Biochimica et Biophysica Acta, 485 (1977) 188—202

BBA 68267

REACTION OF SARCOPLASMIC RETICULUM Ca^{2+}-ATPase IN DIFFERENT FUNCTIONAL STATES WITH 5,5′-DITHIOBIS(2-NITROBENZOATE)

JENS P. ANDERSEN and JESPER V. MØLLER *

Institute of Medical Biochemistry, Aarhus University, 8000 Aarhus C, (Denmark)

(Received April 1st, 1977)

Summary

1. All sulfhydryl groups of a purified Ca^{2+}-ATPase (ATP phosphohydrolase, EC 3.6.1.3) vesicle preparation of sarcoplasmic reticulum from rabbit skeletal muscle were reactive with 5,5′-dithiobis(2-nitrobenzoate) (DTNB). In the absence of substrates, four groups per ATPase molecule (115 000 daltons) reacted with a pseudo first-order constant of 0.18 min^{-1} (0.5 mM DTNB, pH 7.5, I = 0.11, and 20°C), while the remaining 15 groups were characterized by a rate constant of 0.016 min^{-1}. Reaction rates were markedly enhanced by a rise of pH and ionic strength.

2. At 0.1 mM ATP, ADP and Mg · ADP, two of the rapidly reacting groups disappeared, and at 1 mM nucleotide there was in addition a decrease of the modification rate of slowly reacting groups. ATPase activity decreased linearly with the number of modified groups, complete inactivation occurring after reaction of 12 groups. Protection by ATP against DTNB inactivation was accompanied by a reduction in modification rate.

3. Ca^{2+} and Mg^{2+} did not appear to affect modification rates except at high concentrations (0.5—1 mM), where the reaction was stimulated.

4. During phosphorylation (simultaneous presence of Ca^{2+}, Mg^{2+} and 0.1 mM ATP) the number of rapidly reacting groups increased from two to three or four, while the modification rate of slowly reacting groups was reduced.

5. Reaction of DTNB with the low molecular weight substrate, glutathion, was found to be 350—4000 times faster than with the sulfhydryl groups of ATPase. The reaction was similarly affected as that of ATPase by ionic strength and pH, but not by the substrates of ATPase.

6. It is suggested that the disappearance of two rapidly reacting sulfhydryl groups by adenine nucleotide is caused by a masking effect of bound nucleotide, while the decrease in modification rate of slowly reacting groups

* To whom correspondence should be addressed.
Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EGTA, ethylene glycolbis(-aminoethyl-ether)-*N*,*N*′-tetraacetic acid.

during phosphorylation and at high adenine nucleotide concentrations is attributed to conformational changes of the protein.

Introduction

Active accumulation of Ca^{2+} in vesicles prepared from sarcoplasmic reticulum of skeletal muscle is dependent on a Ca^{2+} and Mg^{2+} activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) [1—3]. In intact preparations of sarcoplasmic reticulum vesicles transport of 2 mol Ca^{2+} ordinarily requires hydrolysis of 1 mol ATP [2,3]. Two high-affinity binding sites for Ca^{2+} ($K_{Diss} \approx$ 0.3—0.5 μM) have been demonstrated [4—6]. These sites may be involved in translocation since Ca^{2+} transport, phosphorylation and ATP hydrolysis occur in the same concentration range (0.1—1 μM) at which the binding sites are occupied by Ca^{2+}. If so, translocation of Ca^{2+} must involve movement of the Ca^{2+}-binding sites from the sarcoplasmic facing side of the membrane to the aqueous phase in contact with the inner side of the membrane. In contrast, both phosphorylation and dephosphorylation appear to occur on the sarcoplasmic side of the membrane [7,8], and the phosphorylation site is thus presumably localized in a different region on the protein than are the high affinity Ca^{2+}-binding sites. The simultaneous requirement of Ca^{2+} and Mg · ATP for both Ca^{2+} transport and phosphorylation of the ATPase suggests a concerted effect of these two compounds to produce a conformation of phosphorylated protein which is different from that found in the absence of substrates. Such a change in the state of the protein could be the basis for active transport of Ca^{2+}. In agreement with this view Ikemoto [5] has demonstrated decreased affinity of phosphorylated protein for Ca^{2+}, which accounts for the different activities of Ca^{2+} on the two sides of the membrane caused by the active transport process.

In agreement with a central role of the phosphorylated intermediate in the transport process Francois [9] detected slower exchange of labile hydrogen in Ca^{2+}-transporting vesicles of sarcoplasmic reticulum as compared to preparations examined under non-transporting conditions. Evidence of conformational changes accompanying phosphorylation of the protein has also been obtained by electron spin resonance (ESR) [10,11] and fluorescence [12] changes in suitable spectroscopic probes covalently attached to sulfhydryl groups of the ATPase. Landgraf and Inesi [13] from earlier ESR measurements reported an effect of ATP per se at high nucleotide concentrations (1—5 mM). This finding was confirmed by Pang et al. [14], but these authors attributed the effect to residual phosphorylation in their preparations. More recently small effects of Ca^{2+} alone on the ESR probe spectrum [15], intrinsic fluorescence [16], and sulfhydryl reactivity [17] of ATPase have been reported. That a major reorganization of the membrane protein in relation to Ca^{2+} transport probably does not take place is suggested by the failure to observe changes in the circular dichroism spectrum of the protein during transport [18].

In conclusion, the above-mentioned studies support the view that detectable conformational changes probably occur in relation to Ca^{2+} transport. In the present study we have explored the possibility of using the rate of modification

rate of the sulfhydryl groups of sarcoplasmic reticulum Ca^{2+}-ATPase for detecting conformational changes during the functional cycle of the transport protein. In order to assess the usefulness of DTNB for this purpose, the paper includes a study of various factors affecting modification rates of the ATPase and a low molecular weight sulfhydryl compound (reduced glutathione). The presence of a considerable number of reactive sulfhydryl groups in sarcoplasmic reticulum vesicles was first reported by Hasselbach and Seraydarian [19]. In recent papers Murphy [17] and Thorley-Lawson and Green [20] have also published observations on the reaction of sarcoplasmic reticulum Ca^{2+} ATPase under different conditions. The results obtained by the different groups of investigators are considered in the Discussion section.

