The Influence of Detergents on the Ca^{2+}- and Mg^{2+}-Dependent Adenosine Triphosphatase of the Sarcoplasmic Reticulum

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During the stepwise solubilization of sarcoplasmic reticulum vesicles with detergents, the following changes in the structural and enzymatic properties of the preparation are observed:

1. The viscosity of the vesicular suspension initially rises. This change is accompanied by the formation of elongated tubules. Subsequently the membranes are completely desintegrated, resulting in a considerable reduction of the viscosity.
2. A decrease in the activity of the Ca^{2+}-dependent ATPase, which is restored after complete solubilization.
3. A decrease in the change of intrinsic tryptophan-fluorescence on removal of calcium ions, which is also restored after complete solubilization.
4. A decrease of the calcium affinity of the ATPase.
5. A decrease in the amount of phosphorylated protein formed by the incorporation of inorganic phosphate. On the other hand, the amount of phosphoprotein formed from ATP is not affected during solubilization.
6. The dependence of the initial rates of phosphoprotein formation from inorganic phosphate on either magnesium or inorganic phosphate at low concentrations of the respective ligand changes from an S-shape profile to a normal hyperbolic profile after solubilization.

Introduction

The Ca^{2+}- and Mg^{2+}-dependent ATPase of sarcoplasmic reticulum can be obtained as a monomeric enzyme by the use of non-ionic detergents, such as $C_{12}E_8$ [1]. The monomeric ATPase shows most of the enzymatic properties of the native enzyme, especially full ATPase activity and phosphoprotein formation from ATP [1, 2]. On the other hand, it has been suggested, that the ATPase exists as an oligomeric enzyme within the membrane [3–8]. To examine whether there exist some properties of the ATPase, which might be dependent on protein-protein interactions, the influence of detergents on sarcoplasmic reticulum vesicles was investigated. It was found that the ATPase activity, the formation of phosphorylated enzyme from inorganic phosphate (P_i) or from ATP, the dependence of ATPase activity on calcium concentrations, and the decrease of intrinsic tryptophan-fluorescence on addition of EGTA, are strongly dependent on the kind and the concentration of detergent present in the reaction medium. The effects which $C_{12}E_8$, Triton X-100, octylglucoside and myristoylglycerophosphocholine exert on the formation of phosphorylated protein from P_i or from ATP and their influence on the calcium affinity are discussed in view of a cooperation between neighbouring ATPase molecules. In addition, the influence of detergents on ATPase activity and on the decrease of the intrinsic fluorescence on removal of calcium indicates that these properties of the ATPase are fully restored in the completely solubilized enzyme.

Abbreviations: $C_{12}E_8$, dodecyl octaethylene glycol monoether; octylglucoside, octyl-β-D-glucopyranoside; Mops: 3-(N-morpholino)propansulfonic acid; enzyme: Ca^{2+}-ATPase (EC 3.6.1.3).

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Materials and Methods

[^{32}P]phosphate, (γ-^{32}P)ATP and ^{45}Ca were from Amersham Buchler, Braunschweig (FRG). ATP was obtained from Pharma Waldhof GmbH, Düsseldorf (FRG). Lasalocid (X-537A) was a generous gift from Hoffmann-La Roche Ltd., Basel (Switzerland). Myristoylglycerophosphocholine and octyl-β-D-glucopyranoside were from Calbiochem, Lahn-Giessen (FRG). Dodecyl octaethylene glycol monoether ($C_{12}E_8$) was purchased from Nikko Chemicals, Tokyo (Japan). Triton X-100 was obtained from Carl Roth KG, Karlsruhe (FRG). All other chemicals were p.a. grade.

Sarcoplasmic reticulum vesicles were prepared according to the procedure described by Hasselbach and Makinose [9] as modified by de Meis and Hasselbach [10]. Protein concentration was determined by the Biuret method with Kjeldahl calibrated standards, or by the method of Lowry *et al.* [11]. Phospholipids were extracted according to Folch *et al.* [12] and inorganic phosphate was determined as described by Chalvardjian and Rudnicki [13].

Steady state phosphorylation by inorganic phosphate was performed as described by Beil *et al.* [14] in solutions containing increasing amounts of [^{32}P]-phosphate, 10 mM $MgCl_2$, 20 mM histidine, pH 6.0, 10 mM EGTA and the given concentrations of detergent at 30 °C. Phosphorylation was started by the addition of protein (final concentration 0.4 mg · ml^{-1}) and the phosphorylated enzyme was precipitated with an equal volume of 10% Cl_3AcOH-50 mM phosphoric acid. The protein was pelleted by centrifugation and repeatedly washed on a glass wool filter.

Vesicles were phosphorylated from (γ-^{32}P)ATP in a solution containing 0.1 mM (γ-^{32}P)ATP, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 40 mM KCl, 20 mM histidine, pH 7.0, 0.4 mg · ml^{-1} protein and given concentrations of detergent at 20 °C. The phosphorylated enzyme was precipitated, pelleted and washed as described above.

