Effect of Non-Solubilizing SDS Concentrations on High Affinity Ca^{2+} Binding and Steady State Phosphorylation by Inorganic Phosphate of the Sarcoplasmic Reticulum ATPase

Elisabeth Fassold, Wilhelm Hasselbach, and Bernd Küchler

Max-Planck-Institut für Medizinische Forschung, Abteilung für Physiologie, Jahnstraße 29, D-6900 Heidelberg, Bundesrepublik Deutschland

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In this investigation low, non-solubilizing concentrations of the strong anionic detergent SDS were used to perturbate the interaction of Ca^{2+} and P_i with their respective binding domains on the sarcoplasmic reticulum Ca-transport ATPase. Rising SDS concentrations produce a two-step decline of Ca^{2+}-dependent ATP hydrolysis. At pH 6.15, SDS differently affects high affinity Ca^{2+} binding and phosphorylation by inorganic phosphate and releases the "mutual exclusion" of these two ligand binding steps. The degree of uncoupling is considerably more pronounced in the presence of 20% Me_2SO.

The reduction of Ca^{2+} binding by SDS is demonstrated to be a result of decreased affinity of one of the two specific high affinity binding sites and of perturbation of their cooperative interaction. Higher SDS partially restores the original high Ca^{2+} affinity but not the cooperativity of binding. Phosphorylation exhibits a higher SDS sensitivity than Ca^{2+} binding: Increasing SDS competitively inhibits and then completely abolishes phosphoenzyme formation. Thus, SDS binds to the phosphorylation domain, evidently involving the Lys_{352} residue of the ATPase molecule; this is accompanied by a more unspecific concentration-dependent SDS effect, probably mediated by hydrophobic force, which, finally, suppresses phosphorylation.

Me_2SO does neither qualitatively affect the SDS-dependent chemical properties of the vesicular material nor the SDS-dependent perturbation of the investigated reaction steps.

Introduction

Mild detergents, such as cholate/deoxycholate, $C_{12}E_8$ or Triton X-100, when applied to solubilize microsomes [1–6], yield enzymatically active ATPase preparations as long as specific precautions are obeyed [1, 5, 7, 8]. In higher concentrations these detergents replace membrane lipids nearly completely, but do not penetrate the domains of the transport protein essential for activity. In contrast, solubilization by the ionic detergent SDS readily inactivates the enzyme [9], a consequence to be expected from the extensive unfolding of the ATPase protein molecule. However, the protein structure is not indiscriminately disrupted by SDS: It has previously been shown that, on the one hand, high affinity Ca^{2+} binding is retained after solubilization [10], while, on the other hand, non-solubilizing SDS concentrations prevent fluorescein isothiocyanate from specific binding to the Lys_{515} residue in the nucleotide binding domain [11, 12]. We therefore assumed that SDS, by selectively interfering with distinct substrate binding steps, might provide a tool to gain information on the mechanism of the "mutual exclusion" (see later) of high affinity Ca^{2+} binding and phosphorylation by inorganic phosphate, which is the basis for the functioning calcium transport enzyme.

Under the prevailing conditions and the applied SDS/protein ratios SDS combines biphasically with the ATP lipoprotein complex as described for other proteins [13]. Below a SDS/protein ratio of 1, the concentration range prevalent during this investigation, the complex readily sediments and only peripheral proteins are released from the membrane.

Early reports, stating that enzyme activation requires high affinity Ca^{2+} binding to the cytoplasmic surface of the protein (*e.g.* [14–16]), have recently been followed by detailed studies on the mechanism of Ca^{2+} binding itself [17, 18]. These studies were complemented by model-hypotheses [19, 20] based on the structural arrangement of the binding domains [21]. Binding of two Ca^{2+} ions (as well as re-

Abbreviations: Me_2SO, dimethyl sulfoxide; CMC, critical micelle concentration; $C_{12}E_8$, dodecyl octaethylene glycol monoether; FITC, fluorescein isothiocyanate.

Enzyme (IUB Recommendations 1984): calcium-transport ATPase (EC 3.6.1–38).

Reprint requests to Prof. Hasselbach.

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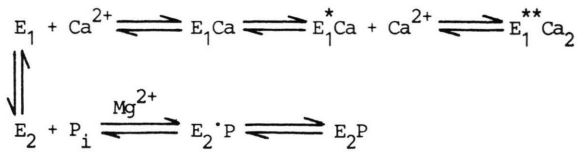
lease) has been proposed to proceed sequentially and cooperatively (Scheme 1), due to an originally distinct accessibility of the two binding sites which lie in close proximity to each other (*cf.* [20]). The enzyme is then in a conformation which exclusively accepts the terminal phosphate of ATP but not inorganic phosphate. Phosphorylation by inorganic phosphate only occurs if the enzyme is devoid of Ca^{2+} bound with high affinity or *vice versa* (Scheme 1, [22, 23]). This correlation is referred to as "mutual exclusion" [24–26]. It exhibits characteristic pH- and Me_2SO-dependent coupling ratios (see Results and Discussion) if EP formation is performed in the presence of varying free Ca^{2+} concentrations.

Scheme 1.



The apparent affinity of phosphate is enhanced at slightly acidic pH in K^+-free media (reduction of the k_{off} rate [27–29]) and vastly augmented by the addition of Me_2SO [30], so that the enzyme can be phosphorylated by phosphate in the presence of relatively high free Ca^{2+} concentrations. This was taken into account resp. utilized in the course of this investigation.

We illustrate that increasing SDS leads to an affinity shift for Ca^{2+}, thereby disclosing the two high affinity binding sites. Concomitantly their interaction during the binding process is abolished. Further we show that SDS is a competitive inhibitor of phosphorylation and that this effect is progressively superimposed by an influence exerted by unspecific SDS binding. The latter, finally, abolishes phosphorylation. Above that SDS uncouples the "mutual ligand exclusion", an effect especially evident in the presence of 20% Me_2SO.

Materials and Methods

Sarcoplasmic reticulum vesicles were isolated from rabbit skeletal muscle by a procedure similar to the one introduced by Hasselbach and Makinose [31]. In detail: Front and hind leg as well as back muscles of one rabbit – about 450 g – were removed into ice water and then ground in a meat grinder. This, like all the following operations, was carried out at 4 °C in the cold room. The minced meat was mixed with 1500 ml of a solution containing 100 mM KCl, 2 mM EDTA, 2.5 mM KH_2PO_4 and K_2HPO_4 each, and 0.1 mM phenylmethylsulfonylfluoride. The mixture was homogenized in portions in a Waring Blender for 2 min.

Myofibrils were sedimented by centrifugation at 6000 rpm for 30 min (Sorvall rotor Type GS-3). The supernatant was filtered through two layers of mull and subsequently centrifuged at 10,000 rpm for 30 min (Beckman rotor Type 19). The resulting pellet was enriched in "heavy" sarcoplasmic reticulum microsomes which could be isolated if desired according to Hasselbach and Migala [32]. The supernatant of the 10,000 rpm run containing the "light" fraction of sarcoplasmic reticulum vesicles was again filtered through mull and centrifuged at 19,000 rpm for 70 min. The resulting pellet was suspended in 400 ml of a solution containing 600 mM KCl, 2 mM ATP, and 2 mM $MgCl_2$ and centrifuged at 40,000 rpm for 55 min (Beckman rotor Type 45 Ti). The pellet was homogenized in 200 ml of a 100 mM KCl solution and centrifuged at 40,000 rpm for 30 min; this purification step was repeated once. For the purpose of the present investigation, during which all experiments were carried out in virtually K^+-free media, the final pellet was thoroughly homogenized in 30 ml of a buffer solution containing 40 mM Tris · maleate pH 7.0 and 20 mM $MgSO_4$ (instead of 20 mM Tris · HCl pH 7.0 and 100 mM KCl, the standard homogenization medium). Before an experiment was started on the day of or after the isolation procedure, residual KCl from earlier isolation steps was further diminished by dialysis of the vesicular suspension against the 100-fold volume of the same solution.