Materials and Methods

Materials. DTNB, ATP (disodium salt), ADP (monosodium salt), phospho*enol*-pyruvate (monopotassium salt), and pyruvate kinase (from rabbit muscle, 400—500 units/mg) were purchased from Sigma Chemical Co., Miss. Glutathione in reduced form was obtained from Boehringer, Mannheim, and [γ-^{32}P]ATP (500—3000 mCi/mmol) from The Radiochemical Centre, Amersham.

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle according to the method of Meissner et al. [21]. Proteins other than Ca^{2+}-ATPase were removed from the 26.5—30% sucrose fraction of the second zonal centrifugation by extraction with deoxycholate and KCl according to method 2 of Meissner et al. [21]. The resulting vesicular preparation showed a major band of ATPase by dodecyl sulfate polyacrylamide electrophoresis, while the other bands found in sarcoplasmic reticulum were faint and estimated not to constitute more than 5% of the ATPase. The Ca^{2+}-dependent phosphorylation capacity as measured at 0° C by the procedure described below was 7—9 nmol/mg protein. The phospholipid content, analyzed according to the procedure of Bartlett [22], was 0.45 mg/mg protein, and the vesicles were leaky to Ca^{2+}. Electron microscopic observation of preparations negatively stained with uranylacetate showed that most of the ATPase vesicles had a spherical shape with rather uniform size (400—800 Å diameter).

Reaction of Ca^{2+}-ATPase with DTNB. The reaction of sarcoplasmic reticulum and purified ATPase vesicles with DTNB was followed by measuring the increase in light absorbance at 412 nm [23]. The incubation media usually contained 0.5 mM DTNB, 0.01 M imidazole (pH 7.5), 0.1 M KCl, 0.1 mM EGTA or EDTA, and other additions as described in the legends of the figures. To 2.5 ml of incubation medium (20° C) in an optical cuvette was added 0.01—0.03 ml of ATPase or sarcoplasmic reticulum vesicles to a final concentration of about 0.06 mg protein/ml. The sample was placed in a cuvette holder of a Zeiss PMQ II spectrophotometer at 20° C. The light absorbance of the sample was read 5—10 s after mixing, and then at appropriate intervals, usually over a period of 1 h. DTNB was present in a 50-fold molar excess as compared with the concentration of reactive sulfhydryl groups. Control experiments established that the increase in light absorbance at 412 nm was proportional to the vesicle concentration. The light-scattering properties of added vesicles did not measur-

ably affect the increase in absorption at 412 nm resulting from the formation of nitrothiobenzoate.

Reaction of glutathion with DTNB. The modification rate of reduced glutathion by DTNB was studied with an Aminco-Morrow stopped flow apparatus. A solution containing DTNB (1 mM) was mixed at 20° C with a solution of freshly prepared, reduced glutathione (25 mM), having the same electrolyte composition as the DTNB solution. Light absorption was registered by a Beckman DU spectrophotometer, and displayed on a Tektronix Dual Beam oscilloscope.

Phosphorylation. Incorporation of ^{32}P into protein was measured after incubation of ATPase vesicles (0.02 mg protein) in 1 ml of 0.01 M imidazole (pH 7.5), 0.1 M KCl, 0.1 mM Ca^{2+}, 1 mM Mg^{2+}, and 0.1 mM ATP, containing [γ-^{32}P]ATP. Phosphorylation was stopped after 6 s by addition of 25 ml ice-cold 4% trichloroacetic acid containing 2.5 mM ATP and 5 mM phosphate, filtered through a Millipore filter (pore size 0.45 μm), and washed with 35 ml 5% trichloroacetic acid. Control samples were treated in exactly the same way, except that 0.1 mM Ca^{2+} in the phosphorylation medium was substituted for 1 mM EGTA. The radioactive content after solubilization of the filters in 10 ml Bray mixture [24] was measured by liquid scintillation counting. Ca^{2+} dependent phosphorylation was calculated after subtraction of counts in the control filters from those of the phosphorylated samples.

Other methods. ATPase activity of DTNB treated ATPase vesicles was assayed by measurement of the formation of inorganic phosphate [25] at 20° C in a medium containing 0.01 M imidazole (pH 7.5), 0.1 M KCl, 5 mM Mg · ATP, and 0.1 mM Ca^{2+}. Protein concentration was determined by the method of Lowry et al. [26] as previously described [27]. Dodecyl sulfate polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn [28]. The level of contaminating Ca^{2+} in the solutions used for modification of ATPase was estimated to be around 5 μM by a Ca^{2+} sensitive electrode (Ca-selectrode, Radiometer, Copenhagen), calibrated with Ca^{2+} standard solutions as described by Ruzicka et al. [29].

Calculations. The number of sulfhydryl groups per ATPase modified by DTNB was calculated from the concentration of reacted DTNB (absorbance coefficient 13 600 $M^{-1} \cdot cm^{-1}$ [23]), and the molar concentration of protein (molecular weight 115 000 [30]). The number of modified groups as a function of time was analyzed in terms of modification constants and classes of reactive sulfhydryl groups. Since DTNB is present in large excess the unmodified fraction of a particular sulfhydryl group at time t may be represented by a pseudounimolecular reaction scheme, provided that the process is unaffected by the modification of other reactive groups. Under specified experimental conditions (DTNB concentration, pH, temperature, etc.) we obtain

$$N_\infty - N_t = \sum_{i=1}^{j} n_i e^{-k_i t}$$

where N_t and N_∞ are the numbers of sulfhydryl groups modified at time t and infinite time respectively, j is the number of classes of sulfhydryl groups with similar modification constants, and n_i and k_i are the number and an average value for the pseudounimolecular modification constant of the ith group.

Results

Reaction of Ca^{2+}-ATPase with DTNB in absence of substrates

Modification of ATPase vesicles carried out in presence of 0.5 mM DTNB over a period of 3 h indicated a total number of reactive groups of approx. 16 mol per 10^5 g protein, which corresponds to about 19 reactive sulfhydryl groups per ATPase molecule (115 000 daltons). The same number of reactive sulfhydryl groups was obtained after solubilization of ATPase vesicles in 3% dodecyl sulfate as can be seen from Fig. 1. In the case of sarcoplasmic reticulum vesicles a total of 11 mol reacted per 10^5 g protein, while there were around 13 mol of reactive sulfhydryl groups after solubilization of the vesicles in dodecyl sulfate. Thus, in contrast to sarcoplasmic reticulum, all sulfhydryl groups of the purified ATPase preparation appear to be reactive. The lower degree of modification of sulfhydryl groups of sarcoplasmic reticulum solubilized by dodecyl sulfate may be accounted for by the presence of other proteins like calsequestrin and the high affinity Ca^{2+} binding protein which according to amino acid analysis has a lower content of cysteinyl residues than ATPase [21,31,32].

