# Transient Activation of the $Ca^{2+}$-ATPase from Sarcoplasmic Reticulum during Thiol Modification by 5,5′-Dithiobis(2-nitrobenzoate)

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In the reaction of sarcoplasmic reticulum membranes with excess 5,5′-dithiobis(2-nitrobenzoate) (DTNB) some new features were observed:

1) The $Ca^{2+}$-dependent ATPase activities of increasingly modified preparations were considerably enhanced during the initial stage of thiol blockage. A maximum (130–160% of the control activity) was reached when about 1.5–2 mol thiol groups per $10^5$ g vesicular protein had reacted, in the absence of ATP and detergent. At higher extents of modification inactivation occurred. Purified ATPase behaved principally similar to native sarcoplasmic vesicles.

2) In the presence of $Mg^{2+}$ and ATP the activity maximum (up to 180% of control) was broadened and shifted towards a higher degree of thiol blockage. Concomitantly the modification and inactivation rates were considerably reduced.

3) Glycerol (10–30%, v/v) slightly enhanced the ATPase activity maximum and reduced the rate of inactivation essentially only by decreasing the DTNB modification rate.

4) In the presence of sufficient myristoylglycerophosphocholine for solubilization no activation was observed.

5) The steady state level of phosphoprotein from ATP was raised to about 150% of the control level 10 s after addition of DTNB (about 1/2 thiol blocked), followed by a linear decrease with the number of thiols labeled, while the $Ca^{2+}$-dependent ATPase activity of preparations modified under equivalent conditions ($10^{-4}$ M $Ca^{2+}$ and $2 \times 10^{-3}$ M $Mg^{2+}$ present) showed a broader maximum corresponding to 1.5 thiols blocked.

## Introduction

It has been discovered long ago that the calcium pump of the sarcoplasmic reticulum is sensitive to the blocking of the transport protein's thiol residues [1, 2]. In the meantime extensive studies with different thiol reagents under various conditions have been carried out. Reagents employed include N-ethylmaleimide [2–6], 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [7–11], iodoacetamides [12–15] and mercurials (*e.g.* the fluorescent S-mercuric N-dansyl-cysteine [16]).

In the purified $Ca^{2+}$-ATPase, which contains approximately 22 cysteinyl residues per 100000 daltons [17, 18], about 15–17 can be titrated with DTNB in the presence of sodium dodecylsulfate, while in the native state about 11–13 are reactive. 4 thiol groups appear to be buried in the apolar regions of the ATPase molecule [18].

Several kinetically distinguishable classes of thiol groups have been assigned from their rate of reaction with N-ethylmaleimide or DTNB. In spite of somewhat controversial results regarding the distribution of the kinetic classes and the functionally critical number of thiols there has been general agreement on a linear decrease of enzyme activity with increasing blockage of the thiols of the second most reactive class. Recently a distinction of 2 essential thiol groups out of 4, one responsible for phosphoenzyme formation and one for its decomposition, could be made using low concentrations of N-ethylmaleimide [5, 6]. – Spin-labeled iodoacetamide derivatives, on the other hand, can react with several thiol groups without causing an appreciable loss of activity [13, 14]. Thus different reagents appear to react preferentially with different thiol groups of the ATPase protein.

Other energy transducing enzymes have also been shown to be affected by thiol reagents. Thus the $(Na^+ + K^+)$-ATPase [19–21] and proton-translocat-

*Abbreviations:* DTNB, 5,5′-dithiobis(2-nitrobenzoate); EGTA, ethyleneglycol bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid; TES, N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid. *Enzyme:* $Ca^{2+}$-ATPase (EC 3.6.1.3).

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ing ATPases (*e.g.* fungal plasma membrane [22], mitochondrial coupling factor B [23]) have been treated with several thiol reagents. Usually an inhibition of enzyme activity is observed. In the case of myosin, however, a severalfold activation of the $Ca^{2+}$-ATPase occurs, when the first thiol group on each myosin globular head is modified by N-ethylmaleimide while at the same time the $K^+$-ATPase activity is suppressed (*e.g.* [24, 25]). Blockage of the second thiol inactivates the $Ca^{2+}$-ATPase as well. On the other hand it has been demonstrated that thiol blocking as such does not necessarily produce inactivation. Thus introduction of a small group (like $-CN$) does not inhibit the $Ca^{2+}$- or $Mg^{2+}$-ATPase of myosin even after blocking of virtually all thiol groups [26], whereas bulky or charged substituents inactivate. Activations during thiol blocking have been described with other enzymes as well (comp. *e.g.* aspartate aminotransferase [27]). Conformational changes have been made responsible for these effects in all cases.

We have now observed an increase of the $Ca^{2+}$-dependent ATPase activity as well as the phosphoenzyme level from ATP phosphorylation in the initial stage of blocking of the thiol groups of native sarcoplasmic reticulum membranes with DTNB. Such an activation has not been described before with sarcoplasmic reticulum. Apparently in the sarcoplasmic ATPase, too, some kind of conformational change occurs during the modification of the first thiol group. Only at higher extents of thiol blocking the expected inactivation was observed. In this study we have explored different conditions of thiol blocking with the thiol reagent DTNB, in search of an explanation for this transient activation effect.