The protein concentration was determined after dialysis by the Biuret method standardized by Kjeldahl nitrogen determinations.

Experimental Procedures

For all experiments (except the ATP hydrolysis rate measurements and the CMC determination, see later) reaction media stock solutions were prepared containing (final concentrations) 40 mM Tris · maleate buffer pH 6.0, 20 mM $MgSO_4$, no or varying $^{40}Ca^{2+}$ or $^{45}Ca^{2+}$, EGTA, and $^{31}P_i$ or $^{32}P_i$, and no or 20% Me_2SO, as given by the rationale of the respective

experiment and as indicated in the legends of the Fig. Since an addition of 20% Me_2SO caused an alkalization of the media to about pH 6.85 the pH of the Me_2SO-containing solutions had to be adjusted. The stock solutions were divided into single assay aliquots, and varying SDS concentrations and vesicles to a final concentration of 1 mg·ml^{-1} were added. A final pH of 6.15 was prevalent in all experiments.

SDS binding

The measurement was carried out in duplicats. 3 ml of 0.5 M sucrose in 4 ml Beckman Ultraclear centrifuge tubes were overlayed carefully with 1 ml assay media containing [^{35}S]SDS. The vesicles were centrifuged through the sugar phase at 55,000 rpm for 30 min (Beckman rotor Type SW 60 Ti). The distribution of [^{35}S]SDS after centrifugation was determined in aliquots of the assay top layer, the sugar phase as well as the solubilized (14% SDS) pellet.

Protein and phospholipid content of the pellet

The same procedure was carried out as described for SDS binding except that non-radioactive SDS had been added to the assay media. After centrifugation the liquid phases were discarded whereas the pellets were resuspended in 2 ml 100 mM KCl and spun down again at 55,000 rpm for 30 min (Beckman rotor Type 70.1 Ti). Protein in the pellets was determined by the Kjeldahl procedure. For the determination of phospholipids the pellets were digested in perchloric acid and the resulting P_i was measured according to Bartlett [33, 34].

SDS-PAGE

Complete incubation media (2 ml each, containing 2 mg vesicular protein) were centrifuged at 55,000 rpm for 30 min. The resulting pellets were solubilized in warm 5% SDS and treatment buffer according to the Laemmli procedure [35] to a final volume of 2 ml. Traces of protein in the supernatants were precipitated by addition of 0.2 ml 100% trichloroacetic acid and centrifugation was performed at 4000 rpm for 20 min. The precipitates were washed once by resuspension in 2 ml H_2O and another centrifugation run as above. Finally the precipitates were, likewise, solubilized in warm 5% SDS and treatment buffer to a final volume of 0.1 ml. The pretreated samples were divided into aliquots and stored at −18 °C until electrophoresis was performed, usually in-between 24 h.

Continuous gradient (5/15%) Laemmli slab gels were prepared and 20 µl protein samples (for one exception see legend to Fig. 2B) were electrophorized for about 1 h.

Determination of CMC

Reaction media were prepared, containing 40 mM Tris·maleate pH 6.0, 20 mM $MgSO_4$, 100 µM $CaCl_2$, no or 20% Me_2SO, and 50 µM 7-chlorotetracycline. The fluorescence was measured in a SLM fluorescence spectrophotometer; the excitation wavelength was 390 nm, the emission wavelength 530 nm. The base line was thus established and, subsequently, SDS was added in µl-steps until an upward shift of the record indicated the commencement of micelle formation (for determination of CMC by spectral shift recording see *e.g.* [36]).

ATP hydrolysis rate

The assay media for the ATPase activity measurements contained (final concentrations) 40 mM Tris·maleate buffer pH 6.0, 20 mM $MgSO_4$, 15 µM Ca-ionophore A 23187, 1 mM (Ca^{2+}-independent or basal activity) or no, 150 or 250 µM EGTA, no (see before) or 100 µM Ca^{2+} (Ca^{2+}-dependent activity), 0.1 mg vesicles·ml^{-1}, and varying SDS concentrations. The resulting free Ca^{2+} concentrations were 100, 13, and 5.6 µM [37]. The reaction was started by addition of 1 mM Tris·ATP pH 6.0 and terminated after 1 or 2 min by addition of 4 ml 6% trichloroacetic acid. The P_i liberated was determined spectrophotometrically according to Rockstein and Herron [38].

For the ATPase activity measurements in the presence of 20% Me_2SO the assay media contained 0.5 instead of 0.1 mg vesicles·ml^{-1}. Here the times of reaction were 2 or 4 min.

Ca^{2+} binding

The reaction media stock solutions generally contained 0.1 mM $^{45}Ca^{2+}$. Varying concentrations of free Ca^{2+} were achieved by addition of 0–0.4 mM EGTA to aliquots. The assay volume was 4 ml each. Immediately after completion of the single assay media 0.5 ml samples were removed for liquid scintillation counting (total cpm $^{45}Ca^{2+}$, I). 2 ml were pipetted into Beckman Ultraclear centrifuge tubes and spun

down at 55,000 rpm for 30 min (Beckman rotor Type 70.1 Ti). 0.5 ml of the resulting supernatant, containing the unbound fraction of $^{45}Ca^{2+}$ were removed for liquid scintillation counting. To the residual assay media, 1.5 ml, 30 µl 14% SDS were added to completely solubilize the vesicles and another sample of 0.51 ml was counted (total cpm $^{45}Ca^{2+}$, II). By taking two total cpm $^{45}Ca^{2+}$ samples in this order we could exclude a falsification of results which might have stemmed from SDS-induced aggregation during the mixing and removal period and, thus, an uneven distribution of vesicular material. The mean of the samples I and II minus the cpm $^{45}Ca^{2+}$ in the supernatants yielded the bound fraction of Ca^{2+}.

Free Ca^{2+} concentrations were calculated using the Ca·EGTA complex binding constants by Schwarzenbach and Flaschka [37], which have been reported to be unaffected by 20% Me_2SO [39].

Since at certain characteristic points the measured ratio bound Ca^{2+} *versus* total Ca^{2+} proved to be very low, we established controls in which the measurements were repeated in the presence of 5 times higher vesicle and SDS concentrations (same $mg \cdot mg^{-1}$ ratios) in the otherwise identical reaction media.

Phosphorylation

The reaction media stock solutions generally contained 1 mM EGTA and 0.03–5 mM $^{32}P_i$. Here varying concentrations of free Ca^{2+} were achieved by addition of up to 1.2 mM $CaCl_2$ to single assay aliquots. The completed reaction mixtures were left standing under stirring for 4 min before phosphorylation was terminated by addition of 10 ml 10% trichloroacetic acid. Protein-bound $^{32}P_i$ was determined as described by Fassold *et al.* [40].

Phosphorylation was carried out in duplicates if the hydroxylamine-sensitivity of the formed phosphoenzymes was to be investigated. The trichloroacetic acid-precipitated phosphoprotein samples were repeatedly resuspended and centrifuged in 2% trichloroacetic acid and, finally, 10 ml H_2O. Half of the samples were dissolved in 1 ml conc. formic acid and their $^{32}P_i$ content was determined in a Packard scintillation counter. To the other half 1 ml 0.4 M hydroxylammonium chloride in 0.2 M Na-acetate buffer pH 5.2 was added, and the mixture was left standing at room temperature for 20 min. Then 10 ml 3% perchloric acid were added and, again, the samples were prepared for liquid scintillation counting by repeated washing procedures as above, and, finally, dissolution in 1 ml conc. formic acid. The hydroxylamine-insensitive fraction of total phosphoenzyme was thus determined.