Fig. 1 shows the reaction rate of ATPase vesicles with DTNB at different concentrations of the modifying reagent at pH 7.5 and I 0.11. The curves at the highest DTNB concentrations are characterized by a much steeper rise during the first 5—10 min than in the following part of the reaction. A similar set of curves was obtained in presence of sarcoplasmic reticulum vesicles. With a total number of 19 reactive sites per 115 000 daltons, analysis of the results

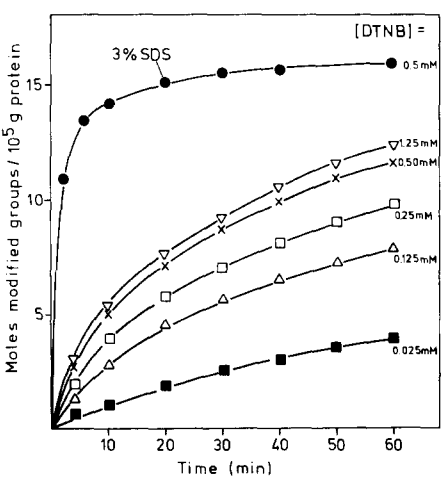

Fig. 1. Effect of DTNB concentration on modification of Ca^{2+}-ATPase. The rate of reaction was followed by the absorption increase at 412 nm as described under Methods, after addition of ATPase vesicles (0.06 mg protein/ml) to a medium consisting of 0.01 M imidazole (pH 7.50), 0.1 M KCl, 0.1 mM EDTA, and various concentration of DTNB: ■, 0.025 mM; △, 0.125 mM; □, 0.25 mM; ×, 0.5 mM; ▽, 1.25 mM. The filled circles (●) show the reaction in 0.5 mM DTNB and 3% dodecyl sulfate (SDS). In this case the medium contained 0.1 M NaCl instead of 0.1 M KCl. The crosses denote the mean value of 15 experiments, and the curve corresponding to these results was drawn according to Eq. 1: $(N_\infty - N_t) = 4 \times e^{-0.18t} + 15 \times e^{-0.016t}$, i.e. $n_1 = 4$, $k_1 = 0.18$ min^{-1}; $n_2 = 15$, $k_2 = 0.016$ min^{-1}. The other points in the figure denote the averages obtained in 5 experiments.

on ATPase vesicles according to Eqn. 1 indicates that in the higher range of DTNB concentration the reaction is satisfactorily described in terms of two classes, the most rapidly reacting class consisting of four sulfhydryl groups. At 0.5 mM DTNB the average modification constants of the two classes are 0.18 min^{-1} and 0.016 min^{-1}. It should be noted that the modification constants are not proportional to the DTNB concentration, and therefore the reaction does not follow a bimolecular reaction scheme. On the contrary, Fig. 1 demonstrates that the reaction rate approaches zero-order dependence on DTNB at a concentration of the reagent of 1.25 mM. The reaction of DTNB with the low molecular weight substrate glutathion was, on the other hand, found to be strictly bimolecular. In the same electrolyte medium and at a DTNB concentration of 0.5 mM the modification rate constant was 62 min^{-1} at 20° C. The reactivity of sulfhydryl groups of ATPase is thus around 350—4000 times slower than that of glutathione. These observations and the relatively high reactivity in dodecyl sulfate (Fig. 1) indicate that the membrane structure imparts a severe restriction on the modification rate of sulfhydryl groups. A possible reason for the saturation of the reaction at high DTNB concentrations is that the reaction proceeds via modification agent bound to the membrane rather than by a collision type of reaction.

Fig. 2 shows the effect of pH on the DTNB reaction with Ca^{2+}-ATPase. A sigmoidal relation is observed between the number of groups modified after a reaction period of 10 and 30 min as a function of pH. Since DTNB reacts with the deprotonated form of sulfhydryl groups [33,34] this result is consistent

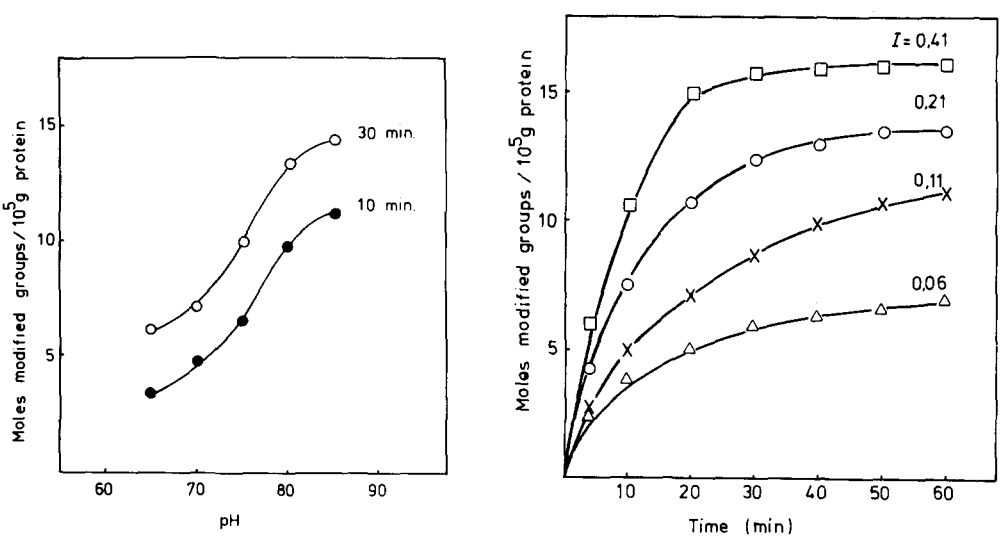

Fig. 2. Modification of Ca^{2+}-ATPase by DTNB at different pH. Modification rates of ATPase vesicles (0.06 mg protein/ml) were measured in 0.5 mM DTNB, 0.1 M KCl, 0.1 mM EDTA and 0.01 M imidazole, adjusted to various pH values. The number of groups per ATPase molecule modified after 10 (•) and 30 min (○) are shown in the figure. Each point denotes the mean of 5 experiments.

