The Inhibition of the Calcium Transport ATPase of the Sarcoplasmic Reticulum by Fluorescamine: Evidence for an Oligomeric Functional Unit of the Calcium Transport System

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Sarcoplasmic Calcium Transport, Fluorescamine Labelling

The labelling of the protein moiety of the sarcoplasmic calcium transport ATPase by fluorescamine suppresses calcium transport, calcium dependent ATPase activity, protein phosphorylation by [γ-^{32}P]ATP and [^{32}P]phosphate at different extent of amino group substitution. For the hydrolysis of para nitrophenylphosphate by the calcium transport ATPase it is shown that the relationship between the extent of amino group labelling can considerably be altered by the temperature and the presence of ethyleneglycol. It is shown that the amino residues of the phosphatidylethanolamine moiety do not contribute to the inhibiting effect of fluorescamine labelling. The observations suggest that the different functions of the calcium transport system are based on the cooperation of a varying number of calcium transport ATPase molecules.

Introduction

Fluorescamine which reacts specifically with free amino residues has recently been used for the localization of the protein and lipid components in sarcoplasmic reticulum membranes [1–4]. Under controlled conditions allowing only a low degree of labelling, it could be shown that 60–70% of the surface of the transport ATPase which is not embedded in the lipid bilayer is exposed to the cytoplasm [1, 2]. Furthermore, a highly asymmetric distribution in the lipid matrix was found for the phosphatidylethanolamine component; 80% of which is present in the external membrane leaflet [2, 3]. This distribution fully agrees with the results obtained by other methods [5, 6]. In the course of our studies, it was observed that the structure as well as various functions of the reticulum were affected by fluorescamine labelling. As observed for other reagents substituting various functional groups of the protein, inhibition of the calcium transport system by fluorescamine was correlated with the degree of labelling. The publication of these observations was prompted by a recent study of Hidalgo [7] who indicated that the loss of the ability of sarcoplasmic reticulum vesicles to store calcium, might be related to the substitution by fluorescamine of the amino group of phosphatidylethanolamine and that this loss was not due to an increased membrane permeability of the vesicles. The results summarized in this report show that different functions of the calcium transport system vary differently with the extent of substitution of amino groups in the transport protein. Furthermore, it is shown that the relationship between the degree of amino group labelling and the alteration of one and the same function can considerably be affected by the assay conditions. The observations can most plausibly be explained by the assumption that the different functions of the calcium transport system depend differently on the interaction of a limited number of molecules of the transport enzyme.

Abbreviations: EGTA, ethyleneglycol bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid; SDS, sodium dodecylsulfate; DCCD, dicyclohexylcarbodiamide.

Enzymes: Ca^{2+}-dependent ATPase, ATP phosphohydrolase (EC 3.6.1.3).

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Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from white rabbit skeletal muscle according to the procedure described by Hasselbach and Makinose [8] as modified by de Meis and Hasselbach [9]. The preparations were labelled with fluorescamine as described by Hasselbach *et al.* [1]. 10 mg vesicular protein was suspended in 5 ml 0.1 M KCl, 0.05 M sodium borate buffer pH 8.5. Fluorescamine dissolved in acetone was added, in a volume never exceeding 50 µl, to the solution with vigourous stirring. Aliquots of the solution were immediately transferred to the assay media. The number of

labelled amino residues in the protein was determined as described [1]. The protein was precipitated and the lipid was extracted with ether ethanol (1:1, v/v). The protein precipitate was washed with 70% acetone and subsequently dissolved in 0.1% SDS, 0.05 M sodium borate. The fluorescence intensity was measured after appropriate dilution at wave lengths of 390 nm for excitation and 480 nm for emission. The fluorescence intensity was calibrated by the fluorescence of a vesicular preparation which had been reacted quantitatively with fluorescamine [1].

The preparations were phosphorylated with [γ-^{32}P] ATP in solutions containing 0.1 mM [γ-^{32}P] ATP, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 40 mM KCl, 20 mM histidine pH 7.0, and 0.4 mg protein · ml^{-1} at 20 °C. The phosphorylation was terminated by adding an equal volume of 10% TCA-50 mM phosphoric acid. The protein was pelletted by centrifugation and repeatedly washed on a glass wool filter.

The calcium dependent ATPase activity was measured in a solution containing 5 mM ATP, 5 mM $MgCl_2$, 40 mM KCl, 0.1 mM $CaCl_2$, 0.1 mg protein · ml^{-1}, 50 µmol Lasalocid as described by Ronzani *et al.* [10]. The calcium independent splitting was determined in the presence of 2 mM EGTA and in the absence of calcium ions.

