} was increased to 3 mM and even further, up to 10 mM, the time required for ATP exhaustion was reduced (final portion of the trace in Figure 4C). Conversely, when the experiment

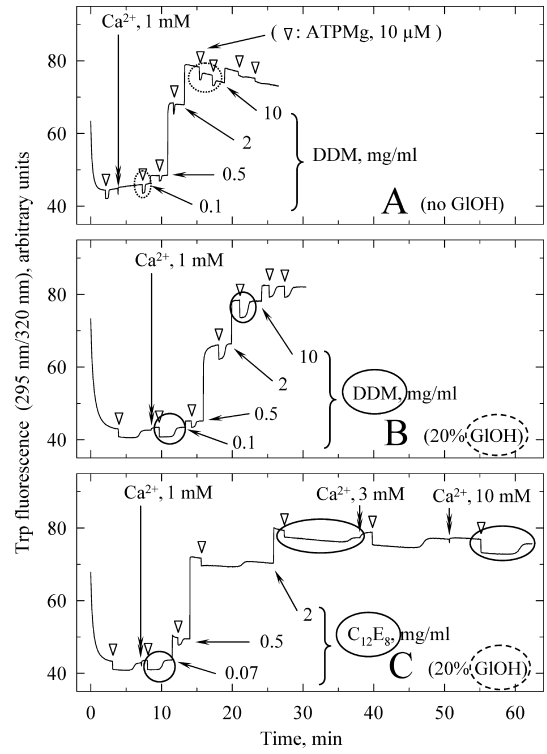

FIGURE 4: Turnover-dependent changes in Trp fluorescence at pH 6 in the absence of KCl, in the absence or presence of glycerol, in the presence of various concentrations of DDM or $C_{12}E_8$, and in the presence of various concentrations of Ca^{2+}. Here, Trp fluorescence was measured with 0.1 mg/mL SR vesicles in a buffer containing 150 mM MesTris, 100 mM LiCl and 20 mM $MgCl_2$ at pH 6 and 20 °C, in the absence (panel A) or presence (panels B and C) of 20% glycerol (here, v/v); 100 μM Ca^{2+} was then added (the corresponding fluorescence level was taken as the 100% level), followed by 2 μg/mL of ionophore A23187, i.e., calcimycin, which induced rapid (but not instantaneous) fluorescence quenching through resonance ("Förster") transfer. The illustrated trace starts here. The next step consisted of adding 10 μM Mg·ATP (upside-down triangle), followed by 0.9 mM Ca^{2+} to raise the Ca^{2+} concentration to 1 mM, and then Mg·ATP again (upside-down triangle again). Next, detergent (DDM for panels A and B and $C_{12}E_8$ for panel C) was sequentially added at the final total concentrations indicated (0.1, 0.5, 2 and 10 mg/mL for DDM; 0.07, 0.5, and 2 mg/mL for $C_{12}E_8$), with repeated additions of ATP between the detergent additions to test the turnover-dependent signals (upside-down triangles). At the end of the experiment illustrated in panel C, 2 mM and then an extra 7 mM Ca^{2+} were added, bringing the total Ca^{2+} to 3 mM (10 μM ATP was also added at each step, upside-down triangles). Values were corrected for the small changes in intensity resulting from dilution on the addition of ligands.

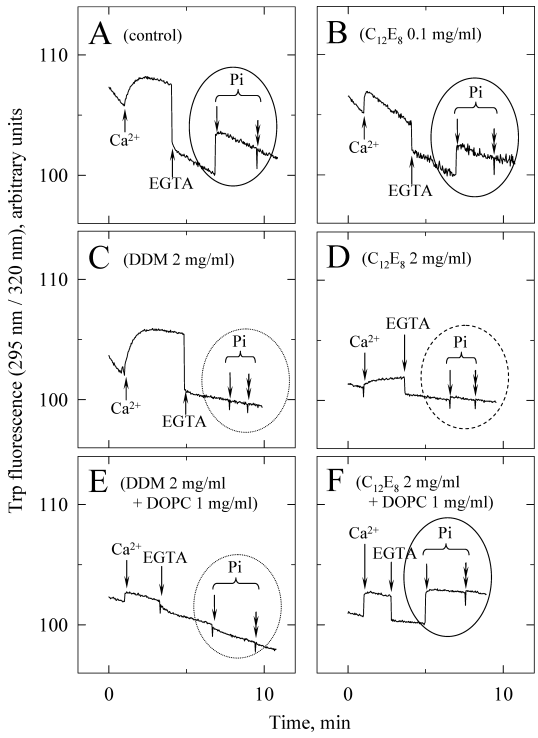

FIGURE 5: Pi-dependent changes in Trp fluorescence for membrane-embedded or detergent-solubilized Ca^{2+}-free SERCA1a. Effect of additional lipids. SR vesicles were suspended at 0.1 mg/mL protein (corresponding to about 0.05 mg/mL of SR lipid) in a buffer containing 150 mM Mes-Tris, 20 mM Mg^{2+}, 15% DMSO and 20% glycerol (w/w) at pH 6 and 20 °C, either in the absence of detergent (panel A) or together with one of the following five agents or combinations: 0.1 mg/mL of $C_{12}E_8$ (panel B), 2 mg/mL DDM (panel C), 2 mg/mL $C_{12}E_8$ (panel D), 2 mg/mL DDM + 1 mg/mL DOPC (panel E), or 2 mg/mL $C_{12}E_8$ + 1 mg/mL DOPC (panel F). Ca^{2+} (1 mM) was then added, followed by 10 mM EGTA, and last Pi, in two amounts of 2 mM each (single arrow, then double arrow). Values were corrected for the small changes in fluorescence intensity resulting from dilution on the addition of ligands.

was conducted in the presence of 100 μM Ca^{2+} only, turnover-dependent fluorescence signals were not detectable at all for the solubilized SERCA1a, even at 0.5 mg/mL $C_{12}E_8$, and turnover-dependent signals only appeared after the Ca^{2+} concentration was raised to millimolar concentrations (data not shown). Note that this stimulation of ATPase activity by high Ca^{2+} concentrations occurs *despite* the inhibition expected, at these concentrations, to result from the substitution of Ca·ATP for the normal substrate, Mg·ATP (*51*).