Fig. 3. Modification of Ca^{2+}-ATPase as a function of ionic strength. Modification rates were measured in 0.5 mM DTNB, 0.01 M imidazole (pH 7.50), 0.1 mM EDTA and various concentrations of KCl: △, 0.05 M KCl; X, 0.1 M KCl; ○, 0.2 M KCl; □, 0.4 M KCl. Each point denotes the mean of 5 experiments.

with the view that the reaction takes place with $-S^-$ groups of ATPase having apparent pK values around 7.5—8, although an effect of pH on the structure of ATPase is not excluded. Fig. 3 shows that the modification rate is also very sensitive to changes in ionic strength. At I = 0.41 the reaction runs to completion in around 50 min, while only approx. 1/3 of the sulfhydryl groups have reacted with DTNB at I = 0.06 during the same time period. Analysis according to Eqn. 1 indicates an approx. 10-fold decrease in modification rates by lowering the ionic strength from 0.41 to 0.06. By comparison, the modification constant of gluthathion was reduced 3-fold by the same reduction of ionic strength. The decrease in reactivity at low ionic strength may in part by attributed to a change of the apparent pK of the sulfhydryl groups which in the case of gluthathion by spectrophotometric titration [35] was found to be 8.95 at I = 0.41 and 9.1 at I = 0.06. Another factor which probably contributes to enhanced reactivity of ATPase is the attenuation of electrostatic repulsion at high ionic strengths between negatively charged DTNB and ATPase, as is generally observed in the interaction of charged ligands and proteins [36] in accordance with the Debye-Hückel theory for electrolytes [37].

Effect of adenine nucleotides on sulfhydryl reactivity

Fig. 4 shows that ATP or ADP added at concentrations of 0.1 or 1 mM in presence of 0.1 mM EDTA inhibits the modification of sulfhydryl groups by DTNB, while a very modest effect is observed at nucleotide concentrations of 10 μM. Analysis of the rate curves indicates that at 0.1 mM the number of rapidly reacting groups is reduced from four to two with no apparent change in the rate constants of the two classes of sites. At 1 mM there remain two rapidly reacting sulfhydryl groups with unaltered reactivity, while the modification constants of the slowly reacting groups are reduced to less than half of those observed in absence of nucleotide. The same inhibitory effect of ATP was also observed in modification experiments on sarcoplasmic reticulum vesicles and in presence of 10 mM EDTA to ensure that phosphorylation was efficiently prevented from taking place. Measurement of phosphate liberation in the reaction media containing ATPase vesicles showed that less than 5% of added ATP was hydrolyzed during 1 h. A similar set of curves as those of Fig. 4 was obtained in presence of 0.5 mM free Mg^{2+} and various concentrations of ADP, except that reactivities both in the absence and presence of nucleotide were somewhat higher, due to the presence of Mg^{2+} (see below). Thus the effect of 0.1—1 mM nucleotide on sulfhydryl reactivity seems to be independent of the γ-phosphate group and of complexation of the nucleotide with Mg^{2+}. Finally it may be mentioned that ATP had no effect on the reaction of DTNB with glutathione.

Nucleotide protection of Ca^{2+}-ATPase against inactivation by DTNB

Fig. 5 shows that 0.1 and 1 mM ATP protects against inactivation of ATP hydrolysis and phosphorylation by DTNB. But when ATPase activity is plotted as a function of the number of modified sulfhydryl groups in presence and absence of ATP no difference in the inactivation process is noted (Fig. 6). Thus the protective effect of ATP may be ascribed solely to the slowing of the modification reaction in presence of nucleotide, and there is no evidence for

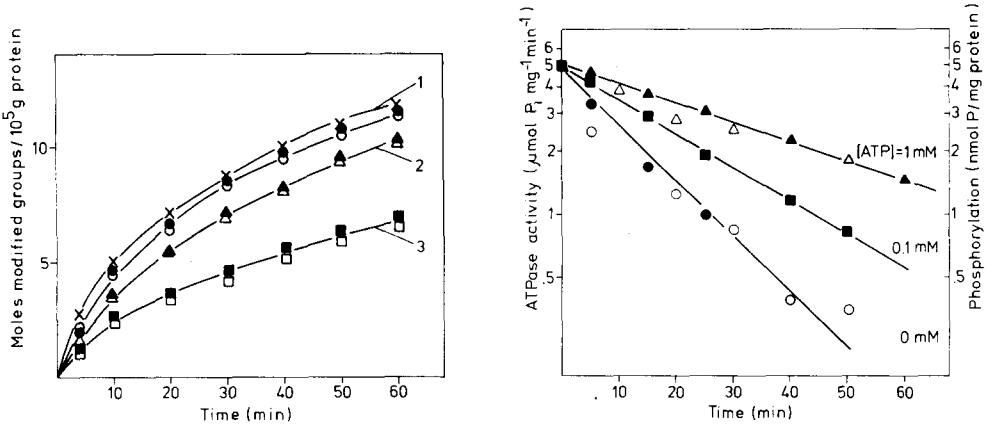

Fig. 4. Modification of Ca^{2+}-ATPase by DTNB in presence of ATP or ADP. Modification rates of ATPase (0.06 mg protein/ml) were measured in 0.5 mM DTNB, 0.01 M imidazole (pH 7.50), 0.1 M KCl, 0.1 mM EDTA and various concentrations of ATP (open symbols) or ADP (filed symbols): ■,□, 1 mM nucleotide; ▲,△, 0.1 mM nucleotide; ●,○, 10 μM nucleotide; X, without added nucleotide. The curves have been drawn according to the following values for number and modification rate constants of sulfhydryl groups in the two classes: curve 1: $n_1 = 4, k_1 = 0.18\ min^{-1}; n_2 = 15, k_2 = 0.016\ min^{-1}$; curve 2: $n_1 = 2, k_1 = 0.18\ min^{-1}$; $n_2 = 17, k_2 = 0.015\ min^{-1}$; curve 3: $n_1 = 2, k_1 = 0.18\ min^{-1}; n_2 = 17, k_2 = 0.007\ min^{-1}$; Each point denotes the mean of 5 experiments, except the crosses which represent the average of 15 experiments.