Materials

Me_2SO was obtained from Merck-Schuchardt (Hohenbrunn b. München, F.R.G.), [^{35}S]SDS, $^{45}Ca^{2+}$ and $^{32}P_i$ came from Amersham Buchler (Braunschweig, F.R.G.), Tris·ATP came from Sigma Chemicals, the Ca-ionophore A 23187 from

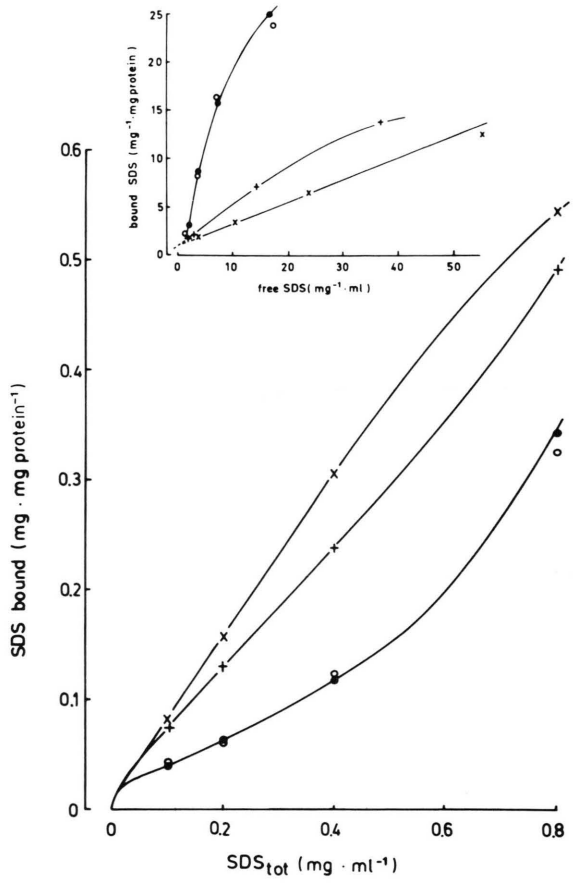


Fig. 1. SDS-binding to the sarcoplasmic reticulum membrane. Assay media containing 40 mM Tris·maleate pH 6.0, 20 mM $MgSO_4$, 100 µM $CaCl_2$, 1 mg vesicles·mg^{-1}, no (●, ×) or 20% (○, +) Me_2SO, and varied concentrations of [^{35}S]SDS, were centrifuged at 55,000 rpm for 30 min through 3 ml 0.5 M sucrose. The resulting [^{35}S]SDS content in the assay top layer, the sugar phase, and the subsequently solubilized pellet was measured by liquid scintillation counting. Note (Table I) that the pellet comprised at least 86% of the vesicular proteins and phospholipids. [^{35}S]SDS content in the pellet: ●, ○; in the pellet plus sugar phase: ×, +. Inset: Double-reciprocal plot of mg SDS bound·(mg protein)$^{-1}$ *versus* mg SDS-free·ml^{-1}.

Calbiochem-Behring (La Jolla, U.S.A.), and 7-chlorotetracycline was obtained from Serva (Heidelberg, F.R.G.). Hydroxylammonium chloride was purchased from Merck (Darmstadt, F.R.G.). Low and high molecular weight protein standards were obtained from Bio-Rad Laboratories (München, F.R.G.). All other chemicals were analytical grade.

Results

SDS binding to vesicles

Complete reaction media were centrifuged through a 0.5 M sugar solution in order to reliably exclude trapping of the SDS (used as [^{35}S]SDS)-containing assay media in the pellets. SDS-binding data in Fig. 1 reveal that a small fraction of SDS was bound to the pellet with high affinity (about 0.02 mg·resp. 70 nmol·(mg protein)$^{-1}$). Increasing the SDS/protein concentration ratio to about 0.4 resulted in a rather flat increment of the binding curve. Above that SDS concentration binding pronouncedly increased. An equal number of SDS molecules bound to the pellet if 20% Me_2SO was present in the medium. The large fraction of SDS which was detected in the sugar phase, however, was diminished by Me_2SO. In the absence of Me_2SO the combined SDS fractions, pellet and sugar phase, yielded a hyperbolical binding curve with saturation characteristics as deducible from the straight line obtained by double reciprocal plotting (inset Fig. 1). An apparent maximum of 1 mg SDS bound to 1 mg protein (or 3.5 μmol·mg^{-1}).

The same kind of evaluation in the presence of Me_2SO exhibited a non-linear relationship in the low SDS concentration range, as observed for SDS binding to the pellet regardless of Me_2SO.

SDS micelles were not spun down: The radioactively marked sediment at the SDS/protein ratio of 2 was negligible, reflecting also extensive lysis of the lipoprotein complex (absence of Me_2SO, compare Table I).

Protein and phospholipids in the pellet

For an adequate assignment of the measured sedimented fraction of SDS, we clarified whether SDS addition affected the total concentrations of protein and phospholipids in the pellet. In relation to subsequent experiments this had to be ascertained likewise for pellets obtained with SDS after plain centrifugation (without sugar layer) of complete assay media.

Table I. SDS-dependent recovery of protein and phospholipid after sedimentation of vesicles. Reaction media as in Fig. 1, non-radioactive SDS was used. Plain or centrifugation through 0.5 M sugar was performed as in Fig. 1. The resulting pellet was resuspended in 100 mM KCl and centrifuged again. Protein in the sediment was determined by the Kjeldahl procedure; phospholipids, respectively their P_i content, were measured according to Bartlett [33, 34].

SDS/protein ratio	Recovered protein [percent]		Recovered phospholipid [percent]	
		20% Me_2SO		20% Me_2SO
0	100	88	100	92
0.1	92	90	92	90
0.4	90	96	93	95
0.8	86	96	95	96
1.2	64	89	80	96
1.6	16	81	27	88
2.0	7	35	4	51

Table I exhibits that little protein was lost into the supernatant with increasing SDS up to at least 0.8 mg·mg^{-1}; at this concentration the loss comprised only about 14% in the Me_2SO-free medium. In the presence of 20% Me_2SO the recovery of protein in the pellet without added SDS was somewhat lower than in its absence (the reason for which is not clear) while increasing SDS improved the yield, probably due to intensified aggregation of the lipoprotein complex. Also a higher SDS/protein ratio was needed for solubilization.

The phospholipids in the pellet (Table I, columns 3 and 4) exhibited a similar SDS concentration dependence. A moderate delay of lysis with increasing SDS as compared to the protein was observed.

Protein composition of pellet and supernatant

Although there was no marked variation in the ATPase content of the pellets up to a SDS/protein ratio of 0.8 (Fig. 2A) or 1.2, respectively, if Me_2SO was present (not shown), density measurements of the stained gels yielded that the ATPase content rises in the pellet with increasing SDS. This, of course, also follows from Fig. 2B, where ATPase protein is virtually lacking in the supernatants at 0.4 and 0.8 mg SDS·mg^{-1}.

At the same SDS concentrations without Me_2SO the peripheral vesicular membrane proteins of 45,000–60,000 Da (calsequestrin, high affinity Ca^{2+}

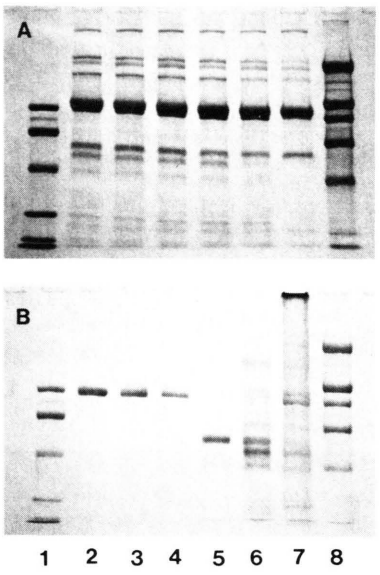


Fig. 2. Protein composition of the pellets and the supernatants after centrifugation. Reaction media as in Fig. 1, no Me_2SO present, non-radioactive SDS concentrations: 0, 0.1, 0.2, 0.4, 0.8, 1.2 mg·mg^{-1} (lanes 2–7). For further procedure see Methods. A: pellets, B: 20-fold concentrated supernatants. Lane 1 and 8: low and high molecular weight standards (MW: 92,500, 66,200, 45,000, 31,000, 21,500; 200,000, 116,250, 92,500, 66,200, 45,000). Note exception on lane 7, B: 10 µl instead of 20 µl protein-containing solutions, as in all other cases, were applied.

binding protein, and intrinsic glycoprotein) started to appear in the supernatants. In contrast, there was a complete lack of protein traces when 20% Me_2SO was present (not shown).