Note that similar experiments were also performed in another medium known to favor accumulation of E2P or E2 species at steady state, namely, at alkaline pH in the presence of a high Mg^{2+} concentration (*48*). In this medium, unfortunately, the additional presence of 40% glycerol under the same conditions was not sufficient to reduce to zero the irreversible inactivation of SERCA1a known to occur (Figure 7E in ref (*48*)) in the absence of glycerol and presence of solubilizing concentrations of detergent (see Figure S3A in the Supporting Information, and corresponding comments). Back to the pH 6 situation, note also that Ca^{2+} release experiments performed under similar conditions (data not shown, but see a short description in the Supporting Information) were consistent with the Trp fluorescence results and the here-demonstrated very poor affinity of SERCA1a for Ca^{2+} in the presence of $C_{12}E_8$ at pH 6.

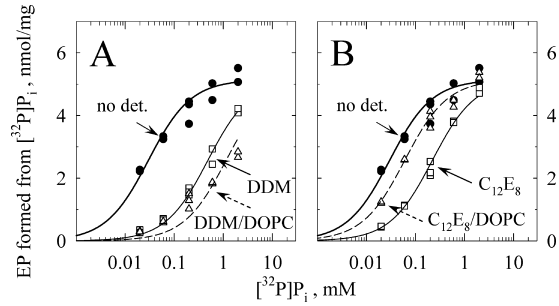

FIGURE 6: Formation of E2P from [^{32}P]Pi in membrane-embedded or detergent-solubilized Ca^{2+}-free SERCA1a, as deduced from standard acid quenching experiments. SR vesicles were suspended at 0.1 mg/mL protein in a buffer containing 150 mM Mes-Tris, 20 mM Mg^{2+}, 2 mM EGTA, 15% DMSO and 20% glycerol (w/w) at pH 6 and 20 °C, either in the absence of detergent (closed circles, panels A and B), or together with one of the following five agents or combinations: 2 mg/mL DDM (squares, panel A), 2 mg/mL DDM + 1 mg/mL DOPC (triangles, panel A), 2 mg/mL $C_{12}E_8$ (squares, panel B), or 2 mg/mL $C_{12}E_8$ + 1 mg/mL DOPC (triangles, panel B). Various concentrations of [^{32}P]Pi were then added, followed after 30 s by acid quenching and filtration. Observed EP levels were fitted to Michaelian curves with a common EP max (5.14 nmol/mg).

Effects of Pi or Fluoride Salts on Trp Fluorescence in Ca^{2+}-Free and Glycerol-Protected Detergent-Solubilized ATPase. We then attempted, in the combined presence of glycerol and detergent, to detect the formation of ADP-insensitive phosphoenzyme (E2P) from Ca^{2+}-free solubilized SERCA1a, as was shown to be possible for membrane-embedded SERCA1a (*10, 32*). Initial attempts using a pH 7, KCl-containing medium, supplemented with 20% DMSO to make the medium more favorable to E2P formation (*52, 53*), did not allow us to observe any Pi-dependent fluorescence changes in the presence of detergent (DDM or $C_{12}E_8$), either in the absence or in the presence of glycerol (data not shown). We then attempted to create different favorable conditions, by leaving potassium out of the medium and buffering it at pH 6, either in the absence of DMSO but presence of 40% glycerol, or in the presence of 15% DMSO and 20% glycerol (DMSO itself afforded partial protection against detergent-induced denaturation). In the latter situation, the rise in Trp fluorescence concomitant with E2P formation was easily detectable in the absence of detergent (see Figure 5A), but in the presence of detergent (either DDM or $C_{12}E_8$) there was again little evidence for any change in Pi-dependent fluorescence (Figure 5, C and D), although with $C_{12}E_8$, very small but reproducible changes in Trp fluorescence could still be observed. This only very small signal was disappointing, because E2P formation has been reported to remain possible in the presence of some of the most common detergents, although less favorable than in the absence of detergent (see, for instance refs (*30, 44, 54*),).

As the conditions for these fluorescence experiments, namely, low protein and high detergent concentrations, presumably lead to SERCA1a delipidation, we then wondered whether the residual presence of phospholipid together with detergent would help to make Pi-induced changes detectable. We found that this was indeed the case, but remarkably, only in the presence of $C_{12}E_8$ (Figure 5F) and not of DDM (Figure 5E). From the point of view of the fluorescence responses to Pi addition, the incubation of SR vesicles with a 2:1 (w/w) mixture of $C_{12}E_8$ and DOPC,

obtained from an optically clear, concentrated 2:1 mixture (in which the lipid had therefore been almost fully solubilized) gave results not very different from those obtained after incubation of the same SR vesicles with a small, only minimally solubilizing amount of $C_{12}E_8$ alone, i.e., when 0.1 mg/mL $C_{12}E_8$ was added to 0.1 mg/mL SR protein and therefore about 0.05 mg/mL SR lipid (so that the overall detergent to lipid ratio was similar in both experiments): in both experiments, the Pi-induced rise in Trp fluorescence was quite satisfactory (Figure 5, F and B). Note that in both cases, the Ca^{2+}-dependent changes also displayed smaller amplitude and faster kinetics than in SR vesicles in the absence of any detergent (compare panels A, B and F in Figure 5). Results similar to those illustrated in Figure 5, A,F (in the presence of lipid) were in fact also obtained in the *absence of glycerol* (data not shown), presumably because the presence of lipid together with detergent was sufficient to protect solubilized SERCA1a from EGTA-induced irreversible denaturation (*19*−*21*).