To measure the kinetics of phosphoprotein formation, a quench flow apparatus was used, as recently described [15]. The reaction time was varied between 22 and 260 milliseconds by changing the flow rate in the reaction chamber. The performance of the apparatus was tested by studying the kinetics of the hydrolysis of 2.4 dinitrophenyl acetate with potassium hydroxide at 20 °C, as described by Barmann and Gutfreund [16]. The obtained values of the second order rate constant of 50–58 M^{-1} · sec^{-1} are in good agreement with the values reported in the literature [16, 17]. The substrate syringe of the quench flow apparatus contained 2–30 mM [^{32}P]phosphate, 5 mM EGTA, 40 mM Tris-maleate at pH 6.0 and 2–30 mM $MgCl_2$. The enzyme syringe had all the components of the substrate syringe minus $MgCl_2$ and contained microsomal protein at a concentration of 1 mg · ml^{-1}. The temperature was adjusted to 20 °C. The reaction was stopped with icecold 10% Cl_3AcOH.

The calcium-dependent ATPase activity was measured in a solution containing 20 mM histidine, pH 7.0, 5 mM ATP, 5 mM $MgCl_2$, 40 mM KCl, 0.1 mM $CaCl_2$, $5 \cdot 10^{-5}$ M X-537A, 0.4 mg · ml^{-1} protein and the given detergent concentration as described by Ronzani *et al.* [18]. In those cases, where the detergent produced precipitations in the medium for P_i determination, ATP-splitting was stopped with an equal amount of 2% sodium dodecylsulphate instead of 6% Cl_3AcOH.

The calcium concentrations were obtained using different $CaCl_2$ to EGTA ratios and the final calcium concentration was calculated according to Schwarzenbach [19].

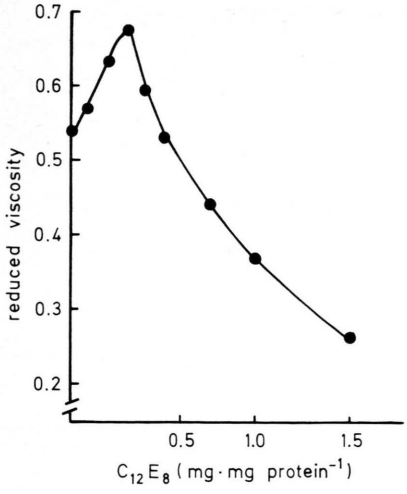
The binding of calcium to sarcoplasmic reticulum vesicles was measured as follows: Sarcoplasmic reticulum vesicles (5 mg · ml^{-1}) in 1.5 ml buffer solution containing 20 mM imidazol, pH 7.0, 40 mM KCl, 2 mM $MgCl_2$ were dialyzed 70 h at 4 °C against 15 ml of the above buffer, containing 10^{-4} M $^{45}CaCl_2$ and various amounts of EGTA. Samples of dialysis buffer and of vesicles were counted for radioactivity From the difference in the amount of ^{45}Ca present in the vesicle solution and in the dialysis buffer, the amount of Ca^{2+} bound to the vesicles was calculated. The experiment was repeated in the presence of 2.5 mg · ml^{-1} myristoylglycerophosphocholine in the vesicle solution as well as in the dialysis buffer.

Intrinsic tryptophan-fluorescence was measured with a SLM 4800/A spectrofluorometer (SLM Instruments Inc., Urbana, Illinois, USA). Sarcoplasmic reticulum vesicles (0.1 mg · ml^{-1}) were incubated in 2.5 ml of a continuously stirred solution containing 20 mM Mops, pH 7.4, 80 mM KCl, 0.5 mM $CaCl_2$, 0.45 mM EGTA. Changes of the fluorescence intensity on addition of various amounts of EGTA (0.1 M solution) were detected at an excitation wavelength of 285 nm and an emission wavelength of 330 nm. Slits were at 4 nm and 8 nm, respectively [8, 20].

The viscosity of the vesicles (20 mg · ml^{-1}) containing increasing amounts of detergent was determined with a modified Ubbelohde viscosimeter at 20 °C. The buffering solution contained 20 mM Tris-maleate, pH 7.0 and 0.1 M KCl.

Results

When increasing amounts of detergents, such as Triton X-100, $C_{12}E_8$, myristoylglycerophosphocho-