The second Me_2SO-entailed difference was, that at a SDS/protein ratio of 1.2 less than half as much protein was detected in the supernatant sample with Me_2SO, reflecting the depressed solubilizing effect of SDS by Me_2SO (see Table I).

Additional electrophoretic studies established (not shown) that the SDS-dependent protein distribution between pellets and supernatants was unchanged under phosphorylation conditions with/without Me_2SO, Ca^{2+} or EGTA.

CMC for SDS in the reaction medium

The method, taking advantage of the fluorescence changes of the Ca-chlorotetracycline complex on micelle formation, is based on the principle described by Benzonana [36]. The CMC was found to be in the low range 0.125–0.15 mg SDS·ml^{-1} (about 0.5 mM) at both proton concentrations when Me_2SO was absent. This is considerably lower than reported values at comparable ionic strength and temperature obtained with other methods [41, 42]. However, the reported measurements have been carried out in solutions of higher salt content.

With Me_2SO the CMC increased to about 0.5 mg·ml^{-1}. This could explain why Me_2SO delays solubilization by SDS.

ATP hydrolysis at different free Ca^{2+} concentrations

In the absence and presence of Me_2SO SDS addition caused an immediate steep decay of ATPase activity (not shown). An intermediary plateau of activity was reached, the height and width of which depended on free Ca^{2+}. Practically no activity was detected at 0.8 mg SDS·mg^{-1}. 20% Me_2SO reduced the ATP hydrolysis rate to a fifth (see also [43]).

Effect of SDS on high affinity Ca^{2+} binding

SDS addition up to 0.4 mg·mg^{-1} reduced Ca^{2+} binding regardless of the free Ca^{2+} present (Fig. 3A). This effect was most pronounced at an inter-

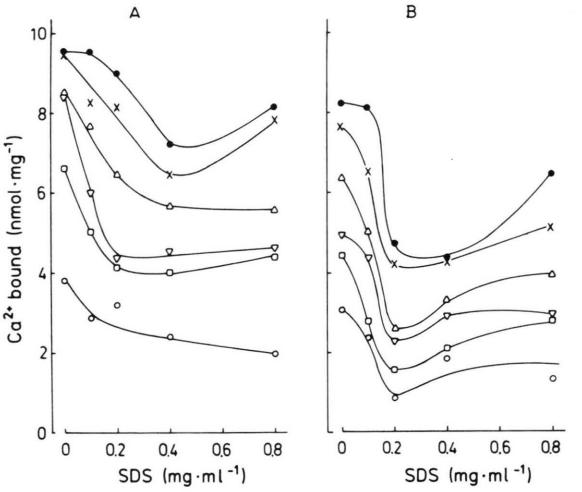


Fig. 3. Effect of SDS on high affinity Ca^{2+} binding. The reaction media contained 40 mM Tris·maleate buffer pH 6.0, 20 mM $MgSO_4$, 0.1 mM $^{45}Ca^{2+}$, 0–0.4 mM EGTA, (A) 0 or (B) 5 mM P_i, 0–0.8 mg SDS·ml^{-1}, and 1 mg vesicular protein·ml^{-1}, added in that order. Aliquots were removed either for liquid scintillation counting of total cpm $^{45}Ca^{2+}$ added or for sedimentation of the protein plus bound $^{45}Ca^{2+}$ by centrifugation (see Methods for details). $^{45}Ca^{2+}$ in the supernatants was likewise determined by liquid scintillation counting. The resp. difference between the total cpm $^{45}Ca^{2+}$ added and $^{45}Ca^{2+}$ in the supernatant was plotted as nmol Ca^{2+} bound·mg^{-1}. SDS concentration dependence at different free Ca^{2+} concentrations (µM): 100 (●), 54 (×), 20 (△), 8 (▽), 4.5 (□), 1.6 (○).

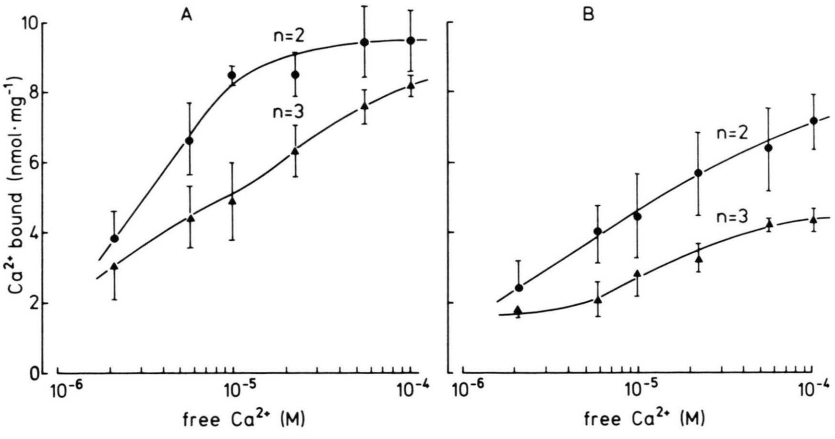


Fig. 4. Ca^{2+} concentration, P_i and SDS dependence of Ca^{2+} binding. The data are selected from Fig. 3. The reaction media contained no SDS (A) or 0.4 mg SDS·ml^{-1} (B), and no P_i (●) or 5 mM P_i (▲) in addition to the components listed in the legend of Fig. 3. The vertical bars represent SEM.

mediate free Ca^{2+} concentration. Along with a further increase of SDS Ca^{2+} binding increased again as long as the free Ca^{2+} exceeded 10 μM. Plotting of selected data from Fig. 3A against the free Ca^{2+} concentration (Fig. 4A and B, upper curves) revealed that in the absence of SDS half-maximum binding was obtained at about 3 μM free Ca^{2+}, while at 0.4 mg SDS·mg^{-1} about 10 μM free Ca^{2+} were needed; saturation of the two high affinity binding sites was not achieved under these conditions. Evidence of a two-step Ca^{2+}-dependence of binding became obvious at a SDS/protein ratio of 0.8.

Plotting of the results according to Scatchard (not shown) yielded that at 0.4 mg SDS·mg^{-1} the Ca^{2+} affinity of one binding site was about ten times lower than that of the other which was virtually unchanged. So was the total binding capacity of about 10 nmol Ca^{2+}·mg^{-1}.

The analysis according to Hill (not shown) revealed that in the absence of SDS, Ca^{2+} was bound non-cooperatively at the prevailing pH 6.15 (slope 0.9) and that with increasing SDS the slope declined. At a SDS/protein ratio of 0.8 it clearly showed a three-step profile.