Calcium transport was measured at 20 °C as described by Makinose and Hasselbach [11], in solutions containing 0.5 mM $^{45}CaCl_2$, 0.5 mM EGTA, 5 mM potassium oxalate, 5 mM $MgCl_2$, 5 mM ATP, 20 mM histidine pH 7.0. Calcium loaded vesicles were removed by filtration after appropriate reaction times by filtration through Sartorius filters 4.5 µM and the ^{45}Ca activity remaining in the filtrate was determined.

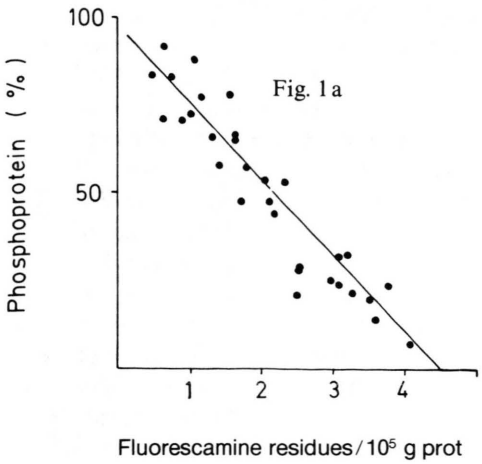
Phosphorylation by inorganic phosphate was performed as described by Beil *et al.* [12], in solutions containing 10 mM [^{32}P]phosphate, 10 mM $MgCl_2$, 20 mM histidine pH 6.0, 10 mM EGTA, 0.4 mg protein · ml^{-1} at 30 °C. The phosphorylated protein was precipitated with an equal volume of 6% TCA–10 mM phosphoric acid. The precipitated protein was collected on a glass wool filter and repeatedly washed.

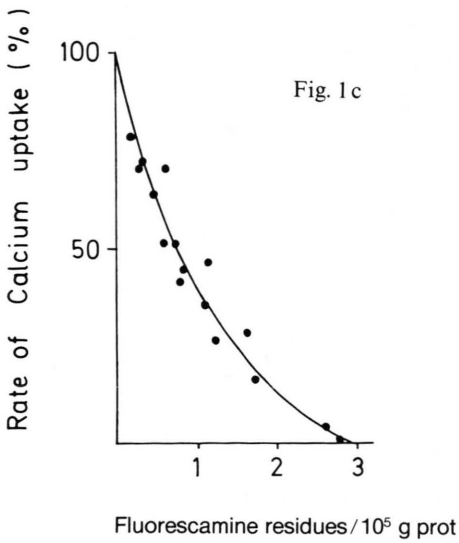
Nitrophenylphosphatase activity was measured in solutions containing 10 mM $MgCl_2$, 0.1 M NaCl, 20 mM histidine pH 7.0, 0.2 mM $CaCl_2$, 20 µM Lasalocid, 3 mM paranitrophenyl phosphate and 0.2 mg protein · ml^{-1}. Where indicated, 30% ethyleneglycol was present. The reaction was quenched by addition of 0.5 ml of 5% SDS–1 M Na_2HPO_4 to 4 ml reaction solution. The liberated nitrophenol was measured spectrophotometrically at 400 nm. The calcium independent activity was determined in the presence of 2 mM EGTA. The dinitrophenyl phosphatase activity of sarcoplasmic reticulum vesicles was measured spectrophotometrically at 420 nm. The composition of the reaction medium is given in the legend of Fig. 4.

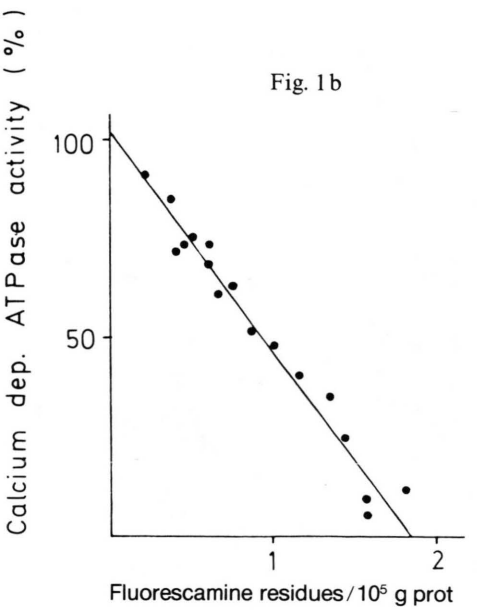
ATP was obtained from Boehringer, Mannheim (FRG), [^{32}P]phosphate was bought from Amersham Buchler, Braunschweig (FRG), [γ-^{32}P] ATP was prepared as described by Glynn and Chapell [13]. The calcium ionophore Lasalocid (X 573 A) was a generous gift of Hoffmann-La Roche Ltd., Basel (Switzerland). Fluorescamine was purchased from Hoffmann-La Roche, Weil am Rhein (FRG). Disodium paranitrophenyl phosphate was obtained from Serva, Heidelberg (FRG). Lutidine 2–4 dinitrophenyl phosphate was synthesized by Dr. H. Husseini according to F. Ramirez and J. F. Maracek [14]. All chemicals were P. A. grade.