## Materials and Methods

Sarcoplasmic reticulum vesicles from rabbit skeletal muscle were prepared according to Hasselbach and Makinose [28] as modified by de Meis and Hasselbach [29]. Purified ATPase was prepared by treatment with low concentrations of deoxycholate according to [30] and stored at $-20\,°C$ (about 30 mg protein/ml in 50% glycerol). DTNB was obtained from EGA-Chemie (Steinheim, FRG), dithioerythritol was from Merck (Darmstadt, FRG) and L-α-myristoylglycerophosphocholine and $Ca^{2+}$ ionophore A-23187 were from Calbiochem (Gießen/Lahn, FRG). $Ca^{2+}$ ionophore X-537A (Lasolocid) was a gift from Hoffmann-La Roche (Basel, Switzerland). [$\gamma$-$^{32}$P]ATP and $^{45}CaCl_2$ were purchased from Amersham Buchler (Braunschweig, FRG).

Protein concentration was determined by the Biuret method, standardized by Kjeldahl, or from the absorption at 280 nm in 1% sodium dodecylsulfate [8].

### *Reaction of sarcoplasmic reticulum membranes with DTNB*

The reaction of sarcoplasmic vesicles with DTNB was followed spectrophotometrically according to the method of Ellman [31]. The incubation medium contained 0.4–0.45 mg protein/ml, 50–100 mM Tris. Cl, pH 7.8 or 8.4 (if not otherwise stated) and salts or other additions as mentioned in the legends. As turbidity changes were observed under some conditions during the first few minutes after putting the vesicles into the medium, DTNB (a 0.06 M solution in histidine buffer, pH 7) was added 10 min later to 3 mM (a minimal 50 fold excess to obtain pseudo-first-order rates and minimize cross-linking reactions) and the increase of the absorbance at 412 nm was recorded *vs.* time at 25 °C. The reference cuvette contained all components except protein. On addition of DTNB a small drop of pH and in most cases a transient or constant turbidity decrease were observed. Since turbidity changes in both directions during the reaction could not be neglected under the conditions, each assay was repeated identically, this time recording at 550 nm, where the produced thiophenolate anion does not absorb.

For the evaluation of the results the curve at 550 nm was subtracted from the curve at 412 nm, using an empirical factor ($f$412/550) for the ratio of "protein absorbances" due to different light scattering at the 2 different wave-lengths (measured before the addition of DTNB in each experiment). The number of DTNB modified thiol groups ($N_{SH}$) at a given time was calculated from the corrected absorbance increase

$$N_{SH} = \frac{\Delta E_{412}\,(\text{corr.})}{\varepsilon_{412} \times C\ \text{protein}}$$

where $\Delta E_{412}$ (corr.) $= \Delta E_{412} - \Delta E_{550} \times f$412/550 and $\varepsilon_{412} = 13600$ [31].

The factor $f$ 412/550 was usually around 2, the exact value slightly depending on the medium composition. It had to be assumed, that these relative absorbances remain approximately constant during the modification reaction. This evaluation method as such was checked by applying the same procedure to vesicles adding histidine instead of DTNB. The deviation from the expected $\Delta E_{412}$ (corr.) = 0 was at no time greater than a value corresponding to about 0.1 SH group.

When ATPase activities or phosphoenzyme levels were determined parallel spectrophotometrical evaluations, as described above, were carried out with the same vesicle preparation.

For the determination of the total number of free thiol groups of a vesicle preparation the medium additionally contained 1% sodium dodecylsulfate.

*ATPase activities*

In order to determine the dependence of the ATPase activities on the extent of thiol modification by DTNB sarcoplasmic vesicles (about 5–6 mg) were incubated at 25 °C as described for the spectrophotometrical evaluation. After starting the reaction with DTNB at time 0, aliquots (1 ml) of the reaction mixture were pipetted at different time intervals into individual ATPase assays (10 ml), each containing 0.15 mM dithioerythritol to stop the reaction, 20 or 40 mM histidine pH 7.0 (depending on whether pH 7.8 or 8.4 was used for the modification), 5 mM ATP, 5 mM $MgCl_2$, 40 mM KCl, 0.1 mM $CaCl_2$ and usually $3 \times 10^{-5}$ M $Ca^{2+}$ ionophore X-537A for the total activities, in order to maintain comparable permeability conditions. Some experiments were performed with ionophore A-23187 ($10^{-5}$ M) instead or with closed vesicles for comparison. The protein concentration in the assay was 0.04–0.045 mg/ml, the final pH about 7.1–7.2, temperature 22–24 °C. The rates of ATP splitting were determined from the differences between the values at 2 and 6 min. Eventual additions to the DTNB reactions, *e.g.* glycerol or myristoylglycerophosphocholine, being diluted 1:10 in the ATPase assays, did not influence the results.

Since the basal ATPase activities were found to be independent of the extent of modification, one or two controls for each vesicle preparation were sufficient. They were determined in the presence of 1 mM EGTA instead of $CaCl_2$ and were subtracted from the total activities to obtain the $Ca^{2+}$-dependent values. The ATPase avtivities of purified ATPase preparations were measured without ionophore or with A-23187; the corresponding basal activities were zero. Control ATPase activity determinations (vesicles preincubated under the modification conditions, omitting DTNB) were included in each series to obtain the corresponding 100% value.

*Phosphorylation by [$\gamma^{32}$P]ATP*

Steady state phosphorylation of vesicle preparations modified to varying extents were performed directly in separate DTNB modification assays, starting with a mixture of 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM Tris. Cl, pH 7.8, and 0.4 mg protein/ml at room temperature. DTNB was added to 3 mM at the time 0, the reaction stopped by dithioerythritol (1.6 mM) at the desired time (10 s to 8 min), immediately followed by the addition of [$\gamma^{32}$P]ATP to 0.1 mM. The phosphoenzyme was precipitated with 10% trichloroacetic acid/50 mM phosphoric acid at 0 °C and further treated as described in [32]. Some samples were yellowish which did not affect the results, however.