Ca^{2+} binding was impaired by 5 mM P_i in the absence of SDS (Fig. 4A, lower curve), except at low free Ca^{2+} where the decline was not significant. Similarily to SDS P_i lowered the affinity of one of the two Ca^{2+} binding sites 6–7-fold (Scatchard plot, not shown). The high affinity of the other site was maintained. The SDS dependence of the Ca^{2+} binding pattern (Fig. 3B) was similar in principle to the one shown in Fig. 3A. The reduction of bound Ca^{2+} was more pronounced and reached its lowest level already at a SDS/protein ratio of 0.2. Further, under phosphorylation conditions SDS progressively in-

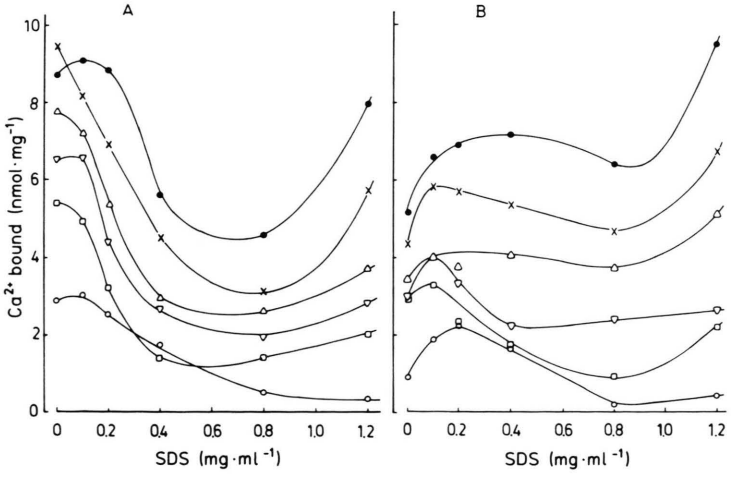


Fig. 5. Effect of SDS on high affinity Ca^{2+} binding in the presence of Me_2SO. The reaction media and experimental procedure were as given in the legend of Fig. 3 except that (A) 0 or (B) 1 mM P_i and 20% Me_2SO was present. SDS concentration dependence at different free Ca^{2+} concentrations (μM): 100 (●), 57 (×), 26 (△), 13 (▽), 8 (□), 3 (○).

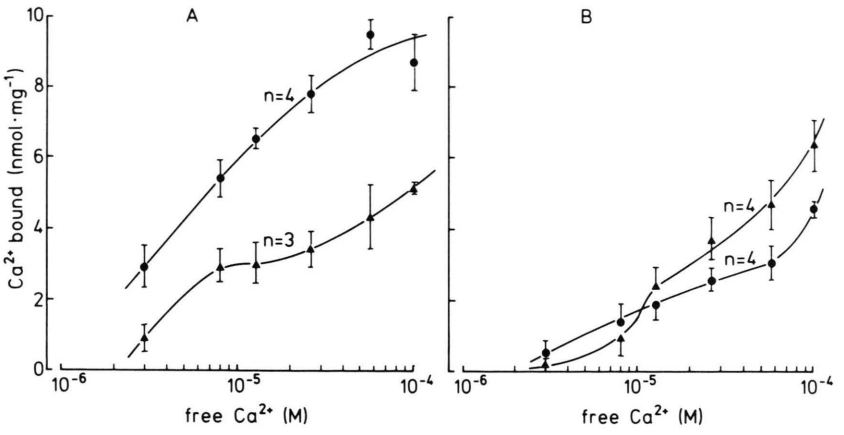


Fig. 6. Ca^{2+} concentration, P_i and SDS dependence of Ca^{2+} binding in the presence of Me_2SO. The data are selected from Fig. 5. The reaction media contained no SDS (A) or 0.8 mg SDS·ml^{-1} (B), and no P_i (●) or 1 mM P_i (▲) in addition to 20% Me_2SO and the components listed in the legend of Fig. 3.

duced an affinity reduction of both binding sites (Fig. 4B, lower curve), which, of course, represents a remarkable difference to the effect of SDS in the P_i-free medium.

Saturating Ca^{2+} binding was achieved also in the presence of 20% Me_2SO (Fig. 5A) despite a minor reduction of Ca^{2+} affinity (compare upper curves Fig. 6A and 4A). While the apparent dissociation constant of Ca^{2+}, K_{app}, was shifted from about 3 μM to 6–7 μM by Me_2SO, the affinity of both binding sites was equal (Scatchard plot, not shown). With Me_2SO the SDS-mediated binding minimum and subsequent increase occurred at higher SDS/protein ratios. Both, plotting of the data against the free Ca^{2+} concentration (Fig. 6A and B) and according to Scatchard support our presumption that the activity of Ca^{2+} ions is lowered by Me_2SO.

The slopes obtained by the analysis of the SDS-induced changes according to the Hill equation were steeper throughout in comparison to the Me_2SO-free media (not shown).

Under phosphorylation conditions, with 1 mM P_i in the Me_2SO-containing assay (Fig. 5B), Ca^{2+} binding was strongly affected. In the absence of SDS its reduction by P_i was significantly intensified as compared to the Me_2SO-free medium (compare Fig. 4A and 6A, lower curves). Increasing SDS, in contrast to all other experimental conditions, yielded a more or less marked increase of bound Ca^{2+} at all free Ca^{2+} concentrations, followed by an intermediary decline and, finally, a second increase (Fig. 5B).

From the Hill plot it could be deduced that the SDS-dependent reduction of cooperativity between the two Ca^{2+} binding sites, as observed

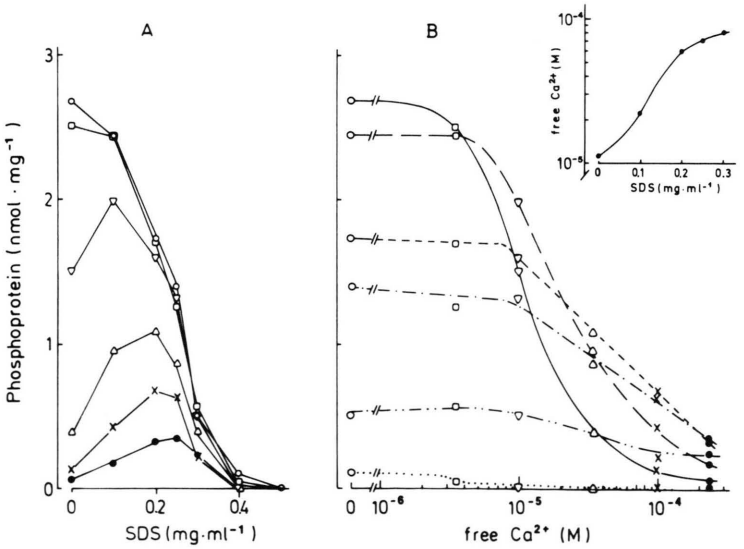


Fig. 7. Effect of SDS on the steady state level of phosphoprotein obtained with inorganic phosphate. The assay media contained 40 mM Tris·maleate buffer pH 6.0, 20 mM $MgSO_4$, 1 mM EGTA, 5 mM $^{32}P_i$, 0–1.2 mM Ca^{2+}, 0–0.5 mg SDS·ml^{-1}, and 1 mg vesicular protein·ml^{-1}. The reaction was terminated after 4 min by addition of 10 ml 10% trichloroacetic acid, and protein-bound P_i was determined as described by Fassold *et al.* [40]. A. SDS concentration dependence at different free Ca^{2+} concentrations (μM): 242 (●), 98 (×), 35 (△), 10.3 (▽), 3.5 (□), 0 (○). B. Ca^{2+} concentration dependence at different SDS concentrations (mg·ml^{-1}): 0 (——), 0.1 (— —), 0.2 (– – –), 0.25 (–·–) 0.3 (—··—), 0.4 (·····). Inset. SDS-mediated rise of the free Ca^{2+} which causes half-maximum inhibition of steady state phosphorylation (K_i).

under all preceding experimental conditions, was abolished when both 20% Me_2SO and 1 mM P_i were contained in the assay medium (not shown).