Results

The results of Fig. 1 illustrate the relation between the extent of fluorescamine labelling of the membrane protein in native vesicular preparations and the resulting decline in different membrane activities: calcium transport, calcium dependent ATPase activity, phosphoprotein formation from ATP and inorganic phosphate. Figs. 1 a and 1 b show that the calcium dependent phosphorylation of the transport protein by ATP as well as the calcium dependent ATPase activity appear to be linearly correlated with fluorescamine substitution. While the abolition of phosphoprotein formation requires the blockage of four amino residues per 100 000 g protein, the calcium dependent ATPase activity is already completely inhibited when only two residues are substituted by fluorescamine. In contrast to phosphoprotein formation from ATP and calcium dependent ATP hydrolysis, calcium transport and the incorporation of inorganic phosphate in the absenc of calcium ions exhibit inhibition profiles which are nonlinear (Figs. 1 c, 1 d). The initially steeply declining profile flattens as the extent of labelling increases. Half inhibition of calcium transport and phosphoprotein formation from inorganic phosphate







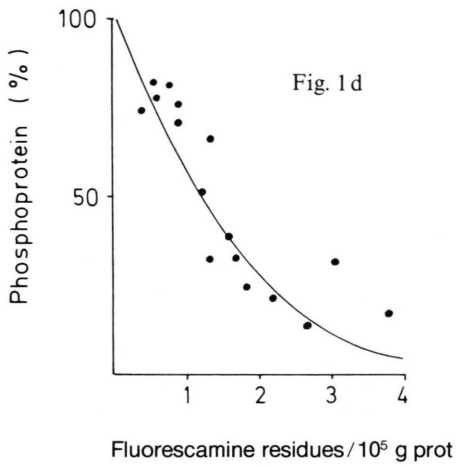
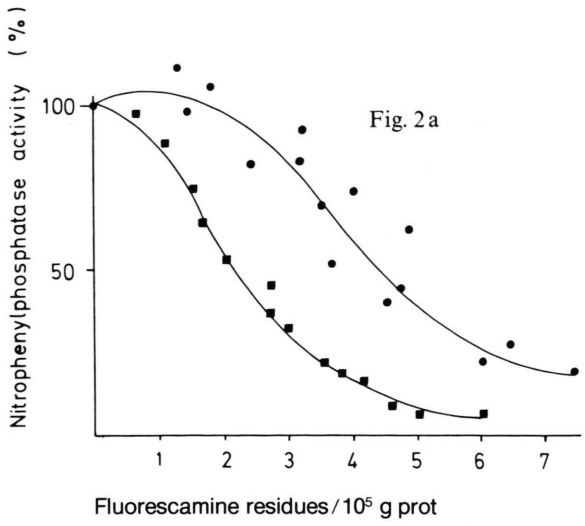


Fig. 1. The abolition of different functions of the sarcoplasmic calcium transport produced by amino group substitution with fluorescamine. a) Phosphorylation of the transport protein by [γ-^{32}P] ATP. The steady state level, reached after a reaction time of 30 sec, is plotted on the ordinate. 100% = 2.3 – 4.0 nmol · mg^{-1}. b) Calcium dependent ATPase activity measured at 20 °C is plotted on the ordinate. 100% = 0.5 – 0.7 µmol · mg^{-1} · min^{-1}. c) Rate of calcium transport at 20 °C. 100% = 0.9 – 1.4 µmol · mg^{-1} · min^{-1}. d) Phosphoprotein formation of the transport protein by phosphorylation with inorganic phosphate. 100% = 1.9 – 2.4 µmol · mg^{-1}. Abscissa: number of amino residues substituted in the membrane protein per 100 000 g protein. Conditions of substitution and the determination of the different functions are described in Materials and Methods.

is reached when 0.7 and 1.2 amino residues per 100 000 g protein, respectively, are substituted.