Controls without DTNB were phosphorylated the same way. Yet, since the pH of the phosphorylation reaction mixtures containing DTNB was lower than the pH of the control (by $\approx 0.2-0.3$ units) a series of controls was phosphorylated at pH 7.3, 7.5 and 7.8, to exclude the possibility that the results might be related to incorrect 100% values. Only a negligible increase of the EP steady state level with increasing pH was observed in this pH range (compare also [33]).

*Calcium uptake*

Uptake of $^{45}Ca^{2+}$ was followed in different DTNB modification assays separately. The DTNB reaction (0.4 mg vesicular protein/ml, 3 mM DTNB, 50 mM TES pH 7.5, 0.2 mM EGTA) was stopped by dithioerythritol (to 1.6 mM) at the desired time, then diluted fourfold with the uptake medium, resulting in a final medium composition of 50 mM TES, pH 7.3, 40 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 0.1 mM $^{45}CaCl_2$, 0.1 mM EGTA and 0.1 mg protein/ml, besides the components from the DTNB reaction. $Ca^{2+}$ uptake was started with ATP (to 5 mM) and measured from 30 s to 4 min employing the millipore filtration technique. For scintilla-

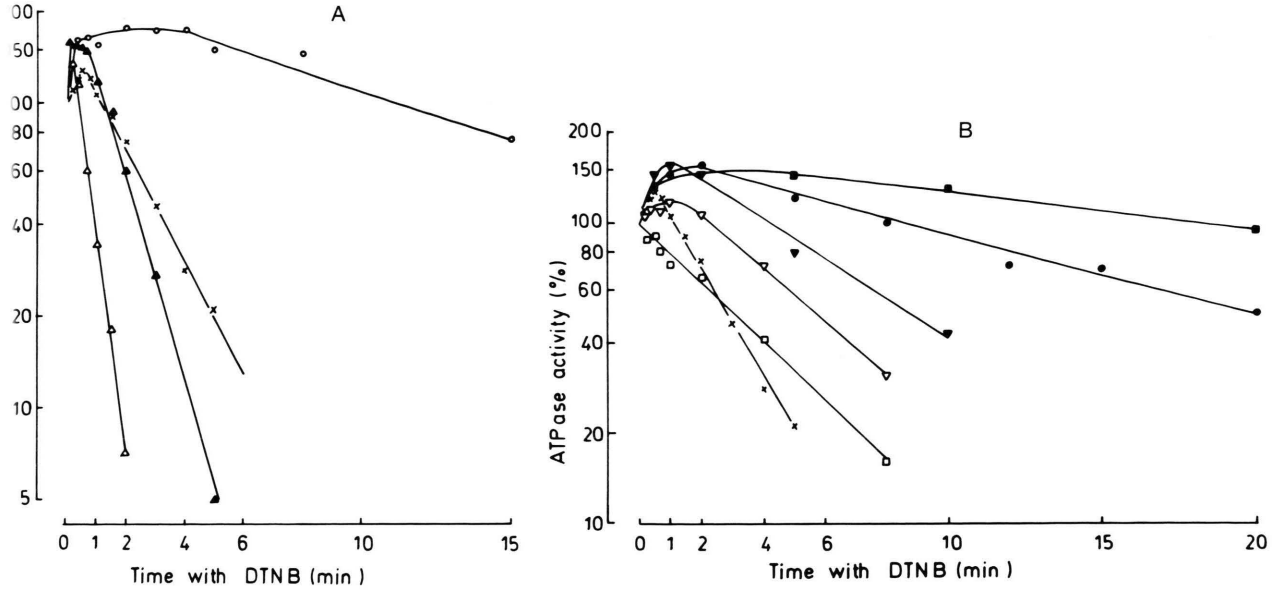


Fig. 1. $Ca^{2+}$-dependent ATPase activity as a function of time with DTNB. DTNB modification was performed as described in Materials and Methods in a medium containing 3 mM DTNB, 0.4–0.45 mg protein/ml, 100 mM Tris · Cl (pH 7.5–8.4) and various additions as given below. For the determination of ATPase activities aliquots (1 ml) were diluted into ATPase assays (10 ml) containing 20–40 mM histidine pH 7, 40 mM KCl, 5 mM ATP, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, $3 \times 10^{-5}$ M $Ca^{2+}$ ionophore X-537A and 0.15 mM dithioerythritol for the total activities (resp. 1 mM EGTA, no $Ca^{2+}$ and no ionophore for the basal activities). The ATPase activities are expressed in percent of the respective control values without DTNB. The control activities of the different vesicle preparations varied between 1 and 2 μmol Pi · $mg^{-1}$ protein · $min^{-1}$ for the total, resp. between 0.1 and 0.2 μmol Pi · $mg^{-1}$ protein · $min^{-1}$ for the basal activity (22–24 °C). (A) (×) 0.2 mM EGTA, pH 7.8 or 8.4 (mean of 8 experiments); (△) 2 mM $MgCl_2$ + 0.2 mM EGTA, pH 8.4; (▲) 0.1 mM $CaCl_2$, pH 8.4; (○) 5 mM ATP + 2 mM $MgCl_2$ + 0.2 mM EGTA, pH 8.4. – (B) (×) as in (A); (▼, ●, ■) glycerol (10, 20, 30%, v/v) + 0.2 mM EGTA, pH 7.8; (▽, □) myristoylglycerophosphocholine [(▽) 1 mg/mg protein, (□) 2.5 mg/mg protein] + 0.1 mM $CaCl_2$ + 2 mM $MgCl_2$, 50 mM Tris · Cl pH 7.5.

tion counting the yellow solutions were decolorized with 60 μl 1 N HCl per 1 ml sample.