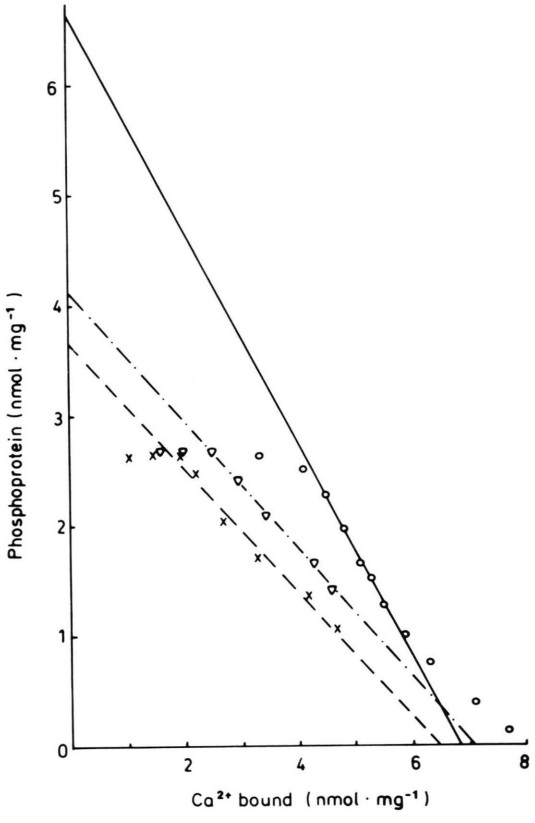


Fig. 8. Effect of SDS on the relation between steady state levels of enzyme-bound Ca^{2+} and phosphoprotein. Single data at corresponding Ca^{2+} and SDS concentrations were taken from Fig. 3B and Fig. 7. The phosphoprotein data at different free Ca^{2+} concentrations presented in this Fig. take into account the depression of steady state phosphorylation in the absence of Ca^{2+} (presence of 1 mM EGTA, Fig. 7 (○)) by the resp. SDS concentration, *i.e.*, the calculated percentage of depression was added to the phosphoenzyme in the presence of Ca^{2+}. SDS concentrations ($mg \cdot ml^{-1}$): 0 (○——○), 0.2 (×——×), 0.3 (▽——▽) (0.3: extrapolated data from Fig. 3).

Effect of SDS on phosphorylation by inorganic phosphate

Both increasing Ca^{2+} in the absence of SDS as well as increasing SDS in the absence of or at low free Ca^{2+} cause a decline of steady state phosphorylation (Fig. 7). In the SDS-free medium half-maximum inhibition occurred at about 11 μM Ca^{2+} (K_i), at which concentration half of the high affinity Ca^{2+} binding sites are saturated (compare Fig. 4A, lower curve), and total inhibition is reached with a free Ca^{2+} concentration of about 100 μM.

The SDS-induced inhibition profiles vary considerably with the free Ca^{2+} in the assay (Fig. 7A) despite the overall complete abolition of phosphorylation at SDS/protein ratios exceeding 0.4. The intermediary increase of protein-bound P_i at free $Ca^{2+} > 4$ μM finds an easy explanation if one considers that SDS also affects high affinity Ca^{2+} binding. Along with the concomitant reduction of bound Ca^{2+} ions SDS

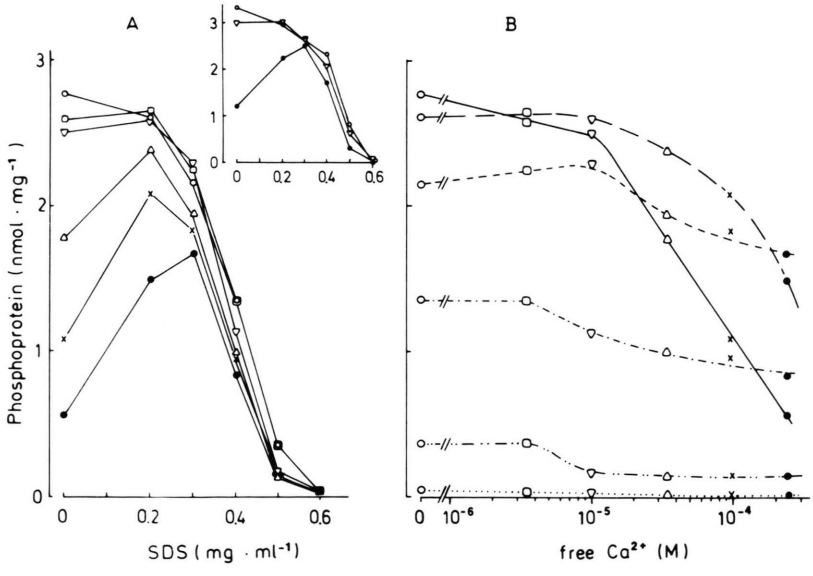


Fig. 9. Effect of SDS on the steady state level of phosphoprotein obtained with inorganic phosphate in the presence of Me_2SO. The assay media were identical to the ones in Fig. 7, except that they also contained 20% Me_2SO and that the $^{32}P_i$ concentration was 1 mM or 3 mM (inset). The experimental procedure was as in Fig. 7. A. SDS concentration dependence at different free Ca^{2+} concentrations (μM): 242 (●), 98 (×), 35 (△), 10.3 (▽), 3.5 (□), 0 (○). B. Ca^{2+} concentration dependence at different SDS concentrations ($mg \cdot ml^{-1}$): 0 (——), 0.2 (— —), 0.3 (– – –), 0.4 (–·–), 0.5 (—··—), 0.6 (·····).

releases the inhibition which Ca^{2+} binding exerts on phosphorylation (see above and Introduction on "mutual ligand exclusion"). Another consequence, the rise of the apparent K_i of Ca^{2+} for phosphorylation with increasing SDS, is depicted in the inset of Fig. 7.

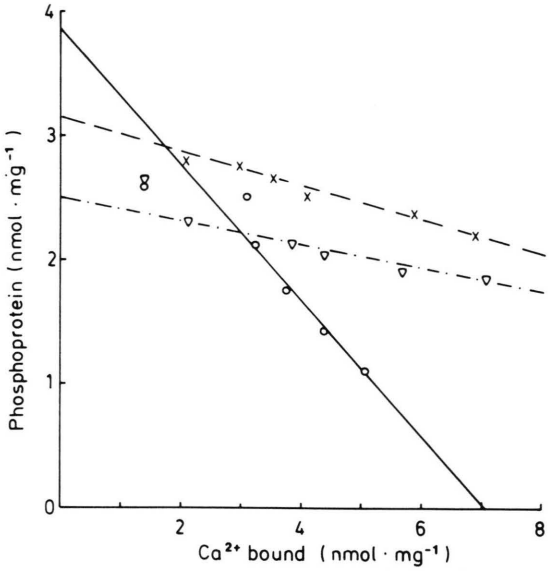


Fig. 10. Effect of SDS on the relation between steady state levels of enzyme-bound Ca^{2+} and phosphoprotein in the presence of 20% Me_2SO. Single data at corresponding Ca^{2+} and SDS concentrations were taken from Fig. 5B and Fig. 9. For calculation of presented phosphoprotein data see legend of Fig. 8, respectively Fig. 9. SDS concentrations (mg·ml^{-1}): 0 (○——○), 0.2 (×——×), 0.4 (▽——▽).

In order to elucidate, whether SDS, in addition to the mentioned effects on Ca^{2+} binding and phosphorylation, also interfered with their "mutual exclusion", we combined Ca^{2+} binding and phosphorylation data, obtained under identical experimental conditions, on one plot. Fig. 8 clearly reveals that this is the case. Hence, the SDS-induced rise of the K_i for Ca^{2+} also results from this uncoupling effect.

In the presence of 20% Me_2SO SDS abolished phosphorylation in the Ca^{2+}-free medium only at detergent/protein ratios above 0.6 (Fig. 9). The SDS-dependent intermediary increase of phosphoenzyme was markedly more pronounced due to the observation that the Ca^{2+} sensitivity of phosphorylation was strongly weakened: the apparent K_i of 65–70 μM Ca^{2+} at zero SDS rose to over 300 μM at 0.2 mg SDS·mg^{-1} (Fig. 9B).