To understand the above results, we measured under the exact same conditions, using standard acid quenching methods, the actual amount of phosphoenzyme (E2P) formed from phosphate, added as [^{32}P]Pi. We found (Figure 6) that the presence of detergent alone increased the concentration of [^{32}P]Pi required for half activation of E2P formation, with a more deleterious effect for DDM than for $C_{12}E_8$. The additional presence of DOPC allowed the recovery of a more favorable situation in the presence of $C_{12}E_8$ (Figure 6B), but not in the presence of DDM (Figure 6A). This result is qualitatively consonant with the fluorescence results. Nevertheless, it appears that, at the concentrations of Pi (2 and 4 mM) used in the Trp fluorescence measurements illustrated in Figure 5, very significant amounts of E2P were formed in the presence of either detergent in the absence of lipid, whereas Pi-dependent changes in fluorescence were minimal. Thus, the combined results in Figures 5 and 6 indicate that the absence of a Pi-dependent Trp fluorescence signal does not necessarily imply the absence of E2P formation, but that only specific conditions (in this case, $C_{12}E_8$ + DOPC) allow Trp fluorescence to be sensitive to this formation. This will be further discussed later. Note that the increased concentration of [^{32}P]Pi required for half activation of E2P formation in the presence of glycerol and detergent was *not* the result of time-dependent partial inactivation, because the (reduced) levels of E2P found in the presence of glycerol and detergent remained stable when the time of contact of SERCA1a with the detergent was varied, on a time scale of a few minutes (data not shown).

As a further check, we studied, under the same conditions, the ability of Trp residues in solubilized Ca^{2+}-free SERCA1a to reveal the putative formation from beryllium fluoride of E2·BeF_3, an E2P analogue, and found results similar to those for E2P detection: Trp fluorescence rose on the addition of beryllium and fluoride in the presence of $C_{12}E_8$ plus DOPC, as it does in membranes (*33, 53*), but did not rise in the presence of DDM plus DOPC (Figure S4 in the Supporting Information).

Effects of Mg-ATP, ATP or Mg^{2+} on Trp Fluorescence in Ca^{2+}-Free Detergent-Solubilized and Glycerol-Protected ATPase. The Trp fluorescence response of solubilized but glycerol-stabilized SERCA1a to nucleotides was also investigated. For this study, as acidic conditions are known to have an unfavorable effect on the affinity of SERCA1a for Mg·ATP (*12*), we reverted to a standard pH 7 situation. Figure 7 shows an experiment in which, after an initial addition of EGTA and 5 mM Mg^{2+} (to be commented upon below), various concentrations of Mg·ATP (ranging from 0.3 to 300 μM) were added to SR vesicles to trigger the anticipated increase in fluorescence caused by Mg·ATP binding to the ATPase. For the highest concentrations of added Mg·ATP, e.g., 300 μM (several such additions were made; see double arrows in Figure 7A), a drop in fluorescence intensity was observed, instead of a rise, because of the inner-filter effect of the UV absorbance of nucleotide at

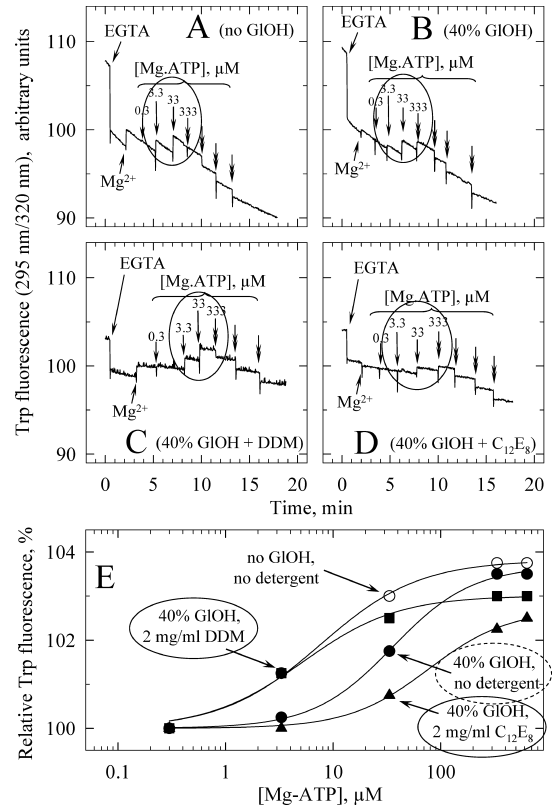

FIGURE 7: Binding of Mg-ATP to Ca^{2+}-free membrane-embedded or detergent-solubilized ATPase in the presence of glycerol, as deduced from Trp fluorescence changes. (A−D) The initial step consisted of adding 2 mM of EGTA to SR vesicles suspended at 0.02 mg/mL in a buffer containing 100 mM KCl, 50 mM Mops-Tris and 100 μM Ca^{2+} at pH 7 and 20 °C (panel A), in the presence, as required, of 40% (w/w) glycerol (panels B−D) and 2 mg/mL detergent (DDM for panel C or $C_{12}E_8$ for panel D). This was followed by the addition of 5 mM Mg^{2+} and then of increasing concentrations of Mg·ATP (final total concentrations are indicated on the figure). Double arrows correspond to additions of 300 μM. Several such additions were made to estimate the inner filter effects due to nucleotide absorbance (double arrows without numbers). Values were corrected for the small changes in intensity resulting from dilution on the addition of ligands, but *not* for the changes due to these inner-filter effects. Note that using 295 nm instead of a shorter excitation wavelength minimizes the inner-filter effect of the nucleotide, and using 320 nm instead of a longer emission wavelength maximizes the Mg^{2+}-induced signal, see ref *11*. (E) After correction for the drift and inner-filter effects, relative fluorescence levels in the presence of the various concentrations of Mg·ATP were plotted for the four conditions above: without glycerol and without detergent (open circles), with glycerol but without detergent (closed circles), or with glycerol and either 2 mg/mL DDM (closed squares) or 2 mg/mL $C_{12}E_8$ (closed triangles). Data points were fitted to equations whose Hill coefficients all fell into the 1.05 ± 0.1 range.