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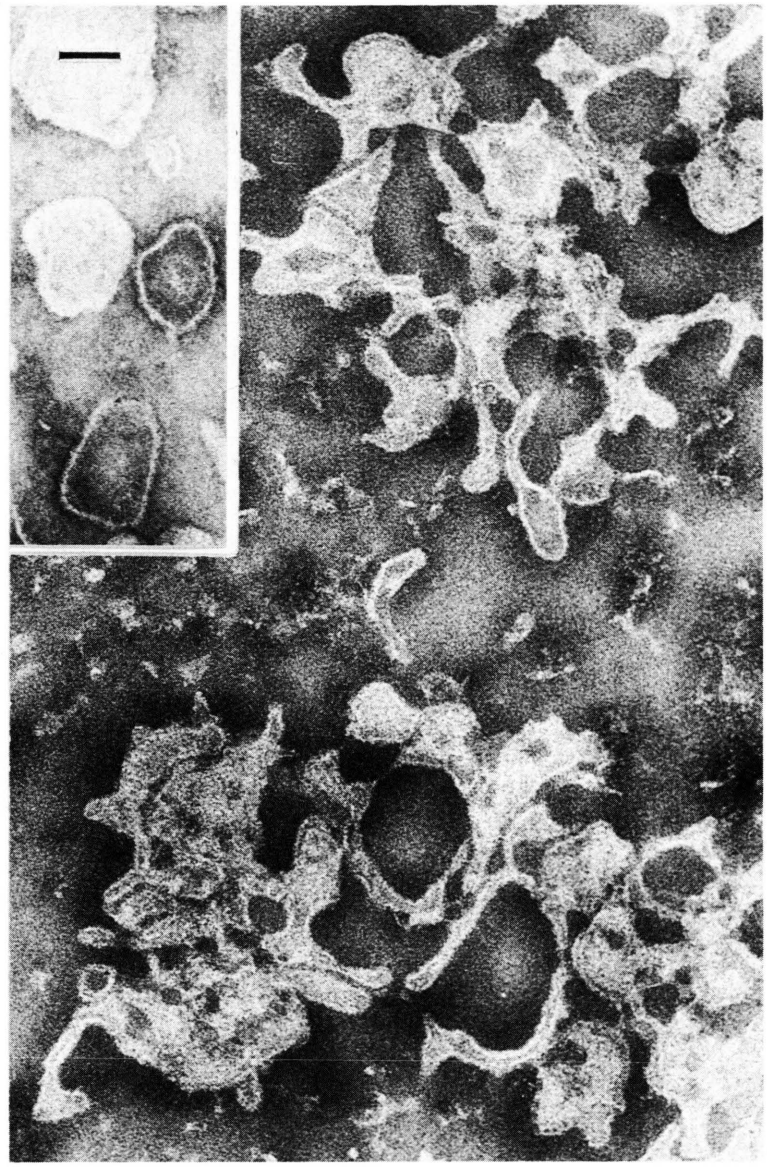

B. Bar 0.8 cm = 0.01 μm = 10 nm.

Fig. 1. Reduced viscosity and shape of sarcoplasmic reticulum vesicles after the addition of detergent. – A. Increasing amounts of $C_{12}E_8$ were added to a solution of sarcoplasmic reticulum vesicles (20 mg · ml^{-1}) in 20 mM Tris-maleate, pH 7.0, 0.1 M KCl. The reduced viscosity was calculated from the results obtained with a modified Ubbelohde viscosimeter. – B. Electron micrograph of sarcoplasmic reticulum vesicles treated with $C_{12}E_8$ (0.4 mg · mg protein^{-1}) and negatively stained with ammonium molybdate. Note that the detergent treated vesicles tend to aggregate. Inset: Native sarcoplasmic reticulum vesicles. The bar represents 100 nm. We thank Dr. B. Agostini for preparing the electron micrograph (*c.f.* 34).

line or octylglucoside are added to suspensions of sarcoplasmic reticulum vesicles, structure and function of the vesicular membranes are distinctly affected at quite low detergent: protein ratios of 0.2 (w/w). The occurring alterations give rise to an initial increase of the viscosity of the suspension (Fig. 1A). In the electron microscope negatively stained preparations reveal that the spherical vesicles have been replaced by membrane sheets and elongated tubules (Fig. 1B). At the same low detergent: protein ratio, the activity of the calcium-dependent ATPase (Fig. 2), the decrease of the tryptophan-fluorescence ($\Delta F/F$) induced by the removal of calcium from the membranes (Fig. 3) and their phosphorylation by inorganic phosphate in the absence of calcium ions (Figs. 5A, B, C) are suppressed by 30–50%. In contrast, the steady state level of phosphoprotein reached during calcium-dependent phosphate transfer from ATP is not affected (Table I, Fig. 5D) When the detergent: pro-

Table I. Influence of detergents on the phosphorylation of ATPase from P_i and from ATP and on ATPase activity.

Phosphorylation and ATPase activity were measured as described under "Materials and Methods".

Detergent	Concentration		ATPase activity		EP[1] from ATP		EP from P_i	
	mg · ml^{-1}	mg · mg^{-1}	µmol · mg^{-1} · min^{-1}		nmol · mg^{-1}		nmol · mg^{-1} at 10 mM P_i	
none	–	–	0.65	100%	2.00	100%	1.70	100%
Triton X-100	0.25	0.68	0.39	60%	1.92	96%	1.03	61%
Triton X-100	0.50	1.36	0.65	100%	1.53*	76%	0.87	51%
Octylglucoside	3.0	7.2	0.42	65%	1.92	96%	0.70	41%
$C_{12}C_8$	1.0	2.5	0.69	106%	1.98	99%	1.20	71%
Myristolyglycero-phosphocholine	0.40	1.0	0.81	125%	1.62*	82%	1.04	60%

* Measured in the presence of 1 mM $CaCl_2$ to avoid the rapid phosphoprotein decay occurring in the presence of 0.1 mM $CaCl_2$.