Fig. 5. ATPase activity and phosphorylation during modification of Ca^{2+}-ATPase with DTNB in presence and absence of ATP. ATPase vesicles (0.06 mg/ml) were modified in the same media as used for the experiments of Fig. 4. At different time intervals 0.20 ml was transferred to 1.80 ml containing 5 mM MgATP, 0.1 mM Ca^{2+}, 0.1 M KCl, 0.01 M imidazole (pH 7.5) at 20° C. ATP hydrolysis was stopped after 2 min by addition of 1 ml 1N perchloric acid. Phosphorylation was determined in separate experiments as described under Methods. Filled symbols indicate ATPase activity, and open symbols phosphorylation of ATPase: ●,○, 0 mM ATP; ■, 0.1 mM ATP; ▲,△, 1 mM ATP. Each point is the average of 4—5 experiments.

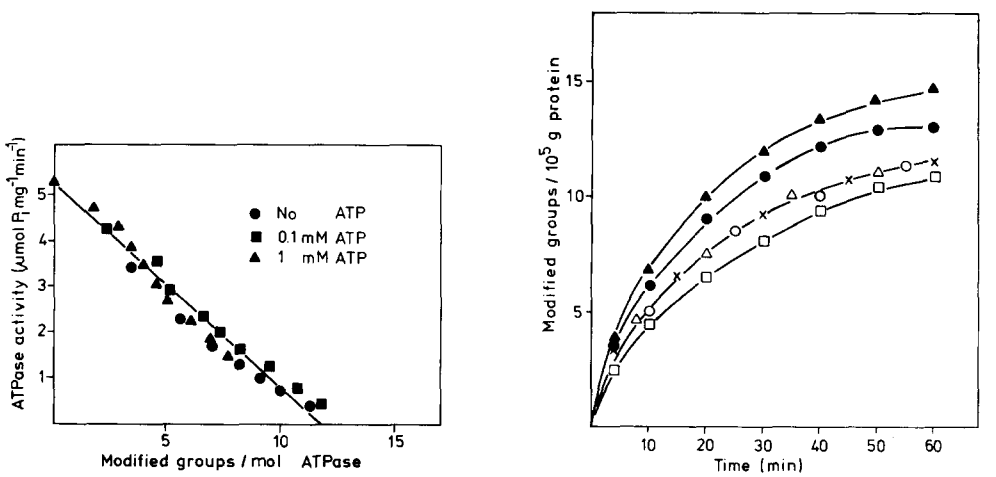

Fig. 6. ATPase activity as a function of number of groups of Ca^{2+}-ATPase modified by DTNB. Modification rates of ATPase were measured in the same media as used for the experiments in Fig. 5, and ATPase activity was determined as described in the legend of Fig. 5. Each point is the average of 4—5 experiments.

Fig. 7. Effect of Ca^{2+}, Mg^{2+}, and Ca · EGTA on modification of Ca^{2+}-ATPase by DTNB. Modification rates were measured in 0.5 mM DTNB, 0.01 M imidazole (pH 7.50), 0.1 M KCl with the following additions: X, 0.1 or 1 mM EGTA; ○, 0.1 mM Ca^{2+}; △, 0.1 mM Mg^{2+}; ●, 1 mM Ca^{2+}; ▲, 1 mM Mg^{2+}; □, 0.1 mM Ca^{2+} and 0.1 mM Ca · EGTA. Each point is the average of 5 experiments.

protection of ATPase activity by masking of one particular rapidly-reacting sulfhydryl group. This point is also apparent from the fact that the reduction of activity as a function of the number of modified cysteinyl residues is gradual, complete inactivation not being approached until after modification of 12 sulfhydryl groups. The non-specific nature of inactivation of ATPase suggested by these findings is also supported by dodecyl sulfate gel electrophoresis which showed that most of the protein was not capable of entering a 7.5% polyacrylamide gel after modification of around 10 sulfhydryl groups. This observation is presumably indicative of extensive cross-linking of ATPase molecules by the bifunctional modification reagent.

Effect of Ca^{2+} and Mg^{2+} on sulfhydryl reactivity of Ca^{2+}-ATPase

In preliminary experiments in which Ca^{2+} activity was controlled by EGTA a distinct inhibitory effect on DTNB modification was noted with addition of Ca^{2+}. However, it was subsequently found that the effect was dependent on the concentration of EGTA in addition to the ratio of Ca^{2+} and EGTA (which in a buffered system determines the concentration of free Ca^{2+}). This observation suggests an inhibitory effect of the Ca · EGTA complex on DTNB modification of sulfhydryl groups. A direct demonstration of such an effect is provided by Fig. 7, from which it can be seen that: (1) the presence of 0.1 or 1 mM EGTA does not affect the DTNB reaction in comparison with medium without added Ca^{2+}; (2) addition of 0.1 mM Ca^{2+} (in absence of EGTA) does not alter the modification rate, either; and (3) the initial rate of modification is reduced in presence of 0.1 mM Ca^{2+} plus 0.1 mM Ca · EGTA. Assuming an apparent stability constant of the Ca · EGTA complex of 10^{-6} M and a level of contaminating Ca^{2+} of 5 μM (Methods) the concentration of free Ca^{2+} in presence of 1—0.1 mM EGTA is of the order $0.05—0.5 \cdot 10^{-7}$ M, which is below the level at which there is appreciable binding of Ca^{2+} to ATPase (K_{diss} for binding of Ca^{2+} to the high affinity binding sites of ATPase being around 0.3—0.5 μM [4—6]). The lack of effect of EGTA as shown in Fig. 7 therefore suggests that binding of Ca^{2+} to ATPase does not noticeably influence sulfhydryl reactivity.

Fig. 7 also shows that Ca^{2+} at a concentration of 1 mM stimulates the modification reaction and that an even more pronounced rate enhancement is obtained in presence of 1 mM Mg^{2+}. None of the divalent cations at this concentration measurably affected the reaction of glutathion with DTNB.