these high concentrations (*36*). For lower concentrations of Mg·ATP, the inner-filter effect was marginal, thus allowing unambiguous evaluation of the "true" effect of Mg·ATP addition on Trp fluorescence. We found that glycerol alone reduced the apparent affinity for Mg·ATP binding (Figure 7B versus Figure 7A), but that the presence of DDM on top of glycerol reversed this effect (Figure 7C versus Figure 7 A and B). The apparent dissociation constants for Mg·ATP binding to DDM-solubilized SERCA1a in the presence of glycerol or to membrane-embedded SERCA1a in the absence of glycerol were fairly similar (5−8 μM at pH 7 and 5 mM Mg^{2+} see Figure 7E). Contrarily to DDM, however, $C_{12}E_8$ did *not* reverse the apparent affinity for Mg·ATP binding, and left this affinity poor, or even poorer (Figure 7D and 7E). The same trend was observed in the absence of Mg^{2+}, i.e., the mere presence of glycerol again made ATP-induced Trp fluorescence changes less easily observable, in terms of both amplitude and affinity, and this loss of affinity for ATP binding was again reversed by DDM, but not by $C_{12}E_8$ (data not shown).

The effect of Mg^{2+} alone in these experiments is worth a word of comment. The effect of Mg^{2+} on the Trp fluorescence of Ca^{2+}-deprived ATPase is thought to result from Mg^{2+} binding either to the Ca^{2+} binding sites or to other sites on the ATPase (*11, 15*). In the absence of detergent, glycerol strongly reduced the Mg^{2+}-dependent rise in Ca^{2+}-free ATPase fluorescence (compare panels A and B in Figure 7), as previously reported (*32*), but DDM again counteracted this reduction by glycerol and made the Mg^{2+}-dependent rise clearly detectable again (panel C in Figure 7). Again, $C_{12}E_8$ behaved differently from DDM, as it did *not* reverse the loss in the Mg^{2+}-dependent rise, and in fact induced further loss (panel D in Figure 7; see also Figure S5 in the Supporting Information, where a higher concentration of Mg^{2+} was tested). The Mg^{2+}-dependent Trp fluorescence changes are therefore consonant with the effects of the same detergents on the apparent affinity for Mg·ATP.

Incidentally, we also tested whether, in the presence of glycerol, nucleotides remained effective in changing the Trp fluorescence of detergent-solubilized *Ca^{2+}-saturated* SERCA1a (the E1 form), as they are, to a moderate extent, for membrane-embedded SERCA1a. ADP (or AMPPCP, not shown) indeed slightly raised Trp fluorescence of both DDM- and $C_{12}E_8$-solubilized SERCA1a (Figure S6 in the Supporting Information). Of course, in this case, glycerol was not indispensable to the success of the experiment, as Ca^{2+} *alone* maintained solubilized SERCA1a in a stable form (*36*). In the presence of glycerol and DDM, addition of ADP and then aluminum fluoride clearly triggered the formation of a species with still higher fluorescence (Figure S6C in the Supporting Information), presumably the Ca_2·E1·ADP·AlF_x species (*36*), but in the presence of $C_{12}E_8$ on top of glycerol, a rise in Trp fluorescence was hardly discernible on the addition of AlF_x after ADP (Figure S6D in the Supporting Information). Note that glycerol alone apparently slowed down the formation of Ca_2·E1·ADP·AlF_x, as illustrated in panel B versus panel A in Figure S6 in the Supporting Information).

FITC Labeling Is Probably Not of Much Use for Studying the Effects of Binding to Solubilized ATPase of Ligands Like Ca^{2+}. At this point we labeled SR ATPase with FITC at Lys515 in the nucleotide-binding domain (*55, 53*), to ascertain whether or not, in the presence of glycerol and DDM or $C_{12}E_8$, FITC fluorescence in the labeled ATPase would retain some of the properties previously described in the absence of detergent. In solubilized and Ca^{2+}-deprived FITC-ATPase, FITC fluorescence did remain stable in the presence of 40% glycerol, in contrast with its previously reported instability in the absence of glycerol (*18*). However, although inactivation of the ATPase was apparently blocked, chelation or subsequent addition of Ca^{2+} only induced small changes in FITC fluorescence, and this was already the case in the mere presence of glycerol and absence of detergent. These somewhat disappointing results are shown as Figure S7 in the Supporting Information, panels A−F. Probably because of this adverse effect of glycerol, FITC-labeled ATPase was not much useful either to detect the possible formation of complexes of Ca^{2+}-free ATPase complexes with vanadate, in the presence of glycerol and detergent (Figure S8 in the Supporting Information, panels A−D).

Trp Fluorescence Experiments with Heterologously Expressed Mutants. In addition to the study of rabbit SR SERCA1a solubilized in different detergents, the study of SERCA1a mutants after their heterologous expression in yeast and subsequent purification in the presence of DDM (*16*) will also benefit from the present results. As an example, we show here experiments performed with the initial aim of confirming, in the presence of detergent, an observation we had previously made with WT and a Ca^{2+} site mutant of SERCA1a, E309Q, after both had been purified and reconstituted in lipids (*15*). Because the E309Q mutant lacks Site II for Ca^{2+} binding but remains able to bind Ca^{2+} at Site I, we had wondered whether this binding was sufficient to induce Trp fluorescence changes. In that earlier work, performed with SERCA1a proteins expressed with a histidine tag, purified and reconstituted, the expressed proteins had suffered to a significant extent from the reconstitution protocol (as shown by the fact that ATPase activities and Ca^{2+}-dependent Trp fluorescence changes were both lower for the WT enzyme than for native SR vesicles). Nevertheless, we had considered significant the fact that, with the E309Q mutant, Ca^{2+}-dependent fluorescence changes were reduced to virtually zero, whereas Mg^{2+}-dependent changes were still observable (*15*).