[1] EP: Phosphorylated protein.

tein ratio is further increased, the membrane structures completely break apart and are solubilized. Except octylglucoside, all other detergents in parallel fully restore the calcium-dependent ATPase activity (Fig. 2) and the decrease of the tryptophan-fluorescence induced by the removal of calcium ions (Fig. 3). As shown in Fig. 4A, the solubilization evidently changes the apparent affinity of the enzyme for calcium ions. While the steep increase

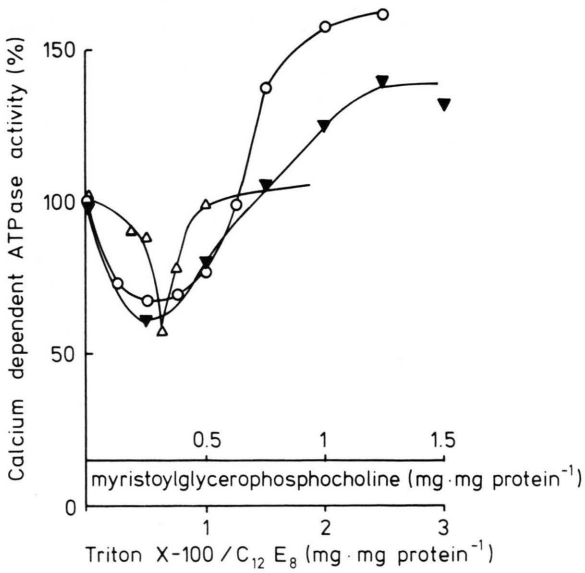

Fig. 2. Dependence of ATPase activity on detergent concentrations. The ATPase activity was measured as it is described under "Materials and Methods" with 0.4 mg · ml^{-1} protein in the presence of increasing amounts of detergent at 20 °C. (△) Triton X-100; (○) $C_{12}E_8$; (▼) myristoylglycerophosphocholine. Normalized activity: 0.65 µmol · mg^{-1} · min^{-1}.

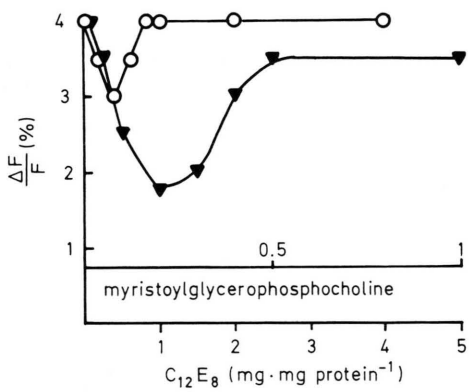

Fig. 3. Influence of detergents on the decrease of intrinsic tryptophan-fluorescence induced by the chelation of calcium ions with EGTA. Sarcoplasmic reticulum vesicles were incubated in a buffer containing 20 mM Mops, pH 7.4, 80 mM KCl, 0.5 mM $CaCl_2$ and 0.45 mM EGTA at 20 °C. Changes in the intrinsic fluorescence ($\Delta F/F$) were measured on addition of 25 µl of 0.1 M EGTA solution (final concentration: 1.45 mM). See "Materials and Methods". (▼) myristoylglycerophosphocholine; (○) $C_{12}E_8$. Excitation wavelength was 285 nm and emission wavelength 330 nm. Slits were at 4 nm and 8 nm, respectively.

of the calcium-dependent ATPase activity within a narrow range of free calcium concentrations is not affected by solubilization, a shift of this range towards higher calcium concentrations occurs (p_{Ca} 6.8 to 6.2). As it can be seen from Table II, this effect is not accompanied by a reduction of the number of high affinity calcium binding sites determined by equilibrium dialysis. The observed change in the apparent calcium affinity of the enzyme does not lead to an alteration of the dependence of the tryptophan-fluorescence decrease on the free cal-

Table II. Binding of calcium to sarcoplasmic reticulum vesicles in the presence and the absence of detergent. Equilibrium dialysis was carried out as described under "Materials and Methods" at the given calcium concentrations in the presence and the absence of 0.5 mg · mg protein^{-1} myristoylglycerophosphocholine.

pCa	Calcium bound to the vesicles [nmol · mg protein^{-1}]	
	Native vesicles	Native vesicles + 0.5 mg · mg protein^{-1} myristoylglycerophosphocholine
4.06	25.5	30.0
5.67	5.6	5.1
6.09	4.7	4.2
6.40	1.6	1.4
6.70	0.2	0.6

cium concentration, if increasing amounts of EGTA are added to a vesicle suspension containing 50 μM free calcium (Fig. 4B) [8]. The fluorescence change monitors in native and in fully solubilized preparations the same apparent calcium affinity, which corresponds to the lower affinity observed for the ATPase activity in solubilized preparations.