The control (without DTNB) was performed at pH 7.3, the same pH as reached in the above assays after DTNB addition.

## Results

### *ATPase activities*

When sarcoplasmic vesicles were reacted with excess DTNB in the presence of 0.2 mM EGTA at pH 7.8 or 8.4 the $Ca^{2+}$-dependent ATPase activity was first enhanced, passing through a maximum (130% of the control activity) about 30 s after DTNB addition, and subsequently inhibited as described elsewhere [7–9]. Fig. 1 A shows the time course of this activation–inactivation process as a semilogarithmic plot. Also shown are the effects of $Mg^{2+}$ and $Ca^{2+}$ ions, as well as of ATP and $Mg^{2+}$. As can be seen, in the presence of 2 mM $Mg^{2+}$ inactivation proceeded more rapidly than with 0.1 mM $Ca^{2+}$ ions, which mainly raised the activity maximum, while at the same time the rates of all three DTNB reactions remained practically unchanged (not shown). ATP, on the other hand, decreased drastically both the rate of the DTNB reaction and the rate of inactivation. The broad activity maximum (about 180% of the control activity) was maintained for several minutes after DTNB addition. Even after 15 min about 80% of the $Ca^{2+}$-dependent ATPase activity were still retained.

Similar curves were obtained in the presence of glycerol, depending on its concentration (Fig. 1 B). The rates of reaction and inactivation were reduced by the same amount, the corresponding activity maxima reaching around 150% of the respective control activities. The effect of glycerol will be discussed below.

Myristoylglycerophosphocholine, however, when applied at a concentration sufficient for complete solubilization, was able to abolish the initial activation of the ATPase by DTNB. While 1 mg myristoylglycerophosphocholine per mg protein still produced a weak activation, in the presence of 2.5 mg per mg protein no more activity increase during DTNB modification was observed (Fig. 1 B). Under these conditions the ATPase protein was reported to be essentially monomeric [30]. The rate of the DTNB reaction was considerably enhanced by increasing concentrations of myristoylglycerophosphocholine. To slow the reaction a lower pH (7.5) was chosen, otherwise a correlation of activity with the number of modified groups would have been impossible in the range where an activation could be expected. Furthermore, compared to the maximum number of thiols reacting in the native enzyme (7–8) the numbers of reactive thiols in the presence of 1 or 2.5 mg myristoylglycerophosphocholine per mg protein were about 10 and 11, respectively.

Table 1 summarizes relevant data on the activation and inactivation of the $Ca^{2+}$-ATPase by DTNB under different conditions of modification. The linear portion of the plots of Fig. 1 A and B allows the calculation of the corresponding rate constants of inactivation as listed in the table. The linearity indicates a first-order loss of $Ca^{2+}$-dependent ATPase activity also described by other authors [7–9].

The kinetic plots of Fig. 1 A and B do not provide any information regarding the number of thiol groups that have reacted under the respective con-

Table I. Activation and inactivation of the $Ca^{2+}$-ATPase by DTNB modification. The experiments were the same as for Figs. 1–3. For the conditions compare the legend of Fig. 1. Conditions for modification and ATPase activity determination of purified ATPase were analogous (except that ionophore X-537A was omitted or replaced by A-23187 in the ATPase assays). The rate constants of inactivation were calculated from the linear portions of the curves shown in Fig. 1 A and B and Fig. 3 A.

| Conditions varied for DTNB modification | | Activity maximum | | | Inactivation rate |
|---|---|---|---|---|---|
| Compounds added | (Starting pH) | % of control activity | Time [min] | Number of SH mod.[a] | $k$ [$min^{-1}$] |
| 0.2 mM EGTA[b] | (7.8 or 8.4) | 129 | 0.5 | 1.5 | 0.42 |
| 2 mM $MgCl_2$ + 0.2 mM EGTA | (8.4) | 135 | 0.2 | 1.6 | 1.59 |
| 0.1 mM $CaCl_2$ | (8.4) | 158 | 0.2 | ≈ 1.4–2 | 0.75 |
| 0.1 mM $CaCl_2$ + 2 mM $MgCl_2$[c] | (7.8) | 164 | 0.7 | 1.5 | 0.42 |
| 5 mM ATP + 2 mM $MgCl_2$ + 0.2 mM EGTA | (8.4) | 178 | 2–4 | ≈ 2–3 | 0.08 |
| Glycerol (%, v/v) + 0.2 mM EGTA | (7.8) | | | | |
| 10% | | 153 | 1 | 1.4 | 0.16 |
| 20% | | 154 | 2 | 1.4 | 0.06 |
| 30% | | 147 | 3–4 | 1.4 | 0.03 |
| Myristoylglycerophosphocholine (mg/mg protein) + 0.1 mM $CaCl_2$ + 2 mM $MgCl_2$[c] | (7.5) | | | | |
| 1 mg/mg protein | | 118 | 1 | ≈ 2 | 0.21 |
| 2.5 mg/mg protein | | no maximum | | | 0.23 |
| 0.2 mM EGTA[c] (without ionophore in ATPase assays) | (7.8) | 159 | 0.5 | 1.8 | |
| 0.1 mM $CaCl_2$ + 2 mM $MgCl_2$[c] (with $10^{-5}$ M A-23187 in ATPase assays) | (7.8) | 133 | 0.75 | 1.8 | |
| Purified ATPase: 0.2 mM EGTA[c,d] | (7.8) | 135 | 1.5 | ≈ 3–4 | 0.08 |
| | | E ~ P level: | | | |
| 0.1 mM $CaCl_2$ + 2 mM $MgCl_2$[c] | (7.8) | 150 | 10 s | 0.5 | 0.23 |