Similar experiments, performed here using SERCA1a tagged with a biotin acceptor domain (BAD), purified by streptavidin chromatography and kept solubilized in the presence of glycerol and detergent (*8*), made it clear that, for the E309Q mutant lacking Site II, Ca^{2+}-dependent changes were indeed reduced to virtually zero, as initially suspected, whereas Mg^{2+}-dependent changes were still observable, although with a reduced amplitude (bottom left trace in Figure 8). A similar conclusion was derived from experiments in which a second Site II mutant, E309A, was used, instead of the E309Q mutant (data not shown). Note that, in the present experiments, Ca^{2+}-dependent relative changes in fluorescence were of similar magnitude for yeast-expressed WT SERCA1a and for control solubilized rabbit SR SERCA1a (middle and top left traces in Figure 8).

Nevertheless, a closer look at the results of these new experiments, compared to those we reported previously (*34*), showed that Ca^{2+}-induced Trp fluorescence changes *not only* were reduced to virtually zero in the E309Q mutant but also were converted to a small signal of opposite sign (see the

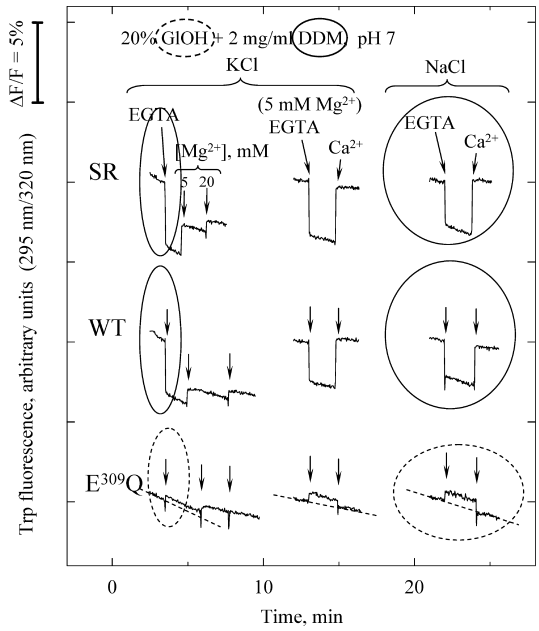

FIGURE 8: Binding of Ca^{2+} to the yeast-expressed SERCA1a mutant E309Q triggers small Trp fluorescence changes, of a sign opposite to that for the corresponding SR or WT SERCA1a changes. SERCA1a (either extracted from native SR, or yeast-expressed as WT or E309Q and purified, middle and bottom traces, respectively) was diluted 20-fold in a medium containing 100 mM KCl (or 100 mM NaCl for the last traces on the right) and 50 mM Mops-Tris at pH 7 and 20 °C (panel A), and supplemented with 20% (w/w) glycerol, 2 mg/mL DDM, and extra Ca^{2+}, whose final total concentration was 100 μM, including the Ca^{2+} added together with the expressed enzyme. The final concentration of SERCA1a was about 10 μg/mL in all cases. Left traces: 2 mM EGTA was added, followed by 5 mM Mg^{2+} and then another 15 mM (to reach 20 mM). Central and right traces (the latter in the presence of NaCl instead of KCl): the medium contained 5 mM Mg^{2+} from the start; 2 mM EGTA was added, followed by 3 mM Ca^{2+}. Values were corrected for the small changes in fluorescence intensity resulting from dilution on the addition of ligands. The traces for SR, WT and E309Q were shifted vertically in relation to each other, for clarity.

dashed line in the bottom left trace of Figure 8), while in the E309A mutant, the residual small signal kept the same sign (not shown, but see below). For E309Q, this signal of opposite sign, of borderline reliability compared with the noise level, was more clearly visible in the presence of Mg^{2+}, added from the start (central traces in Figure 8), and even more clearly when KCl was replaced by NaCl (right traces in Figure 8). Conversely, this small signal was less clear when 290 nm was used as the excitation wavelength, instead of 295 nm (data not shown). Remarkably, in a previous stopped-flow study of the EGTA-induced Trp fluorescence changes in native SR membranes, the presence of NaCl and an excitation wavelength in the long wavelength range (295 nm) had both been found optimal, during dissociation of the two Ca^{2+} ions from their sites, for revealing the transient occupation of an ATPase state with *lower fluorescence than the final equilibrium state* in the absence of Ca^{2+}. This "super-low" fluorescence had been proposed to be due to an E1-like state with only one residual Ca^{2+} ion bound, instead of the two initially bound ions (*35*). A similar proposal had also been previously derived from the observation that in the presence of perturbing concentrations of DDM, i.e., under conditions where cooperativity for the binding of the two Ca^{2+} ions was lost, binding of Ca^{2+} to its binding site of highest affinity in fact *reduced* Trp fluorescence, although Ca^{2+} binding to both sites raised it (*39*). The results of the present experiments, in which only one Ca^{2+} ion can bind to the E309Q mutant, at "Site I", are fully consistent with these previous proposals.