In contrast to the ATPase activity and the tryptophan-fluorescence decrease, phosphoprotein formation from inorganic phosphate remains depressed, when the membranes are solubilized. Figure 5 illustrates phosphoprotein formation from inorganic phosphate of native membranes and of preparations solubilized to different extents, when the concentration of inorganic phosphate is raised. Maximal phosphorylation yield is considerably reduced by solubilization, while the apparent affinity of the enzyme for phosphate seems not significantly altered. It has been excluded that this effect is due to a loss of protein during the washing procedure of the precipitated protein. Filters containing protein phosphorylated in the presence of detergent were loaded with the same amount of protein as filters containing protein phosphorylated in the absence of detergent (0.64 mg/filter). The amount of protein was measured by treating the filters directly as samples for Kjeldahl protein determination proce-

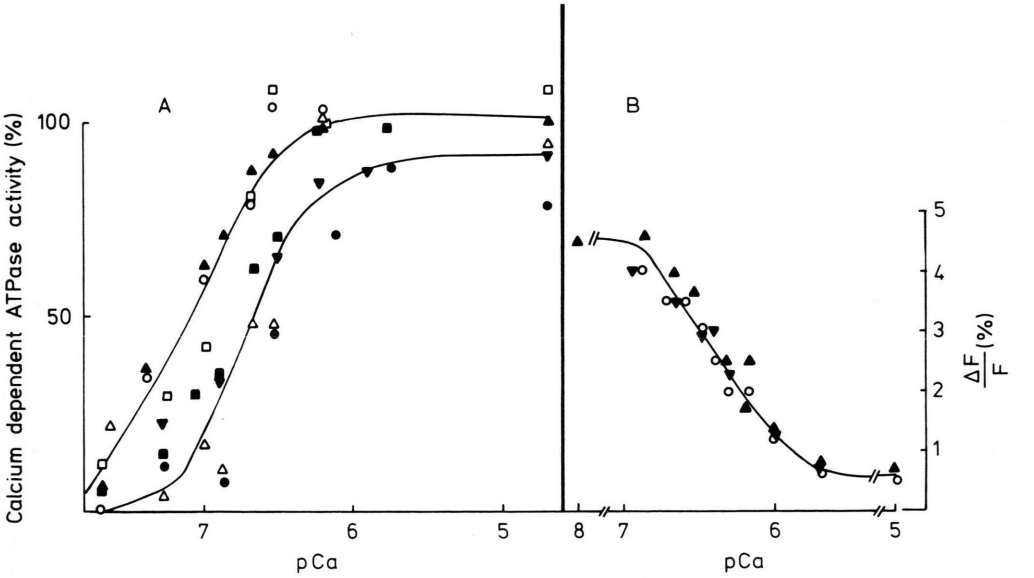

Fig. 4. Influence of detergents on the calcium affinity of the ATPase and on the calcium dependence of intrinsic tryptophan-fluorescence decrease. – A. ATPase activity was measured as described under "Materials and Methods", using various amounts of detergent and increasing free calcium concentrations: (▲) native vesicles; (□) $C_{12}E_8$ (1.25 mg · mg^{-1}); (■) $C_{12}E_8$ (2.5 mg · mg^{-1}); (○) Triton X-100 (0.25 mg · mg^{-1}); (△) Triton X-100 (0.63 mg · mg^{-1}); (●) octylglucoside (7.5 mg · mg^{-1}); (▼) myristoylglycerophosphocholine (1.0 mg · mg^{-1}). Normalized activity for native vesicles and vesicles solubilized with Triton X-100 or 1.25 mg · mg^{-1} $C_{12}E_8$: 0.65 μmol · mg^{-1} · min^{-1}. Normalized activity for vesicles solubilized with $C_{12}E_8$ (2.5 mg · mg^{-1}): 0.92 μmol · mg^{-1} · min^{-1} (comp. to Fig. 2). Normalized activity for octylglucoside: 0.42 μmol · mg^{-1} · min^{-1} (comp. to Table I). – B. Changes of the intrinsic fluorescence were measured as it is described in the legend to Fig. 3. But increasing amounts of a 0.1 M EGTA solution were added to the vesicle suspension with or without detergent, resulting in a final calcium concentration, which is indicated in the figure as pCa. (▲) native vesicles; (▼) myristoylglycerophosphocholine (1 mg · mg^{-1}); (○) $C_{12}E_8$ (10 mg · mg^{-1}).