[a] In terms of mol/$10^5$ g protein. The vesicular protein was assumed to contain about 70–80% ATPase. The total number of vesicular SH reacting with DTNB in the presence of sodium dodecylsulfate was about 12 ± 1.
[b] Mean of 8 experiments.
[c] 50 mM Tris · Cl (instead of 100 mM).
[d] Mean of 3 experiments (without ionophore or with $10^{-5}$ M A-23187 in the ATPase assays practically identical).

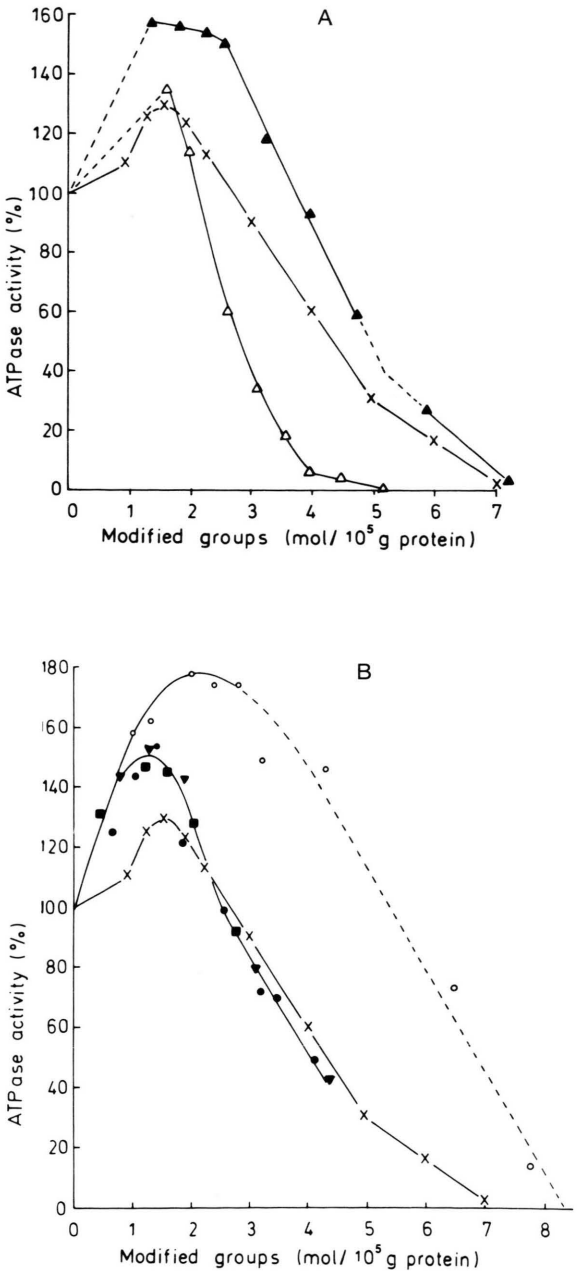


Fig. 2. $Ca^{2+}$-dependent ATPase activity as a function of the number of thiol groups modified by DTNB. The experiments of Fig. 1 were correlated with the number of blocked thiols by spectrophotometrical evaluation of the DTNB reactions, taking into account turbidity changes, as described in Materials and Methods. (A) (×) 0.2 mM EGTA, pH 7.8 or 8.4 (mean of 8 experiments); (△) 2 mM $MgCl_2$ + 0.2 mM EGTA, pH 8.4; (▲) 0.1 mM $CaCl_2$, pH 8.4. (B) (×) as in (A); (▼, ●, ■) glycerol (10, 20, 30%, v/v) + 0.2 mM EGTA, pH 7.8; (○) 5 mM ATP + 2 mM $MgCl_2$ + 0.2 mM EGTA, pH 8.4.

ditions. When correlating the $Ca^{2+}$-dependent ATPase activities with the number of thiols blocked at a given time after DTNB addition, taking into account turbidity changes as described in Materials and Methods, the position of the activity maximum was found to be approximately constant. It corresponded to about 1.5–2 mol thiol modified per $10^5$ g vesicle protein, irrespective of added compounds (with the exception of ATP; compare Fig. 2A and B, as well as Table I). When purified ATPase preparations were used the activation maximum was found at significantly higher extents of modification (3–4 thiols). The height of the maximum and the rate of inactivation with further increasing thiol modification differed considerably, however. The effects of the various added compounds on the activation have already been described above. As soon as about two thiols have been blocked, all curves show a linear portion of activity decrease with an increasing number of thiols modified. In this portion (comprising about 3 thiols) approximately 30–40% activity are lost per thiol blocked. This suggests that only one out of 3 to 4 thiol groups is essential for ATP splitting, which would be in line with some results with N-ethylmaleimide [5, 6]. When a total of about 4–6 thiols was modified a break of the curve was observed (Fig. 2A).