The observations that, on the one hand, Ca^{2+} binding increased in the presence of phosphate and low concentrations of SDS and that, on the other hand, phosphorylation was hardly impaired by high free Ca^{2+} in the same concentration range, indicated a pronounced uncoupling by SDS of the "mutual ligand exclusion": Fig. 10 illustrates that with 20% Me_2SO both reactions proceed almost independent of each other if SDS is added.

Indications of ionic interaction of the strong anion SDS with the ATPase molecule (see before) lead us to investigate whether the deleterious effect of the detergent on phosphorylation might be a conse-

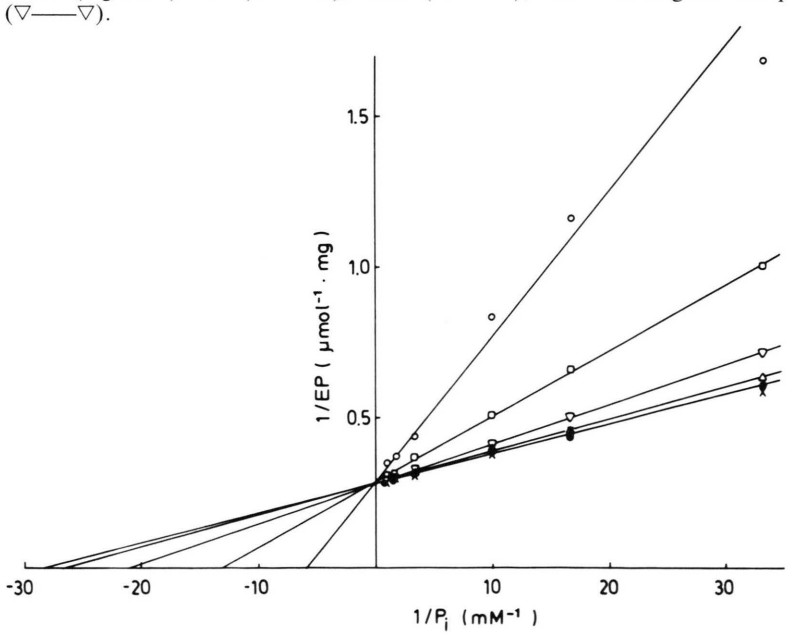


Fig. 11. Competitive inhibition of steady state phosphorylation by SDS in the presence of Me_2SO. The assay media and experimental procedure were as described in the legends of Fig. 9 and Fig. 7. The reaction was carried out in the presence of 1 mM EGTA and absence of added Ca^{2+}. $^{32}P_i$ concentrations (mM): 0.03, 0.06, 0.1, 0.3, 0.6, 1. SDS concentrations (mg·ml^{-1}): 0 (●), 0.03 (×), 0.06 (△), 0.1 (▽), 0.2 (□), 0.3 (○).

quence of SDS binding to the phosphorylation site itself. Phosphorylation experiments were performed by varying the concentrations of SDS and P_i in Me_2SO-containing media in order to exploit the Me_2SO-induced vast increase of the P_i affinity. Fig. 11 proves that SDS > 0.1 mg · mg^{-1} competed with P_i for its binding site and thus increased the K_m from ~ 0.035 mM P_i at zero SDS to 0.17 mM P_i at 0.3 mg SDS · mg^{-1}. Although the inherent implication of plain competitive inhibition, *i.e.* that an elevation of the substrate counteracts the effect of the reaction inhibitor, is corroborated by Fig. 9 A (inset), plotting of the data from Fig. 11 according to Dixon (not shown) did not yield straight lines with a common point of intersection. Hence, another interaction mechanism progressively superimposes competitive inhibition and is finally responsible for complete abolition of phosphorylation.

Hydroxylamine addition caused a decline of the phosphoenzyme to practically zero irrespective of the composition of the reaction media. Hence, neither Me_2SO nor SDS caused or allowed significant unspecific phosphorylation of the ATPase or other proteins in the preparation.

Discussion

This study demonstrates different specific effects of low SDS concentrations on high affinity Ca^{2+} binding and phosphoenzyme formation by P_i. The "mutual exclusion" of these two binding steps is uncoupled, more evidently in the presence of 20% Me_2SO. The overall ATPase is reduced in a two-step manner.

The actual experimental findings could only be reliably evaluated with respect to the applied experimental procedures, after it had been clarified whether or from what concentration onwards SDS affects the integrity of the vesicular material used.

So far investigations of the physicochemical and biochemical properties and effects of SDS on purified or membrane-bound proteins [13, 44–46] have mainly been undertaken for empirical reasons. Recent results from our laboratory [47], showing differential solubility of the sarcoplasmic reticulum vesicular components with low SDS concentrations, were obtained at pH 7.0. A comparison with our findings reveals that at pH 6.15 the SDS-initiated solubilization of the ATPase is shifted to considerably higher SDS/protein ratios. An explanation is provided by the fact that the ATPase protein has its isoelectric point and, consequently, its minimum solubility at an acidic pH [48, 49]. Further, the number of positive hydrophilic charges of the membrane proteins in general increases with decreasing pH, and by that the number of sites with which SDS is believed to interact early by electrostatic force [50]. This would result in a layer of SDS molecules directing hydrophobic chains to the aqueous medium, and solubilization would require higher ratios.

Like other authors [47, 51] we observed a distinct solubility of the membrane proteins. The only weakly associated intravesicular protein calsequestrin [48] was the first to appear in the supernatant (although not quantitatively) followed by other peripheral proteins [52]. The first implies the presence of both sealed and unsealed vesicles.

For practical reasons we determined SDS binding to the sedimentable fraction of vesicles. Accordingly a lower maximum binding ratio (1.0 mg SDS · mg^{-1}) and a less well-defined binding profile were obtained than reported by other authors for purified reduced proteins [13] or partially isolated ATPase [47] by means of the equilibrium dialysis method. The deviations are partly due to the presence of phospholipids and the fact that in our method SDS binding still increases after solubilization of vesicular material.

In the presence of Me_2SO part of the added SDS bound with lower affinity than in its absence. As a strong dipole, Me_2SO diminishes the solvation shell of the membrane components [53] by displacing water molecules especially around positively charged groups. The reduction of water activity [54] increases the tendency for self-association of the ATPase [39] and thereby, maybe, its accessability for SDS. On the other hand, the possibility of a direct interaction between SDS and Me_2SO molecules is corroborated by the Me_2SO-entailed shift of the CMC of SDS in our reaction medium.

Independent of Me_2SO the ATPase activity exhibits a two-step SDS-dependency profile. The intermediary activity plateau prevails over a more or less broad SDS concentration range, depending on the free Ca^{2+} in the medium. Regardless of Ca^{2+} the activity reaches zero at 0.8 mg SDS · mg^{-1}. For a closer analysis investigations are presently under way.

The important consequence of these preceding investigations was that neither the ATPase nor the surrounding phospholipids had been solubilized by SDS/protein ratios up to about 1.0 and that, therefore,

Ca^{2+} and P_i binding studies were performed with an enzyme whose phospholipid environment and structure was only gradually affected.

Based on experimental evidence it has been claimed [17–19] that the binding of two Ca^{2+} ions proceeds sequentially in a cooperative manner (Scheme 1). However, as mentioned in the Introduction, a Hill coefficient > 1 is obtained only at neutral or slightly alkaline pH. In agreement with this tendency, described in more detail by other authors [55, 56], we calculated the Hill coefficient of Ca^{2+} binding in the absence of SDS to be ~ 0.9 at pH 6.15. The disappearance of the cooperative character of Ca^{2+} binding with decreasing pH has been attributed to occupancy of Ca^{2+} binding sites by protons [16].