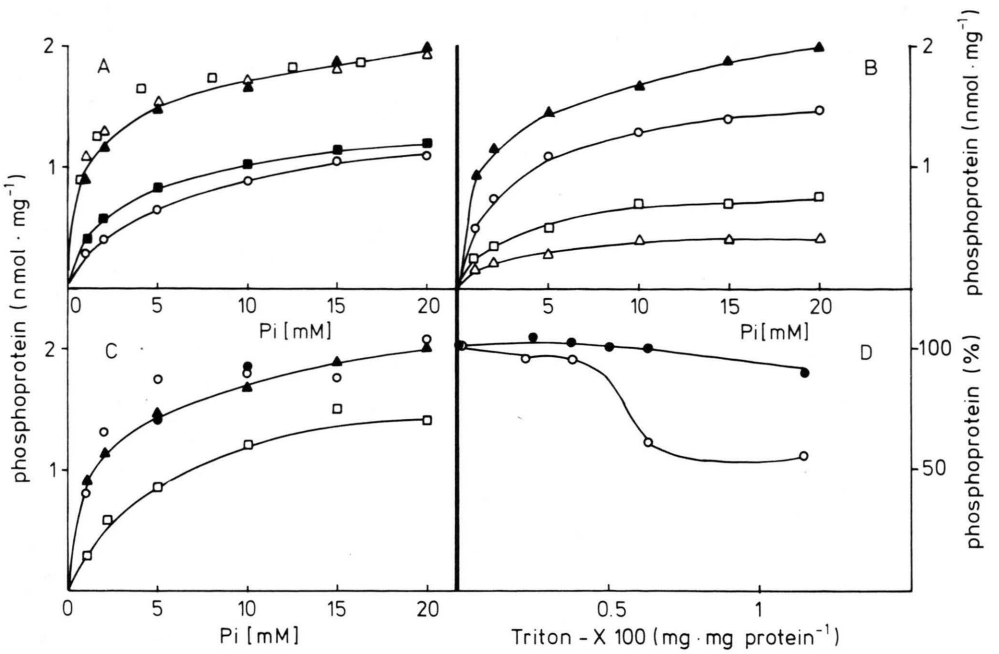

Fig. 5. Influence of detergents on the phosphorylation of sarcoplasmic reticulum vesicles from inorganic phosphate and from ATP. Sarcoplasmic reticulum vesicles (0.4 mg · ml^{-1}) were phosphorylated with increasing amounts of [^{32}P]phosphate as described under "Materials and Methods" in the presence of various concentrations of detergent. (▲) Native vesicles. A. Triton X-100: (□) 0.2 mg · mg^{-1}; (△) 0.39 mg · mg^{-1}; (■) 0.63 mg · mg^{-1}; (○) 1.2 mg · mg^{-1}. B. Same as in A, but various concentrations of octylglucoside were used: (○) 5.0 mg · mg^{-1}; (□) 7.1 mg · mg^{-1}; (△) 10.1 mg · mg^{-1}. C. Same as in A, but various concentrations of $C_{12}E_8$ were used: (●) 0.05 mg · mg^{-1}; (○) 0.26 mg · mg^{-1}; (□) 2.5 mg · mg^{-1}. D. Increasing amounts of Triton X-100 were added to the vesicle suspension and phosphorylation either from P_i or ATP were monitored. Phosphorylation from ATP was measured in the presence of 1 mM $CaCl_2$ at 0 °C due to the rapid decay of phosphorylated protein at high Triton X-100 concentrations. (○) Phosphorylated protein formed from P_i; phosphoprotein formed in the absence of detergent: 2.0 nmol · mg^{-1}. (●) Phoshorylated protein formed from ATP; phosphoprotein formed in the absence of detergent: 2.5 nmol · mg^{-1}.

dure. Furthermore, the decrease of phosphorylated protein formed in the presence of detergent is found to be reversible, since sarcoplasmic reticulum vesicles incubated with 0.3% of octylglucoside yielded 1.7 nmol phosphoprotein per mg protein, if they were diluted 50fold into the reaction mixture containing no detergent instead of 0.7 nmol per mg protein without dilution of the detergent. The same extent of recovery was obtained when the detergent was removed by extensive dialysis.

The finding, that solubilization reduces the amount of phosphoprotein which is formed by incorporation of P_i, while it does not affect the steady state level of ATP-supported phosphoprotein formation (Fig. 5D) indicates, that the incorporation of P_i requires a higher degree of structural integrity of the enzyme's lipoprotein structure than the phosphorylation by ATP. The alteration of the natural lipoprotein assembly does not only affect the phosphoprotein level but also the kinetics of P_i incorporation. Figure 6 shows that the dependence of the initial rates of phosphorylation on the concentrations of the two ligands magnesium and inorganic phosphate exhibits rather complex behaviour. At low and constant concentration of one ligand, rising concentrations of the other ligand activates phosphoprotein formation nonlinearly. The concentration dependence has an S-shape profile. The sigmoidicity of the profile vanishes at fixed high concentration of the respective first ligand when the dependence of phosphoprotein formation on the concentration of the second ligand is measured (Fig. 6). As to the reliability of the rate measurements, we can exclude that the observed slow rates at low substrate concentrations are artefacts due to the limited time resolution of our quench flow device. It measures slow rates more precisely than high rates, while these might be