In the presence of ATP no break was found and activity approached zero, when as many as 8 mol thiol groups per $10^5$ g protein were modified (Fig. 2B). Yet, perhaps the most striking effect of ATP was the shift of the position of the activity maximum towards a higher modification degree. From the kinetic plots of Fig. 1A and B it would have appeared that 5 mM ATP + 2 mM $MgCl_2$ and 20% glycerol in the DTNB reaction mixture had comparable effects on ATPase activity. Now the correlation with the respective number of modified thiol groups reveals a significant difference: Compared to the DTNB assay without addition glycerol causes a higher activation, but leaves the activity maximum at an unchanged position. Different concentrations of glycerol in the DTNB modification assay (10–30%, v/v) led to identical curves, when plotting ATPase activity *vs.* number of modified groups (Fig. 2B). Apparently glycerol only reduces the rate of the DTNB reaction, the same set of thiol groups being blocked with or without glycerol. No specific effect on a single thiol group was detected. ATP

behaved differently. In the presence of $Mg^{2+}$ and ATP activation was still enhanced and concomitantly the maximum was shifted towards a higher degree of thiol blockage (2–3 mol thiol per $10^5$ g protein). Thus the protective effect of ATP described earlier [2, 4, 8–10] cannot be due merely to a reduction of the modification rate. Binding of ATP obviously protects just the groups the substitution of which causes inactivation in the absence of ATP. The slow inactivation still observed must then be due to the modification of a different set of thiol groups.

To make sure the described activation was not an artificial effect of the relatively unspecific ionophore X-537A in the ATPase assays we repeated several experiments with ionophore A-23187 instead or none at all, and also with purified ATPase. The results are included in Table I. In all cases a comparable activation was observed.

The described effects refer to the $Ca^{2+}$-dependent ATPase activities. It should be noted that the $Ca^{2+}$-independent (basal) activities (varying between 0.1 and 0.2 μmol Pi. $mg^{-1}$ protein · $min^{-1}$) were not affected by the DTNB reaction. This has also been shown by Murphy [7].

*Enzyme phosphorylation by ATP*

Steady state phosphorylation from ATP was measured directly in the DTNB assays in the presence of 0.1 mM $Ca^{2+}$ and 2 mM $Mg^{2+}$. The first assay (10 s of contact with excess DTNB) already produced an increase of the steady state phosphoenzyme level by 50%, followed by a linear decrease in the plot log % EP *vs.* time (Fig. 3A).

The $Ca^{2+}$-dependent ATPase activity, measured under the same conditions of DTNB modification, showed a similar time course as without $Ca^{2+}$ or $Mg^{2+}$ ions, except that the activity maximum was somewhat higher (compare Fig. 3A to Fig. 1A and Table I). During the first two minutes of DTNB contact ATP splitting was relatively more enhanced than phosphoenzyme formation, while after that period ATPase activity became more inhibited. In the range where inactivation occurred the phosphoenzyme level declined more slowly, with a rate constant of inactivation about half of that for the reduction of ATPase activity (Table I). This change in the relation between activity and phosphoprotein is difficult to explain and will be discussed later.

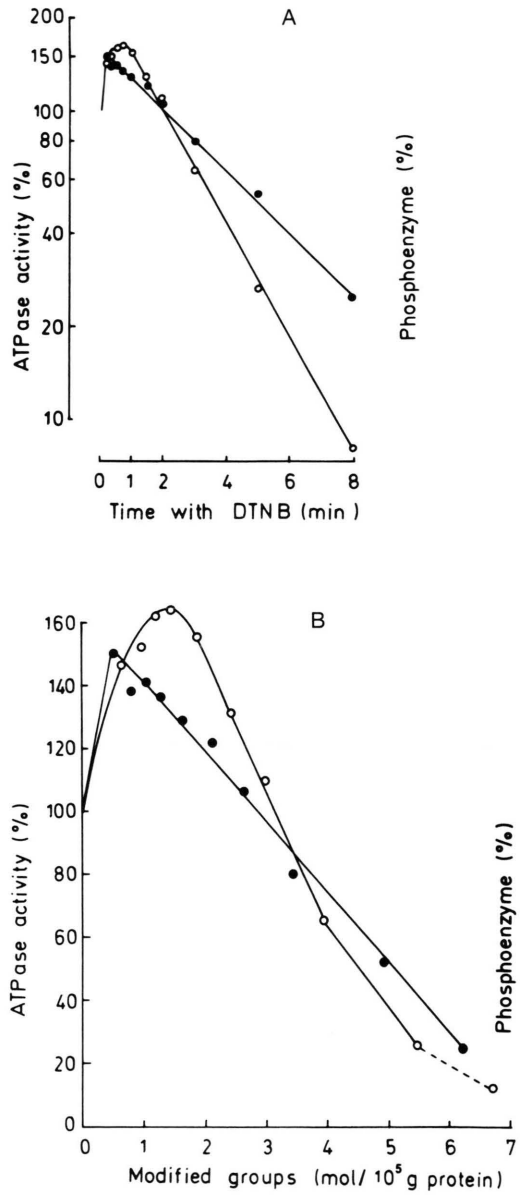


Fig. 3. $Ca^{2+}$-dependent ATPase activity and phosphoenzyme level (A) as a function of time with DTNB, (B) as a function of the number of thiol groups modified by DTNB. The medium for the DTNB reaction contained 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 3 mM DTNB, 0.4 mg protein/ml and 50 mM Tris · Cl pH 7.8. DTNB modification with spectrophotometrical evaluation, determination of ATPase activity and phosphorylation by [$\gamma$-$^{32}$P]ATP were performed as described in Materials and Methods. The values are means of 2 experiments of each kind and are expressed in % of the respective control values without DTNB. 100% correspond to 1.02 μmol Pi · $mg^{-1}$ protein · $min^{-1}$ for the $Ca^{2+}$-dependent ATPase activity and 1.9 nmol · $mg^{-1}$ protein for the phospho-enzyme level (22 °C). (○) $Ca^{2+}$-dependent ATPase activity, (●) phosphoenzyme level.