Effect of phosphorylation on sulfhydryl reactivity of ATPase

The medium used for examination of ATPase in the phosphorylated state contained 0.5 mM DTNB, 0.1 mM Mg · ATP, 0.1 mM Ca^{2+}, 0.5 mM free Mg^{2+}, and an ATP generating system (5 mM phospho*enol*pyruvate and 0.05 mg pyruvate kinase per mg ATPase). In this medium the initial phosphorylation was 4—5 nmol/mg ATPase. Sulfhydryl reactivity was compared with that of an appropriate, non-phosphorylated control. In the series of experiments shown in Fig. 8 the control contained 0.1 mM Mg · ADP instead of 0.1 mM Mg · ATP and pyruvate kinase. The presence of Mg · ADP in the control medium corrects for the nucleotide induced reduction of sulfhydryl reactivity. It is seen from the figure that initially the rate of modification is larger in the phosphorylated sample, but after 12 min the two curves cross each other and the modification

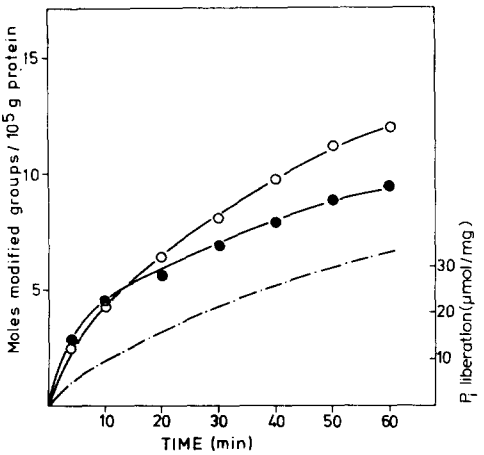

Fig. 8. Modification of Ca^{2+}-ATPase by DTNB in the phosphorylated (•) and non-phosphorylated (○) state. Composition of phosphorylation medium: 0.1 mM ATP, 5 mM phospho*enol*pyruvate, 0.05 mg pyruvate kinase/ml, 0.1 mM Ca^{2+}, 1 mM Mg^{2+}, 0.01 M imidazole (pH 7.50), and 0.1 M KCl. Composition of non-phosphorylation medium: The same as phosphorylation medium, except that 0.1 mM ADP replaces ATP and that pyruvate kinase is omitted. The curves have been drawn according to the following values for the number and modification rate constants of sulfhydryl groups in the two classes: curve 1: $n_1 = 3$, $k_1 = 0.29$ min^{-1}; $n_2 = 16$, $k_2 = 0.011$ min^{-1}; curve 2: $n_1 = 2$, $k_1 = 0.29$ min^{-1}; $n_2 = 17$, $k_2 = 0.019$ min^{-1}. The broken line shows phosphate liberation measured in samples drawn from the phosphorylation reaction mixture at various times. Each point is the mean of 5 experiments.

rate becomes slower in the phosphorylation medium. The broken line showing liberation of inorganic phosphate during the course of the experiment indicates that ATPase activity is present in the phosphorylation medium although at a reduced rate at the end of the experiment. The higher reaction rate observed initially is apparently accounted for by the appearance of a rapidly reacting sulfhydryl group which may be identical to one of those masked by Mg · ADP in the control sample. On the other hand the modification constants of slowly reacting sulfhydryl groups are reduced from 0.019 min^{-1} in the control to 0.011 min^{-1} in the phosphorylated sample, resulting in the lesser degree of modification in the later stages of the reaction.

A similar result was obtained in another series of experiments in which the control medium contained 1 mM EGTA and no Ca^{2+}, and both the control medium and the phosphorylation medium contained ATP, Mg^{2+} and pyruvate kinase. In this case we observed four rapidly reacting sulfhydryl groups in the phosphorylation medium, and the modification rate of the slowly reacting sulfhydryl groups was reduced from 0.019 min^{-1} to 0.011 min^{-1} during phosphorylation.

Discussion

Sulfhydryl reactivity of ATPase in absence of substrates

In agreement with other studies [17,19,20,38] a total number of 11 mol were found to be reactive per 10^5 g protein in sarcoplasmic reticulum vesicles, and this number was increased to 13 mol after solubilization of the vesicles in dodecyl sulfate. Using a purified ATPase vesicle preparation we found 16 mol to be reactive per 10^5 g protein, both in the absence and presence of dodecyl

sulfate, which corresponds to 19 groups per molecule ATPase (M_r = 115 000). Thus all free sulfhydryl groups in our preparation appear to be reactive. Amino acid analyses of the ATPase [21,39] have indicated the presence of about 26 half-cystine residues per molecule protein, of which six appear to be disulfide linked according to the recent results of Thorley-Lawson and Green [20]. By contrast, Murphy [17] reports that all half-cystine residues are reactive after solubilization of sarcoplasmic reticulum membranes in detergent.

The kinetics of sulfhydryl modification indicated the presence of two classes of reactive sulfhydryl groups, in agreement with the original results of Hasselbach and Seraydarian [19]. Under our standard conditions (20° C, pH 7.5, I = 0.11, DTNB 0.5 mM) the two classes were characterized by average modification constants of 0.17 and 0.015 min^{-1}, respectively. Under these conditions reaction rates were 350—4000 times slower than the disulfide interchange reaction of DTNB with reduced glutathion. Other evidence of constraints in the reaction of DTNB with the sulfhydryl groups of ATPase is the finding that the reaction rate apparently approached an upper limit at high DTNB concentrations (Fig. 1).

In the recent studies of Murphy [17] and Thorley-Lawson and Green [20] DTNB modification of sarcoplasmic reticulum vesicles was examined in a way similar to that reported here. Murphy analyzed his results in terms of three classes of reactive sites, consisting of 2, 8 and 7 sulfhydryl groups per molecule ATPase, respectively. The reactivities of the two fastest reacting cysteinyl residues were approx. 40 times higher than those observed here. This suggests a definite difference in the reactivity of some of the sulfhydryl groups of ATPase in the two investigations, although part of the difference could probably be accounted for by the use of a higher temperature (25° C), higher pH (7.8), and higher DTNB concentration (4 mM) in Murphy's study. On the other hand Thorley-Lawson and Green [20] found only two classes of reactive sites which for a preparation of purified ATPase was characterized by a ratio of rapidly reacting to slowly reacting sites (approx. 1 : 4) similar to that found in our study. The rate constants obtained by Thorley-Lawson and Green are comparable to those reported by us when taking into account that Thorley-Lawson and Green performed their measurements at pH 8.4. However, in contrast to our findings they report the presence of approx. four unreactive sulfhydryl groups in their preparations of purified ATPase vesicles.