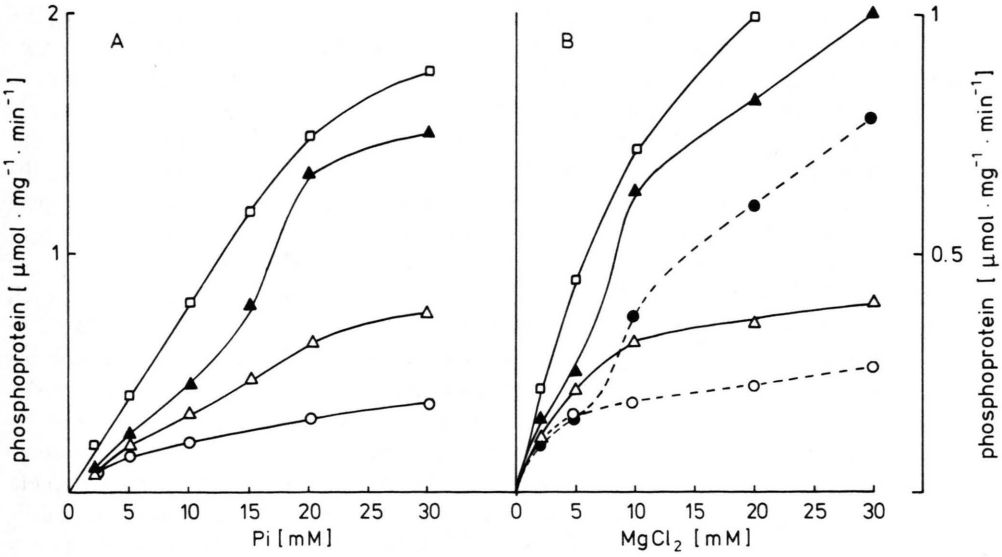

Fig. 6. Influence of Triton X-100 on the kinetics of phosphoprotein formation from inorganic phosphate. A. Initial rates of the formation of phosphorylated protein were determined at various concentrations of P_i as it is described under "Materials and Methods" at 5 mM $MgCl_2$. (▲) Native vesicles; (△) 0.5 mg · mg^{-1} Triton X-100; (○) 1 mg · mg^{-1} Triton X-100. (□) Native vesicles at 20 mM $MgCl_2$. (Rates are 2 times greater than indicated!) B. Same as in A, but a fixed concentration of P_i (5 mM) and increasing amounts of $MgCl_2$ were used. (●) Native vesicles; (○) 0.5 mg · mg^{-1} Triton X-100. Concentration of P_i fixed at 10 mM: (▲) Native vesicles; (△) 0.5 mg · mg^{-1} Triton X-100. (□) Native vesicles at 20 mM P_i. (Rates are 3 times greater than indicated!)

somewhat underestimated. This technical deficiency would not augment, but blur the sigmoidal activity profile. Furthermore, the doubt that the technical deficiency might have given rise to the complex activity profile is largely excluded by the finding that the sigmoidal concentration dependence is completely abolished when sufficient quantities of detergent are added to the assay media, as shown for Triton X-100 in Fig. 6. At detergent:protein ratios of 1 to 3 the activity profiles become normal, reaching saturation at low substrate concentration. Evidently the kinetics of phosphorylation by inorganic phosphate are drastically changed by solubilization of the native vesicle preparation.

Discussion

At increasing concentrations of detergents such as Triton X-100, $C_{12}E_8$, octylglucoside and myristoylglycerophosphocholine, the natural lipids of the sarcoplasmic reticulum membranes are gradually displaced. Quite low concentrations cause the membrane structure to collapse, and simultaneously distinct changes in the reactivity of the calcium transport ATPase occur: Partial inhibition of the calcium-dependent ATPase and depression of the tryptophan-fluorescence signal induced by the removal of calcium. These alterations must result from a limited disturbance of the interaction between the ATPase molecules and their lipid surrounding, because these low detergent concentrations do neither completely solubilize the transport protein nor the membrane lipids. If the detergent-treated vesicles at a $C_{12}E_8$: protein ratio of 0.5 are pelleted, 45% of protein and 33% of the phospholipids are recovered in the supernatant. The inhibition is evidently produced by an unfavourable composition of the detergent doped lipid phase. With the exception of octylglucoside all detergents can largely normalize the activity of the calcium transport ATPase if they become the predominant lipid constituent (see also [8, 21]). The only reaction of the transport enzyme which is not affected by the gradual displacement of the native lipids is its calcium-dependent phosphorylation by ATP measured during steady state. This is in agreement with the fact that the membrane lipids have to be reduced considerably in order to suppress ATP-supported phosphorylation [22, 23]. In contrast, the ability of the transport enzyme to accept inorganic phosphate in the pres-

ence of magnesium and the absence of calcium ions is considerably reduced and it is not restituted like the calcium-dependent ATPase when the enzyme is solubilized at high detergent concentrations. This has been reported by Kanazawa [24] for Triton X-100. But the yield of phosphorylated protein at 37 °C and pH 7.0 was low, even in the absence of detergent and no comparison was made with the phosphorylation of the protein by ATP.