To make sure that the E309Q and E309A mutants had not been denatured during purification, we checked, using the glycerol- and DMSO-containing medium at pH 6 previously used for the experiments illustrated in Figures 5 and S4, that, in the presence of $C_{12}E_8$ and DOPC in a 2:1 ratio (w/w), the putative formation of the E2·BeF_3 species induced rises in Trp fluorescence of fairly similar amplitudes for all SERCA1a proteins, namely, these expressed mutants, the expressed WT SERCA1a, and the rabbit SR SERCA1a. We found that this was indeed the case (right portion of the traces in Figure S9 in the Supporting Information). Note that, as a result of the prevailing $C_{12}E_8$/lipid ratio, Ca^{2+} chelation by EGTA induced Trp fluorescence changes of relatively small amplitude even for native SR SERCA1a (Figure S9A in the Supporting Information) and expressed WT SERCA1a (Figure S9B in the Supporting Information). Nevertheless, for the expressed E309Q mutant, the above-reported change in the sign of the Ca^{2+}-dependent signal was even more apparent (Figure S9C in the Supporting Information) than under the conditions of the experiment in Figure 8. Again, this change of sign did not occur for E309A (Figure S9D in the Supporting Information).

DISCUSSION

When native SERCA1a is solubilized by detergent in the absence of glycerol, the amplitudes of certain ligand-induced changes in its Trp fluorescence may be reduced (see, for instance, ref *23*), and the SERCA1a instability which results from solubilization and delipidation (e.g., in the presence of a large excess of detergent) makes fluorescence measurements even more difficult (see, for instance ref (*18*)). We found here that clear Ca^{2+}-dependent changes again became observable in the presence of glycerol together with solubilizing concentrations of detergent (Figure 13), and this was also true of other ligand-induced changes, although success in some cases depended on the particular detergent used and required the simultaneous addition of lipid (Figures 4−7). In the combined presence of glycerol and detergent, the steady drift in intensity, which under other conditions is not always easily distinguished from ligand-induced changes, became even slower than in the absence of detergent, perhaps because of reduced adsorption of the protein onto the cuvette walls. Thus, the main message conveyed by the present work is that adding glycerol to the medium for experiments with detergent-solubilized SERCA1a is effective not only for ensuring long-term stability of the overall ATPase activity of the solubilized SERCA1a as was long ago demonstrated (see, for instance, refs *17, 27*) but also, more specifically, for making feasible with solubilized SERCA1a most of the Trp fluorescence experiments which in membrane-embedded SERCA1a have proved useful for detecting and quantifying ligand-induced conformational transitions. The protective effect of glycerol on solubilized and delipidated SERCA1a, which is especially visible in the absence of Ca^{2+}, might be partly due to the slowing down by glycerol of the dynamics of the SERCA1a polypeptide chain (*56*), partly to more

general and previously discussed effects of cosmotropes (*57*). Note that we checked that in the presence of glycerol up to 40% w/w, there was not much change in the cmc or in the solubilizing efficiency of DDM or $C_{12}E_8$.

An attractive consequence of this message is that it opens the way to easier Trp fluorescence studies with heterologously expressed SERCA1a mutants, as such studies will be possible without the previous need to reconstitute these mutants into liposomes as in previous studies (*14*−*16*), an additional step during which inactivation hazards may occur. To our knowledge, only one previous study took advantage of this possibility, but it was restricted to the characterization of expressed WT SERCA1a (*13*). Recently, in a different work, we studied the D351A and P312A mutants by such Trp fluorescence measurements in the simultaneous presence of glycerol and detergent, without reconstitution (*8*). Here, as another example, we used the E309Q mutant, which lacks Site II, one of the two sites for Ca^{2+} binding, to prove a suggestion originally derived from both kinetic experiments with native SR vesicles (*35*) and equilibrium experiments performed under conditions in which the cooperativity of Ca^{2+} binding had been lost (*39*). The suggestion was that Ca^{2+} binding to Site I in WT SERCA1a in fact slightly *reduced* Trp fluorescence, implying that Ca^{2+} binding to Site II only was mainly responsible for the generally observed *rise* in Trp fluorescence. The results obtained here with the E309Q mutant fully support this suggestion.

Note, however, that for another Site II mutant, the E309A mutant, EGTA-induced fluorescence changes at pH 7 were again reduced compared to WT, but nevertheless kept the "normal" sign. This observation might at first sight be connected with the fact that the E309Q mutant was previously reported to be *completely* unable to be phosphorylated from ATP, whereas an E309A mutant could be phosphorylated at high enough Ca^{2+} concentrations (*4, 58, 59*). However, it is fair to note that these E309Q and E309A mutants were derived from rabbit and frog SERCA1a, respectively. If the difference in their behavior is nevertheless due to the mutation itself, it would fit very well with our fluorescence data, and both sets of results would imply that some residual binding of Ca^{2+} to the mutated Site II in E309A is possible, but none at all to the corresponding site in E309Q, despite the fact that mutation of E to Q is generally thought to be more conservative than mutation to A. Alternatively, or as a complementary possibility, our results might be discussed in connection with previous findings suggesting, from both proteolytic and TG inhibition studies, that mutation of the E309 side chain could result in unusual properties for the mutant (*60*−*62*). These results could imply that, beyond its role in liganding Ca^{2+}, the side chain at residue 309 might play a significant role in stabilizing or destabilizing interhelical interactions in the transmembrane region in the absence of Ca^{2+}. In rat gastric H^+,K^+-ATPase, residues involved in the cation-binding site have already been suggested to participate in a conformationally sensitive interhelical network (*63, 64*), and related interhelical interactions can indeed be found in the SERCA1a published structures. In SERCA1a in the absence of Ca^{2+}, the different abilities of E, Q and A side chains at position 309 to establish this type of interhelical connection might differ and account for the existence of differences between WT and the various mutants, beyond differences in their Ca^{2+} binding ability.