Increasing SDS causes an intermediary depression of Ca^{2+} binding. Since we found the binding constant of one binding site, *i.e.*, according to Scheme 1, most likely the initial binding step, unchanged in the P_i- and Me_2SO-free medium, the interaction of SDS must be attributed to the binding of the second Ca^{2+} ion and/or the linked conformational transition. The SDS-induced decrease of the Hill coefficient (negative cooperativity) could result from sterical hindrance exerted by SDS binding to the Ca^{2+} binding domain of the ATPase; the two binding sites are supposed to lie in close proximity to each other in the stalk region of the ATPase molecule protected by a "protein envelope" (for ref. see [20]). The affinity of the second site is lowest in the SDS concentration range $0.3-0.4\ mg \cdot mg^{-1}$, where the SDS-dependent ATPase activity profile exhibits an intermediary plateau.

The observed partial restoration of the Ca^{2+} affinity of the second binding site when higher SDS/protein ratios are applied presumably is a result of unfolding of the resp. protein region, *i.e.* increasing independence of the two binding sites and better accessability for the substrate to the second site. This finds its mathematical expression in the observed three-step slope on the Hill plot. It should be recalled that the number of Ca^{2+} binding sites is maintained.

In Me_2SO-containing and SDS-free media we traced a moderate decrease of the combined apparent Ca^{2+} dissociation constant, but an elevated degree of binding site interaction. Partly diverging solvent-induced changes were reported by other authors [39] at pH 7.0. These changes could be attributed to high Mg^{2+} binding [39, 57] which does not play a remarkable role at pH 6.15 regardless of Me_2SO [57, 58]. Under our experimental conditions the Me_2SO-dependent changes are presumably a consequence of the replacement of water molecules both in the solvation shell of Ca^{2+} ions and on the surface of the ATPase (see before).

When SDS was added to Me_2SO-containing media the reduction of total Ca^{2+} binding was shifted to higher SDS/protein ratios (see also Me_2SO-dependent shift of the CMC).

It follows from Scheme 1 and is depicted in Fig. 4A and 6A that P_i in the reaction medium reduces Ca^{2+} binding, *i.e.* a higher free Ca^{2+} concentration is needed to saturate the Ca^{2+} binding sites. The quoted Fig. also reveal an interesting parallelism between the effects of 5 mM P_i, or 1 mM in the presence of Me_2SO, on the one hand (Fig. 4A and 6A, lower curves), and 0.4 mg $SDS \cdot mg^{-1}$, resp. 0.8 with Me_2SO, in the absence of P_i, on the other hand (Fig. 4B and 6B, upper curves). From the Scatchard plots (absence of Me_2SO) it could be deduced that the affinity of the second Ca^{2+} binding site was reduced to the same degree while the affinity of the first site was unaltered. It will be discussed later that very likely an important identical electrostatic interaction accounts at least partially for this similarity. SDS addition to the P_i-containing assay, moreover, causes a progressive decline of the affinity of the first Ca^{2+} binding site which is not reversed by higher SDS concentrations.

In the Me_2SO and P_i-containing but SDS-free media Ca^{2+} binding is quite low, especially at low free Ca^{2+} concentrations, due to the shift of the equilibrium in favor of E_2P [30]. An addition of SDS leads to an intermediary elevation of Ca^{2+} binding and the degree of interaction between the Ca^{2+} binding sites steadily increases with SDS.

This was completely unexpected and could not be reconciled with what we had found until then. We were able to approach a solution when we investigated the reciprocal stoichiometric relation between Ca^{2+} and P_i binding. The presence of 20% Me_2SO alone under the prevailing conditions shifted the ratio from 1 (see also [55]) to 1.85, *i.e.*, 2 Ca^{2+} bound, 1 P_i released (Fig. 8 and 10). An addition of $0.2-0.4\ mg\ SDS \cdot (mg\ protein)^{-1}$ resulted in a shift of the ratio from 1 to 1.7–1.8 in the absence of Me_2SO indicating that SDS moderately counteracted the "mutual ligand exclusion" between Ca^{2+} and P_i. In

the presence of Me_2SO, however, this uncoupling effect of SDS was much more pronounced and the stoichiometric ratio shifted to values around 10.

This shows, in accordance with de Meis and Inesi [26], that the coupling rules depend on the strength of the interaction between the Ca^{2+} binding and phosphorylation domains which appear to be uncoupled by both the solvent and the detergent. Hence, as a consequence of the ready uncoupling by increasing SDS the ATPase molecule at the same time binds as much Ca^{2+} as possible under the prevailing conditions (SDS, Me_2SO), practically irrespective of P_i, and phosphorylation is subject to the interaction by SDS (and Me_2SO) alone and independent of Ca^{2+}.

If one compares Fig. 5A and B one realizes, however, that slightly higher levels of steady state Ca^{2+} binding are obtained at free Ca^{2+} concentrations exceeding 20 μM and at intermediate SDS concentrations in the presence of P_i and Me_2SO. This can only be reconciled with the aforementioned if one assumes (see earlier paragraph) that SDS in the absence of P_i depresses Ca^{2+} binding more strongly than in its presence. Probably this preceding inhibiting effect on Ca^{2+} binding is correlated to binding of SDS itself, simulating P_i binding.

The P_i concentrations used in our experiments exceeded the reported Michaelis-Menten constants both in the absence and presence of Me_2SO (20 mM Mg^{2+}), irrespective of the nature of the true substrate [39, 59]. Under our conditions the K_m of P_i in the Me_2SO-containing and SDS-free medium was found to be ~ 0.035 mM (resp. about 5 μM MgP, calculated with help of the constants given by Punzengruber *et al.* [59]).

A ready inhibition of phosphorylation by SDS (Fig. 7) was observed in the absence of free Ca^{2+} and Me_2SO, while, as observed for Ca^{2+} binding, the same effect was shifted to higher SDS concentrations if Me_2SO was present (Fig. 9). With varying free Ca^{2+} concentrations the parallel reduction of steady state Ca^{2+} binding brought about higher levels of phosphoenzyme, as could be expected in any case, but the reported uncoupling of the two binding reactions caused an even larger increase, esp. in the presence of Me_2SO. A complete suppression of phosphorylation with SDS as observed by us does not occur with other detergents although the affinity of P_i to its binding site has been found to be reduced [6, 60].

The suspicion (see earlier) that SDS and P_i have a common binding site on the ATPase molecule is verified by experimental evidence of competitive inhibition of phosphorylation by the detergent (Fig. 11). However, the Dixon plot yielded that this is progressively superimposed by another effect exerted by unspecific SDS binding such, that maximum phosphorylation can not be achieved in the presence of infinite P_i as soon as 0.1 mg SDS is added per mg protein.

There was a striking similarity between the SDS-dependent decay profiles of NH_2-bound fluorescein [11] and of Ca^{2+}-free steady state phosphoenzyme (Fig. 7). It has been reported that FITC binds to Lys_{515} located in the nucleotide binding domain [12] while the phosphorylation site has been identified to be the partial ATPase sequence $-Ser_{350}-Asp_{351}-Lys_{352}-$ [21, 61]. Although the phosphorylated intermediate after treatment with strong acids has been found to be an acylphosphate (Asp_{351}) [62], it is strongly corroborated by the comparative evidence from our and Swoboda's and Hasselbach's [11] work, that the neighbouring Lys_{352} plays an essential role under physiological conditions in the phosphate binding process. Such an additional intermediate might provide an explanation for the time lag of E_2P formation observed by Pickart and Jencks [25] as well as found the suggestion brought forward by Guillain *et al.* [63] that the initial step of P_i binding is comparatively slow.

A simple correlation between the SDS-dependent ATP splitting rate (see before) and the evidence on steady state levels of bound Ca^{2+} is not obvious. Further elucidating work is in progress regarding the ATP turnover, such as the effects of SDS on ATP binding, phosphoenzyme formation, as well as Ca^{2+} release after the addition of ATP.

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