The correlation of both parameters with the number of modified thiol groups is shown in Fig. 3 B. As can be seen, maximum activation of phosphoenzyme formation occurred when approximately 1/2 mol thiol/$10^5$ g protein had reacted with DTNB.

*Calcium uptake*

In contrast to $Ca^{2+}$-dependent ATPase activity and phosphorylation $Ca^{2+}$ uptake was not activated by low DTNB modification extents. A linear decrease of the amount of $^{45}Ca^{2+}$ stored after 1 min was observed. The corresponding rate constant of inactivation was 0.11 $min^{-1}$. Although blocking of the most reactive thiol group had only a minor effect, further modification drastically reduced $Ca^{2+}$ uptake.

To exclude the possibility that DTNB might simply cause permeability changes in the membrane the following experiment was carried out: Sarcoplasmic vesicles, actively loaded with $^{45}Ca^{2+}$ in the presence of acetyl phosphate and inorganic phosphate, were treated with DTNB under standard conditions up to 8 min and the remaining $Ca^{2+}$ was determined by millipore filtration technique. The releases observed in the presence and in the absence of DTNB were slow and did not significantly differ. This is in line with efflux experiments recently described [34].

## Discussion

Several previous studies dealing with the thiol groups of the sarcoplasmic reticulum have demonstrated that different types of thiol reagents react preferentially with different thiol groups (for a discussion of these aspects see *e.g.* ref. [5]). While the blocking of 2 specific out of 4 thiols with N-ethylmaleimide has been shown to abolish $Ca^{2+}$ uptake, ATP splitting as well as phosphoenzyme formation [5, 6], reaction of 3–4 thiol groups with spinlabeled iodoacetamides did not inhibit activity [13–15]. It was therefore concluded that iodoacetamides in contrast to N-ethylmaleimide reacted primarily with non-essential thiol groups.

DTNB, on the other hand, was reported to impair the catalytic functions of the ATPase enzyme already during modifying the first groups. A linear decrease of log ATPase activity *vs.* time [7–9] or activity *vs.* number of modified groups [9] was demonstrated. An activity increase in the initial stage of DTNB modification was not observed by these authors. In some cases their first measurement might have lain beyong the range where we found the activation. Differences in the conditions of determining ATPase activities could be excluded as a reason, since we found the activation effect with sarcoplasmic vesicles with two different ionophores or none at all and also with purified ATPase.

Our finding of an ATPase activity maximum requires the assumption of two counteracting effects. Thus only at a later stage of progressive thiol modification inhibiting effects become predominant, while in the beginning an activating effect is prevailing. This would imply that in the native vesicles as well as in ATPase we must assume some kind of constraint or inhibition, that is more and more loosened or abolished as soon as a DTNB molecule starts to interact with the most reactive thiol group of an ATPase protein. Evidently this type of restraint, which prevents maximal enzymatic activity in the native structure, can also be abolished by myristoylglycerophosphocholine at sufficient concentration. The lysophospholipid per se interferes with protein protein as well as lipid protein interactions by breaking up the native structure. It cannot be decided whether the changes in protein protein or protein lipid contacts are responsible for the relief of the constraint.

Various observations have been interpreted in the sense that the operating unit of the $Ca^{2+}$ pump consists of at least two or more 100000 dalton polypeptides (a summary of evidences so far is given in ref. [18]; compare also [11, 35–37]). If enzymatic activity is constrained in the oligomer, its dissociation into two subunits could be expected to elevate the ATPase activity.

Møller *et al.* [11, 35] compared the thiol-DTNB reactivity of ATPase in the membranous, oligomeric and monomeric state, adjusted by dodecyl octaethylene glycol monoether treatment, and found characteristic differences in the time course of the reaction. Yet, the authors did not compare ATPase activities at different extents of thiol blockage.

The effects of various ions, especially $Ca^{2+}$ and $Mg^{2+}$, on the kinetics of the DTNB reaction with sarcoplasmic ATPase have been studied extensively (compare [7–9]). We did not find a significant influence of any of these ions on thiol reactivity, in

accord with Thorley-Lawson and Green [8] and Andersen and Møller [9], but in contrast to Murphy [7] who reported a drastic change of the whole distribution of kinetic classes in the presence of 0.1 mM $Ca^{2+}$. We did observe, however, effects of $Ca^{2+}$ and $Mg^{2+}$ on ATPase activity, as described in the Results section. Specific effects of $Ca^{2+}$ have been demonstrated in the reaction of sarcoplasmic vesicles with other thiol reagents and have been explained in terms of changes in enzyme conformation produced by $Ca^{2+}$ binding to the high affinity sites (*e.g.* [5, 16]; compare also [15] and [38] where specific effects of $Ca^{2+}$ in the presence of nucleotides are described).