Sulfhydryl reactivity in presence of adenine nucleotides

Our observations indicated a significant decrease in the disulfide exchange of DTNB and ATPase at high concentrations of adenine nucleotides as previously demonstrated in the reaction of sarcoplasmic reticulum with *N*-ethylmaleimide [19,40]. ATP does not interfere with the reaction of DTNB with gluathion, but in the case of ATPase it could do so by being bound at site(s) adjacent to a reactive sulfhydryl group or by inducing a conformational change in the protein. The same effect of ATP, ADP and Mg · ADP was observed over a range of nucleotide concentrations of 0.1—1 mM. Two rapidly reacting groups were converted to slowly reacting groups at a nucleotide concentration of 0.1 mM. It is apparent from the study of Meissner [4] that ATP and ADP are bound with a much lower affinity than Mg · ATP (K_{diss} = 4 μM at pH 7.3) to the

phosphorylation site. It is therefore possible that the effect observed at 0.1 mM nucleotide is due to protection of one or two sulfhydryl groups which may be located near the phosphorylation site. It is of interest in this connection that a cysteinyl residue has been located two amino acid residues away from the aspartyl group which is phosphorylated by Mg · ATP [41]. The appearance of one more rapidly reacting sulfhydryl group when the protein is in the phosphorylated state (Fig. 8) is consonant with a protective effect of ATP on sulfhydryl reactivity.

Increasing the adenine nucleotide level from 0.1 to 1 mM was accompanied by a decrease in the modification rate of slowly reacting sulfhydryl groups. A similar retarding effect of high concentrations of ATP on the reaction of the enzyme with *N*-ethylmaleimide was observed previously (see Table II of ref. 38 and Fig. 1 of ref. 40). According to Martonosi [42] high concentrations of ATP 1.6 mM) inhibit the incorporation of ^{14}C-labelled *N*-ethylmaleimide into all fragments of ATPase obtained by peptic digestion, suggesting a generalized reduction in the reactivity of most of the sulfhydryl groups. These results are consistent with an effect of high concentrations of ATP on the conformation of the transport protein, resulting in a decreased availability of sulfhydryl groups for reaction with modifying agents. There is also evidence for conformational changes in ATPase at high nucleotide levels, mainly by the ESR technique [13,40]. The functional significance of such a conformational change requires further investigation. The possibility exists that it is related to the stimulatory effect of Mg · ATP on phosphate liberation which occurs at nucleotide concentrations higher than those required to saturate the high affinity phosphorylation site [44—46].

Effect of sulfhydryl reagents on ATPase activity

A pronounced protective effect of ATP on ATPase activity in presence of *N*-ethylamide has previously been demonstrated, and this effect was attributed to protection of a rapidly reacting sulfhydryl group [19,38,40]. In our study, modification with DTNB resulted in a much less pronounced effect on ATPase activity than has been reported for *N*-ethylamide. Thus, inactivation was not complete until after modification of 12 sulfhydryl groups. Under these conditions dodecyl sulfate gel electrophoresis indicated extensive cross-linking between ATPase molecules. We conclude that in our study none of the rapidly reacting sulfhydryl groups proved to be essential to ATPase activity and that, in fact, the inactivation process may well be of a non-specific nature, caused by structural alterations of the whole enzyme. The presence of ATP protected against inactivation, but in accordance with Yoshida and Tonomura [40] we found the same activity decrease when the results were plotted as a function of the number of modified residues in presence and absence of ATP (Fig. 6). In other words, ATP does not appear to protect preferentially a particular sulfhydryl group, important for catalytic activity, against modification. The decline in activity was accompanied by a parallel decline in the phosphorylation of the ATPase, indicating an effect of DTNB on ATP binding and/or phosphoryltransfer to the enzyme.

Effect of Ca^{2+} on sulfhydryl reactivity

The stimulation of DTNB modification of ATPase at high concentrations of

Ca^{2+} (1 mM) presumably must be considered non-specific, since Mg^{2+} exerted a similar effect on sulfhydryl reactivity (Fig. 7). These results are similar to those reported by Thorley-Lawson and Green [20]. By contrast, Murphy [17] reported an enhancement of the reaction rate of slowly reacting sulfhydryl groups at concentrations of free Ca^{2+} close to the concentration range where Ca^{2+} is actively transported by the protein in presence of Mg · ATP. There is also evidence of Ca^{2+} induced conformational changes by ESR [15] and fluorescence spectroscopy [16]. A priori it seems reasonable to assume that interaction of Ca^{2+} with the high affinity sites on ATPase induces a conformational change, since phosphorylation of the ATPase by ATP requires Ca^{2+}, while the enzyme may be partially phosphorylated by inorganic phosphate in the absence of Ca^{2+} and presence of Mg^{2+} [46,47]. However, it seems unlikely that such a conformational change could be the basis for Ca^{2+} movement across the membrane, since there is no evidence for carrier-mediated transport of Ca^{2+} in the absence of ATP.

Effect of phosphorylation on sulfhydryl reactivity

The results obtained on DTNB modification of ATPase in presence of Ca^{2+}, Mg^{2+} and ATP (Fig. 8) showed that during phosphorylation there was (1) a generalized decrease in the modification rates of slowly reacting sulfhydryl groups, and (2) an increase in the number of rapidly reacting sulfhydryl groups from 2 to 3 or 4 per molecule ATPase, resulting in a net increase in the modification rate during the first minutes of reaction. The decrease in the reaction rate of the slowly reacting groups may be assumed to involve a conformational change of the protein in the phosphorylated state, while the increased number of rapidly reacting groups could be related to a release of the inhibitory effect of Mg · ATP as a result of removal of the nucleotide from its high affinity binding site during phosphorylation [4]. The decrease in reaction rate of the slowly reacting sulfhydryl groups of ATPase resembles the results obtained by Francois [9], who observed less hydrogen exchange in Ca^{2+} transporting vesicles. The data of Tonomura and Morales [48] on ascorbate quenching of nitroxide labelled sulfhydryl groups also suggest a less exposed state of the protein during phosphorylation. Ikemoto [12] has reported a decreased fluorescence of *S*-mercuridansylcysteine attached to sulfhydryl groups of ATPase. More information on the structure of the ATPase in the membrane is needed before observations such as these can be incorporated into a model for the Ca^{2+} translocation process.

Conformational changes as a result of modification of sulfhydryl groups

Extensive modification of enzymes, in particular of slowly reacting sulfhydryl groups, often leads to structural changes in the protein [33]. The modification process by itself may thus be expected to lead to conformational changes of ATPase, at least after modification of several sulfhydryl groups. This possibility could limit the use of the DTNB reaction as an indication of conformational changes in the native state. However, there is evidence to suggest that this is not an important factor in the interpretation of the decreased modification rate of the slowly reacting groups of ATPase during phosphorylation in terms of a conformational change. (1) By reference to Fig. 8 it is seen

that appreciable enzymatic activity is maintained during the modification period, indicating that at least part of the protein is in a native-like state at the end of the experiment. (2) The modification rate of slowly reacting sulfhydryl groups of any inactivated and non-phosphorylatable protein might be expected to be altered towards that present in the control sample. But as can be seen from Fig. 8 the modification curves are still diverging at the end of the experiment suggesting that some features of the protein in the phosphorylated state are maintained despite inactivation by DTNB. (3) A difference in modification rate during the modification process must reflect a difference in the original state before addition of DTNB, regardless of any subsequent event that might potentiate such a difference. This situation would arise if, e.g., modification of some sulfhydryl groups resulted in a conformational change which increased the reaction of the remaining unmodified sulhydryl groups.