Even in WT SERCA1a, Trp fluorescence measurements could of course be considered as a mere black box from which little detailed molecular information will ever be derived. For instance, the results in Figures 5 and 6 imply that the absence of a Pi-dependent Trp fluorescence signal does not necessarily imply the absence of E2P formation, and show that only specific conditions allow Trp fluorescence to be sensitive to this formation (in this case, $C_{12}E_8$ + DOPC). A conceivable explanation for this fact could be that some of the Trp residues which normally change fluorescence as a result of the protein transition to a particular new conformation (e.g., E2P) are Trp residues which reside at the interface between the protein, the hydrophobic region of the membrane and the water phase: as a result, the presence of fairly well positioned phospholipid headgroups (and not only highly disordered detergent chains) in this region, close to these Trp residues, might be critical for discriminating between the fluorescence intensities of these Trp residues in the two conformations of the protein. Note, for instance, that, in the presence of Ca^{2+} and AMPPCP, a phospholipid headgroup was found very close to W^{107} of the protein, a Trp residue which together with the transmembrane M2 probably moves significantly during the various conformational transitions of SERCA1a (*68*), and that another phospholipid headgroup was found nearby in one of the Ca^{2+}-free forms of SERCA1a (e.g., 2AGV in the PDB) but *not* after fluoride binding and relocation of M2 (*69*). The ability of additional DOPC to compete with one particular type of detergent close to these residues, but not necessarily with the other, could account for its possible effects on the amplitude and sign of the Trp fluorescence signals during a particular transition.

Irrespective of detailed (and speculative) interpretation, an attractive prospect of our findings is that they open the way to the study of the different effects exerted on SERCA1a by different solubilizing detergents. In fact, only few of the previous reports about the overall ATPase activity of SERCA1a include detailed descriptions of how the various intermediate steps in the catalytic cycle are altered by one or the other detergent under solubilizing conditions (see, for instance, refs *17, 30, 31, 44, 54, 65*). Detailed information about the specific effects of DDM and $C_{12}E_8$ was only obtained when each of these two detergents was added to SR membranes at a concentration below its cmc and was therefore inserted into the membranes without inducing solubilization (see, for instance refs *25, 26, 39, 47*).

Under fully solubilizing conditions, our experiments make it possible to pinpoint specific effects on SERCA1a of the solubilizing concentrations of DDM and $C_{12}E_8$: (1) these concentrations tend to reduce the equilibrium affinity of the ATPase for Ca^{2+} (Figure 1), at least for the Ca^{2+} ion bound to Site II (Figure 8), in ways specific to each detergent, especially at pH 6 where $C_{12}E_8$ has a distinctly unfavorable effect on Ca^{2+} binding (Figures 2−4); (2) $C_{12}E_8$ is more favorable than DDM for SERCA1a phosphorylation from Pi, especially in the presence of added lipid (Figures 5 and 6); (3) conversely, DDM, but not $C_{12}E_8$, allows Mg·ATP and ATP (as well as Mg^{2+} alone, probably) to retain a high affinity for binding to SERCA1a in the presence of glycerol (Figure 7); (4) a striking finding which also emerges from the present work, despite its poor kinetic resolution, is that, at least at pH 6, solubilizing concentrations of DDM reverse

the inhibition by glycerol of the Ca^{2+}-induced transition, whereas the same concentrations of $C_{12}E_8$ *do not* reverse this inhibition and even induce further slowing down under delipidating conditions (Figure 2, A−D; Figure 3, A versus B; Figure 5, A−D). To explain these various results in detail is beyond the scope of the present report, and at this stage, a complete characterization by stopped-flow experiments of the *rates* of the various ligand-induced fluorescence changes in solubilized SERCA1a is still lacking. We can nevertheless already make a fair suggestion about the detergent-induced changes in the rate of what is known as the E2 to E1 transition.

This E2 to E1 transition is generally thought to *precede* Ca^{2+} binding to the E1 form. In SR membranes, we know that glycerol *per se* slows down this transition (Figure 9 in ref (*32*)), while both DDM and $C_{12}E_8$ accelerate it under nonsolubilizing but "perturbing"conditions (*25, 26, 47*); it is also already clear that, at pH 6, the E2 to E1 transition is the main rate-limiting step for the effect of Ca^{2+} addition, and becomes even more so in the additional presence of glycerol (*32*). We suggest that a slowing down of the E2 to E1 transition, exerted by solubilizing $C_{12}E_8$ concentrations (depending on the exact detergent to lipid ratio), is a key event accounting for most of the observations in the present work concerning the differences between the effects of DDM and $C_{12}E_8$.

Our ATPase activity and turnover results at pH 6 in the presence of glycerol provide a first illustration of the probable validity of this suggestion. Under these conditions, the E2 to E1 transition presumably is the main rate-limiting step for overall ATP hydrolysis, and this is why nonsolubilizing concentrations of both DDM and $C_{12}E_8$ *stimulate* overall ATP hydrolysis (Figure 4B,C), in distinct contrast with what is observed under more usual conditions, i.e., at pH 7.5 in the absence of glycerol, conditions under which nonsolubilizing concentrations of both DDM and $C_{12}E_8$ *inhibit* overall activity (*26, 47*): nonsolubilizing concentrations of $C_{12}E_8$ and DDM are known to stimulate the rate of the E2 to E1 transition under a variety of conditions (Figure 10C in ref (*47*); Figure 3 in ref (*26*)), and therefore are expected to reverse the inhibitory effect of glycerol on this step and to stimulate overall ATPase activity in the present experiments. Conversely, the fact that, at pH 6 and with glycerol, overall ATPase activity reverts to very low values in the presence of solubilizing concentrations of $C_{12}E_8$ but not of DDM (Figures 4C and 4B) (and remains low in the presence of $C_{12}E_8$ even after saturation of the Ca^{2+} sites) is consistent with the idea that solubilizing $C_{12}E_8$ is no longer able to do what nonsolubilizing $C_{12}E_8$ was doing, because it no longer accelerates the E2 to E1 transition but probably slows it down. In contrast, at pH 7.5 in the absence of glycerol, $C_{12}E_8$ is known to keep ATPase activity relatively high (whereas DDM strongly inhibits it (*21*)), because under these new conditions, the E2 to E1 transition is no longer rate-limiting.