Regarding the influence of ATP on the thiol reactivity of sarcoplasmic reticulum a comparison of the many different studies is rendered difficult by the variety of conditions that have been applied: ATP alone or together with $Mg^{2+}$ and/or $Ca^{2+}$ and all in different concentrations may be expected to have different effects on thiol reactivity. Yet, in all studies, employing DTNB or N-ethylmaleimide, a "protection" of thiol groups was observed [2, 4, 7–10]. The rate of the reaction was throughout reduced, in some cases the total number of reactive thiol groups was lower and inactivation was retarded. Most reports suggested that the protective effect of nucleotide was produced by a conformational change induced by nucleotide binding. Andersen and Møller [9], however, found no specific effect of ATP on the ATPase activity of DTNB modified preparations, demonstrated by identical curves for the DTNB inactivation of ATPase in the presence or absence of ATP (alone, without $Ca^{2+}$ or $Mg^{2+}$), when plotting activity *vs.* number of modified groups (see Fig. 6 of ref. [9]). Our results, in contrast, show a significant influence of ATP (in presence of $Mg^{2+}$ and EGTA) on the course of the ATPase activity (compare Fig. 2 B). The two curves with and without ATP/$Mg^{2+}$ are significantly different. Apart from the incipient activation that has already been discussed, the "ATP curve" is formally shifted towards the right; later, inactivation proceeds with a similar slope as without ATP. We have assumed that the essential thiols have been rendered less accessible by the binding of ATP and that the retarded reaction of sarcoplasmic reticulum vesicles with DTNB, observed in the beginning, is due to the modification of different and unspecific thiol groups.

The effect of glycerol on DTNB modification in correlation with ATPase activity was investigated for two reasons. It is known, that 20% glycerol is able to protect the activity of sarcoplasmic ATPase preparations during other procedures like detergent treatment [39, 40]. Furthermore, Esmann [41] recently reported full protection of the activity of a $(Na^+ + K^+)$-ATPase from N-ethylmaleimide poisoning by use of a high percentage of glycerol or sucrose. Our results with varying concentrations of glycerol in the DTNB modification assay, however, showed solely a reduction of the rate of reaction; a retardation, not a protection from inactivation at higher extents of thiol blocking. The curves ATPase activity *vs.* number of thiols modified (Fig. 2 B) were identical for different glycerol concentrations and very similar to the curve without glycerol. Somehow glycerol appears to make the normally fast reacting thiol groups less accessible. Possibly the retarding effect of glycerol on the ionic DTNB reaction could be due to the displacement of water by glycerol molecules involving a viscosity increase and leading to steric hindrance. Studies of the influence of organic solvents on sarcoplasmic reticulum [42, 43] have shown that their effects are complex and not fully consistently explained by factors like dielectric properties and hydrophobicity.

A direct comparison of ATPase activity and the steady state phosphoenzyme level at different extents of thiol modification by DTNB (Fig. 3 A and B) has led to results that are difficult to explain. Initially both phosphorylation and ATP splitting are concomitantly activated. This could mean that either the rate of phosphoenzyme formation becomes elevated and consequently more phosphoprotein can be decomposed or that the number of sites that can be phosphorylated is increased. To illustrate the complicated relationship between both processes during thiol modification we have replotted the data of the experiments shown in Fig. 3 A as a semilogarithmic plot of the ratio ATPase activity/phosphoenzyme level ($v$/[EP]) *vs.* time of DTNB contact (Fig. 4). This curve does not show the steep initial rise of both functions though. Values of $v$/[EP] > 1 are obtained during the first two minutes, where ATP splitting is more enhanced than phosphorylation. The maximum is found after approximately one minute, corresponding to 1.5 thiols per 100000 dalton modified. After two minutes ATP splitting becomes more inhibited with

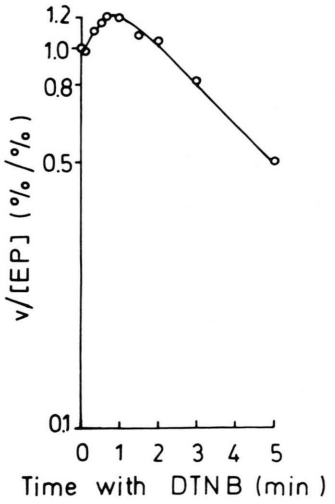


Fig. 4. Semilogarithmic plot of the ratio $Ca^{2+}$-dependent ATPase activity/phosphoenzyme level *vs.* time of DTNB treatment, taken from the experiments shown in Fig. 3. The conditions are given in the legend of Fig. 3.

further progressing thiol blockage, phosphoprotein can be accumulated. This is in line with the results of Yamada and Ikemoto [5] and Kawakita *et al.* [6] that phosphoprotein formation is less sensitive to thiol blocking than phosphoprotein decomposition, respectively $Ca^{2+}$ uptake.

The finding that the relation between enzyme activity and phosphoprotein level changes during the modification with DTNB must mean that all constants of the reaction sequence phosphorylation–phosphate liberation have to change as well. Thus in addition to the proposed increase of the number of enzymatically reactive sites their kinetic properties must have been altered, too. The fact that maximum activation of phosphoenzyme formation is observed when about 1/2 mol thiol per $10^5$ g protein is modified, suggests some kind of a conformational change induced by the modification of the fastest reacting group in the transporting protein unit. We assume that this conformational change subsequently leads to a dissociation of an oligomer into subunits. The concomitantly observed turbidity decrease could be interpreted as in accord with this assumption. This is probably an effect specific for DTNB.

Calcium uptake is not enhanced under conditions giving rise to an elevated ATPase activity and phosphoenzyme level. An increased permeability of the membrane for $Ca^{2+}$ ions cannot be the reason for the inhibition of $Ca^{2+}$ uptake, since a DTNB induced efflux was not observed. Yet, the described effects–conformational change, dissociation, accompanied by an alteration of the kinetic properties, already during the initial stage of thiol modification – can be expected to involve a perturbation of the native structure. And a fully intact structure of whole vesicles is a prerequisite for the functioning of $Ca^{2+}$ transport.

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