The usefulness of sulfhydryl modification rate as an indicator of conformational change

As stated in the Introduction, the impetus for the present paper was exploration of the applicability of the reaction of the sulfhydryl groups of ATPase with DTNB as an indicator of conformational changes of ATPase. Evidence has been presented suggesting conformational changes of the protein in the phosphorylated state and in presence of a high concentration of Mg · ATP by this method. It is evident from the results obtained that modification rates are very susceptible to environmental variables, both of an unspecific (pH and ionic strength) and of a more specific nature. Modification rates may be influenced both as a result of conformational changes and binding of ligands, and an unambiguous discrimination between these two possibilities may not be possible without corroborating evidence from other experimental approaches. On the other hand, structural changes caused by the modification process do not appear to constitute an important obstacle to the use of the method for detection of conformational changes in the intact protein.

Acknowledgment

This investigation has been supported by the Danish Foundation for the Advancement of Medical Science and by the Danish Medical Research Council, to whom we express thanks.

References

1 Hasselbach, W. and Makinose, M. (1961) Biochem. Z. 333, 518—528
2 Hasselbach, W. and Makinose, M. (1963) Biochem. Z. 339, 94—111
3 Weber, A., Herz, R. and Reiss, I. (1966) Biochem. Z. 345, 329—366
4 Meissner, G. (1973) Biochim. Biophys. Acta 298, 906—926
5 Ikemoto, N. (1975) J. Biol. Chem. 250, 7219—7224
6 Fiehn, W. and Migala, A. (1971) Eur. J. Biochem. 20, 245—248
7 Yamada, S., Yamamoto, T. and Tonomura, Y. (1976) J. Biochem. (Tokyo) 67, 789—794
8 Meis, L. and Carvalho, M.G.C. (1970) J. Biol. Chem. 251, 1413—1417
9 Francois, C. (1969) Biochim. Biophys. Acta 173, 86—93
10 Nakamura, H., Hori, H. and Mitsui, T. (1972) J. Biochem. (Tokyo) 72, 635—646
11 Coan, C.R. and Inesi, G. (1976) Biochem. Biophys. Res. Comm. 71, 1283—1288
12 Ikemoto, N. (1976) Biophys. J. 16, 82a

13 Landgraf, W.C. and Inesi, G. (1969) Arch. Biochem. Biophys. 130, 111—118
14 Pang, D.C., Briggs, F.N. and Rogowski, R.S. (1974) Arch. Biochem. Biophys. 164, 332—340
15 Champeil, P., Bastide, F., Taupin, C. and Gary-Bobo, C.M. (1976) FEBS Lett. 63, 270—272
16 Dupont, Y. (1976) Biochem. Biophys. Res. Comm. 71, 544—550
17 Murphy, A.J. (1976) Biochemistry 15, 4492—4496
18 Mommaerts, W.F.H.M. (1967) Proc. Natl. Acad. Sci. U.S. 58, 2476—2482
19 Hasselbach, W. and Seraydarian, K. (1966) Bicohem. Z. 345, 159—172
20 Thorley-Lawson, D.A. and Green, N.M. (1977) Biochem. J., in the press
21 Meissner, G., Conner, G.E. and Fleischer, S. (1973) Biochim. Biophys. Acta 298, 246—269
22 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
23 Ellman, G. (1959) Arch. Biochem. Biophys. 82, 70—77
24 Bray, G.A. (1960) Anal. Biochem. 1, 279—285
25 Fiske, C.H. and Subba Row, Y. (1925) J. Biol. Chem. 66, 375—400
26 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
27 Le Maire, M., Moller, J.V. and Tanford, C. (1976) Biochemistry 15, 2336—2342
28 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
29 Ruzicka, J., Hansen, E.H. and Tjell, J.C. (1973) Anal. Chim. Acta 67, 155—178
30 Le Maire, M., Jorgensen, K.E., Roigaard-Petersen, H., Moller, J.V. (1976) Biochemistry 15, 5805—5812
31 MacLennan, D.H. and Wong, P.T.S. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1231—1235
32 MacLennan, D.H., Yip, C.C., Iles, G.H. and Seeman, P. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 469—477
33 Torchinskii, Y.M. (1974) in Sulfhydryl and Disulfide Groups of Proteins, Ch. 4, Consultants Bureau, New York
34 Legler, G. (1975) Biochim. Biophys. Acta 405, 136—143
35 Benesch, R.E. and Benesch, R. (1955) J. Am. Chem. Soc. 77, 5877—5881
36 Kragh-Hansen, U. and Moller, J.V. (1974) Biochim. Biophys. Acta 336, 30—36
37 Linderstrom-Lang, K. (1924) C. R. Trav. Lab. Carlsberg 15, No. 7
38 Panet, R. and Selinger, Z. (1970) Eur. J. Biochem. 14, 440—444
39 Thorley-Lawson, D.A. and Green, N.M. (1975) Eur. J. Biochem. 59, 193—200
40 Yoshida, H. and Tonomura, Y. (1976) J. Biochem. (Tokyo) 79, 649—654
41 Allen, G. and Green, N.M. (1976) FEBS Lett. 63, 188—192
42 Martonosi, A. (1976) FEBS Lett. 67, 153—155
43 Inesi, G., Goodman, J.J. and Watanabe, S. (1967) J. Biol. Chem. 242, 4637—4643
44 Yamamoto, T. and Tonomura, Y. (1967) J. Biochem. (Tokyo) 62, 558—575
45 Vianna, A.L. (1975) Biochim. Biophys. Acta 410, 389—406
46 Kanazawa, T. and Boyer, P.D. (1973) J. Biol. Chem. 248, 3163—3172
47 Knowles, A.F. and Racker, E. (1975) J. Biol. Chem. 250, 1949—1951
48 Tonomura, Y. and Morales, M.F. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3687—3691