The slowing down by $C_{12}E_8$ (but not DDM), at high, delipidating concentrations, of the rate of the E2 to E1 transition at pH 6, is also likely to account for part if not all of the huge reduction in the affinity for Ca^{2+} of $C_{12}E_8$-solubilized SERCA1a (Figures 2, 3). At pH 6, the E2 to E1 pre-equilibrium which precedes Ca^{2+} binding is indeed generally considered to already shift very much in favor of E2 (in fact E2·H_n) in the absence of glycerol (75, 67), and glycerol probably shifts this equilibrium even further (*32*): thus, any slowing down of the E2 to E1 transition rate, as occurs with solubilizing concentrations of $C_{12}E_8$ (but not of DDM), will directly contribute to a further shift in the equilibrium toward E2, and hence to the observed further shift of $Ca_{1/2}$ values, up to millimolar values (Figures 2, 3). The same is probably true in the absence of glycerol at pH 6, because conventional ATPase activity measurements revealed a similar shift of the $Ca_{1/2}$ value for ATP hydrolysis activation in the presence of $C_{12}E_8$ (Figure 3D). Incidentally, note that these conventional ATPase activity measurements, in the absence of glycerol but presence of a high Mg·ATP concentration, could be performed without suffering from the adverse effect of irreversible ATPase denaturation, presumably because the constant presence of a high Mg·ATP concentration protected the Ca^{2+}-free forms of solubilized SERCA1a from inactivation (*17, 27*) just as efficiently as glycerol did in "turnover" experiments at low ATP concentrations (although presumably via a different mechanism). As mentioned under Results, when lipids were added together with detergent, they also to some extent rendered glycerol superfluous, for similar reasons.

The same differential effect of $C_{12}E_8$ in favor of E2 might also account in part for the fact that formation of E2P (from Pi) or E2·BeF_3 (from fluoride), two species formed from the E2 state of SERCA1a, is more favorable in the presence of $C_{12}E_8$ than in the presence of DDM, especially in the presence of additional lipids (Figures 5 and 6). A similar differential shift by $C_{12}E_8$ (but not DDM) of the E2 to E1 equilibrium in favor of E2, not only at pH 6 but also under other conditions, would also account in part for our observations concerning binding of nucleotides and Mg^{2+} (Figure 7): if one accepts the view that the E1 conformation has a higher affinity for ATP than the E2 conformation, a widely accepted notion in the Na^+,K^+-ATPase field, and the view that Mg^{2+}-dependent changes are accompanied by a shift of the E2 to E1 equilibrium toward E1, as suggested in refs *11*,*66*,*67*, a $C_{12}E_8$-induced shift of this equilibrium in the opposite direction, in favor of E2, would indeed account for the poorer binding of Mg·ATP, ATP and Mg^{2+} (Figure 7 and data not shown) in the presence of $C_{12}E_8$ than in the presence of DDM. Note that, in the absence of detergent, the similar effect of glycerol alone, shifting the E2 to E1 equilibrium toward E2, is also the simplest explanation for the reduced affinity of SERCA1a for Ca^{2+} and Mg·ATP, and the increased apparent affinity for Pi, observed in the presence of glycerol. Conversely, a DDM-induced shift of the E2 to E1 equilibrium toward E1 might contribute to the poorer cooperativity of Ca^{2+} binding at pH 7 in the presence of DDM, compared with $C_{12}E_8$ (Figure 1E and Figure S2E in the Supporting Information), because of the previously discussed view that a major determinant of the cooperativity of Ca^{2+} binding is the balance of this E2/E1 equilibrium (any shift of this equilibrium toward E2 increases cooperativity, see ref *42*): at pH 7, conditions under which the equilibrium is probably not very different from 1 under usual conditions (see, for instance ref (*55*)) and the cooperativity not the highest (*42*), DDM could further reduce the cooperativity for Ca^{2+} by favoring E1 instead of E2.

From a "historical" point of view, the unexpected very large reduction in the affinity for Ca^{2+} of SERCA1a solubilized in the presence of $C_{12}E_8$ at pH 6 probably enables

us, 25 years later, to understand why, in ref (*30*), pCa 4 was *not* sufficient to protect SERCA1a from irreversible activation after SR membrane solubilization with $C_{12}E_8$ at pH 6 (Figures 4, 8 and 9 in ref (*30*)). A large reduction in affinity for Ca^{2+} probably also accounts in part for the fact, long considered somewhat mysterious, that, to achieve appropriate protection of the solubilized Ca^{2+}-bound state of SERCA1a in the first successful crystallization attempts (in the presence of $C_{12}E_8$ at pH 6.1), the addition of unusually high concentrations of Ca^{2+} (and lipid) was found to be required (*6, 29*). The relatively poor affinity of ATPase for Ca^{2+} under these original solubilization conditions probably contributed to this requirement, which should therefore no longer be considered mysterious.

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SUPPORTING INFORMATION AVAILABLE

Supplemental references; methods for measuring the effect of glycerol on the critical micellar concentration and the critical solubilizing concentration of DDM and $C_{12}E_8$; Figure S1 showing protection by glycerol in the case of DDM, too; Figure S2 showing Ca^{2+}-dependent changes in the presence of Mg^{2+}; Figure S3 showing turnover-dependent signals at pH 8 and 10 mM Mg^{2+}, together with a comment on complementary Ca^{2+} release experiments; Figure S4 showing BeF_3-dependent signals; Figure S5 showing Mg^{2+}-dependent changes in Trp fluorescence; Figure S6 showing the effects of ADP and AlF_x in the presence of Ca^{2+}; Figure S7 showing Ca^{2+}-dependent changes in FITC fluorescence in the presence of glycerol and detergent; Figure S8 showing vanadate-dependent changes in FITC fluorescence; and Figure S9 showing the formation of $E2 \cdot BeF_3$ for expressed SERCA1a and mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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