One might suggest that phosphorylation by inorganic phosphate requires the presence of specific factors which might have been removed during solubilization. However, this explanation is largely excluded by the fact that the suppression of phosphoprotein formation by octylglucoside is reversible when the detergent is removed by dilution or dialysis. As another possible mechanism, one might envisage that the loosening of the lipoprotein assembly by the detergent which initiates solubilization interferes with the acceptance of inorganic phosphate by the enzyme. Either only the undisturbed lipid matrix provides the proper reaction conditions for enzyme phosphorylation or after the enzyme has been dislocated in the membrane matrix or removed from it, secondary changes occur which severely reduce phosphate acceptance. Secondary changes affecting phosphoprotein formation are suggested by the observation that membrane preparations which were solubilized with $C_{12}E_8$ continuously lose their calcium-dependent ATPase activity [1, 25]. This decline is especially pronounced at high detergent and low calcium concentrations. The displacement of the activation profile of the calcium-dependent ATPase solubilized with $C_{12}E_8$ to higher calcium concentrations (Fig. 4A) was firstly reported by Møller *et al.* But a rapidly proceeding decay of the enzymatic activity at low calcium levels was not excluded [25]. Under the conditions of this study, however, neither the loss of the ability of the detergent treated preparations to accept inorganic phosphate nor the reduction of the apparent calcium affinity can be attributed to secondary activity changes:

1) All experiments were immediately performed after solubilization at a time where the enzymatic activity is not yet measurably affected.

2) Myristoylglycerophosphorylcholine affects the property of the enzyme like $C_{12}E_8$, although the enzyme solubilized with myristoylglycerophosphocholine remains fully active for at least one day [26]. It also has been reported that the permanent presence of high calcium concentrations can stabilize the enzyme in the presence of high detergent concentrations [8, 27]. We, therefore, have attempted to maintain the phosphate transfer reaction by adding millimolar calcium concentration to the solubilized preparation. However, calcium addition could not preserve the ability of the enzyme to accept inorganic phosphate. The alterations in the enzyme which diminish phosphate incorporation are not related to the loss of cooperative calcium binding in solubilized preparations as reported recently by Verjovsky and Silva [8] because the latter change could be prevented by millimolar calcium concentrations. Yet, the authors report concerning a) the complete disappearance of the fluorescence decrease on addition of EGTA at a $C_{12}E_8$ concentration of about 1 mg · mg protein^{-1} and b) the loss of cooperative calcium binding are at variance with our findings. The first discrepancy may be explained by the fact that Verjovsky used purified ATPase preparations while our experiments were performed with native sarcoplasmic reticulum vesicles. Indeed we found a more pronounced decrease of the fluorescence signal with purified ATPase preparations (75%), although we never succeed to completely abolish the signal. In addition time-dependent denaturation of the protein at low calcium and high detergent concentrations as observed by a decrease in total fluorescence intensity were kept minimal by starting a new experiment for every single fluorescence determination (Figs. 3 and 4B). The initial fluorescence decrement was plotted. The fluorescence decrease could fully be restored at all detergent concentrations used by the readdition of calcium.

The reduction of the calcium affinity ascertained by ATP splitting of native and solubilized membranes is not accompanied by a flattening of the activation profile. Furthermore, identical profiles were obtained for native and solubilized preparations, when monitored by the calcium-sensitive tryptophan-fluorescence signal.

An important argument for the notion that a phosphorylation by inorganic phosphate might require the interaction between a number of sites or protein units, the arrangement of which is disturbed by the detergent, is furnished by the sigmoidal dependence of the initial rate of phosphoprotein formation on the concentration of the two ligands,

phosphate and magnesium. Sigmoidal activity profiles can be furnished by very different reaction mechanisms. Yet, in all reaction models, it is assumed that the substrate interacts with two different binding sites which might exist permanently or only transiently in the same or separate polypeptide chains [28]. That the phosphoprotein level reached at steady state does not exhibit sigmoidicity but conventional concentration dependence indicates that in the reaction sequence only the initial steps need the involvement of two different sites for both ligands. The fact that detergents abolish the sigmoidal behaviour of the reaction suggests that the solubilization interferes with the interaction of the two binding sites for the ligands. It should be noted that sigmoidicity of the initial rate or of the apparent rate constant may also be obtained if phosphorylation reaction follows the scheme proposed by Punzengruber *et al.* [29] and applied by Lacapère *et al.* [30]. However, the results of Lacapère *et al.* measuring intrinsic fluorescence changes did not yield sigmoidal concentration dependence of the apparent rate constants. This discrepancy may be due to the fact that fluorescence changes are not correlated in time with phosphorylation (*cf.* [31]).

A complete dissociation of the calcium transport ATPase complex into separate molecular entities is obviously not required to abolish this interaction essential for phosphorylation by P_i. This conclusion must be drawn from the fact that the ATPase preparations which were delipidated but not solubilized did not regain the ability to accept inorganic phosphate after reconstitution with native phospholipids [26, 32, 33].

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