These observations indicate that different functions of the calcium transport ATPase exhibit differing sensitivities to amino group blockage. Such variation is not only seen with different functions and as shown in Fig. 2, similar differences were found for one and the same function. In these experiments native vesicles were labelled at room temperature under standard conditions. The nitrophenylphosphatase activity was measured in the absence and the presence of ethyleneglycol as indicated in the legends. As shown in Fig. 2 a, the number of amino residues which must be blocked to



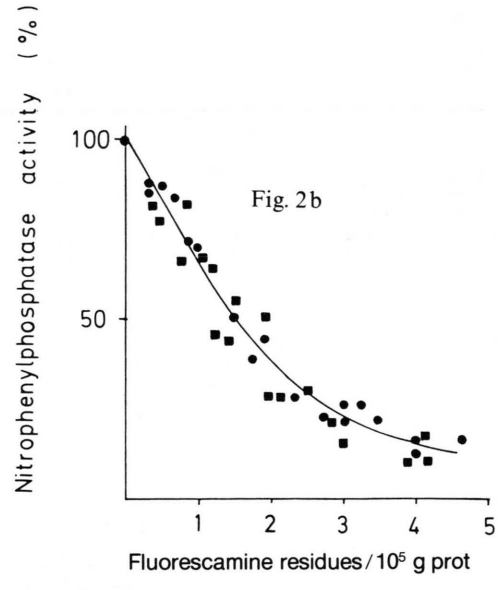


Fig. 2. The abolition of the nitrophenylphosphatase activity of the sarcoplasmic reticulum membranes by amino group substitution with fluorescamine. a) The experiments were performed at 23 °C in the described incubation medium ●; 30% ethyleneglycol were added to the incubation medium ■; 100% activity without ethyleneglycol = 10 nmol · mg^{-1} · min^{-1}; 100% activity with ethyleneglycol = 45 nmol · mg^{-1} · min^{-1}. b) The experiments were performed at 37 °C in normal incubation media ●; the incubation media contained 30% ethyleneglycol ■; 100% activity without ethylene glycol = 35 nmol · mg^{-1} · min^{-1}; 100% activity with ethyleneglycol = 90 nmol · mg^{-1} · min^{-1}.

reduce phosphate liberation by 50%, amounts to 1.5 at room temperature and is approximately 4 at 37 °C. It is further illustrated that at 37 °C, in the presence of 30% ethyleneglycol, the blockage of only two residues reduces the enzymatic activity by 50%. At room temperature, this sensitizing effect of ethyleneglycol seems to be absent (Fig. 2 b). The observed similar sensitivity to fluorescamine labelling of both calcium uptake and calcium dependent ATP splitting supports the concept of a close coupling of the processes and largely excludes the possibility that the reduction of calcium uptake is caused by formation of membrane leaks under the prevailing conditions. Such leaks in fact, can occur, but their formation requires a higher extent of labelling (Fig. 3).

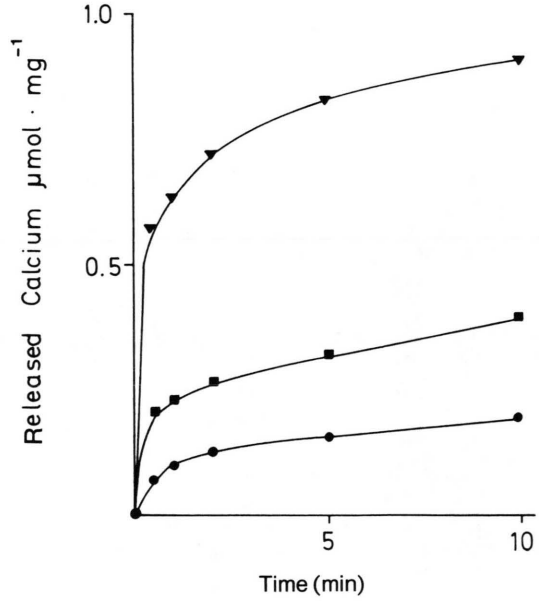


Fig. 3. Calcium release from calcium loaded vesicles at different degrees of amino group substitution. The sarcoplasmic reticulum vesicles were loaded with 0.9 µmol calcium · mg^{-1} in a medium containing 1 mM $CaCl_2$, 10 mM phosphate, 5 mM acetyl phosphate, 3 mM $MgCl_2$, 40 mM KCl, 1 mg protein/ml, pH 7.0, 30 °C. The vesicles were subsequently labelled with fluorescamine in 0.1 M KCl, 0.05 M sodium borate, pH 8.5 and immediately transferred into the release medium containing 40 mM KCl, 20 mM histidine, 5 mM $MgCl_2$, and 2 mM EGTA at 20 °C. Ordinate: µmol calcium · (mg protein)$^{-1}$. Abscissa: time in min. ● unlabelled preparations treated with 0.1 M KCl, 0.05 sodium borate and 50 µl acetone; ■ preparations labelled with 5 fluorescamine residues per 100 000 g protein. 1.2 residues are attached to the protein and 3.8 residues have reacted with the amino groups in the lipid. ▼ preparation labelled with 12 amino residues per 100 000 g. The protein is labelled with 4.3 residues, while the remaining fluorescamine reacted with the amino lipids.

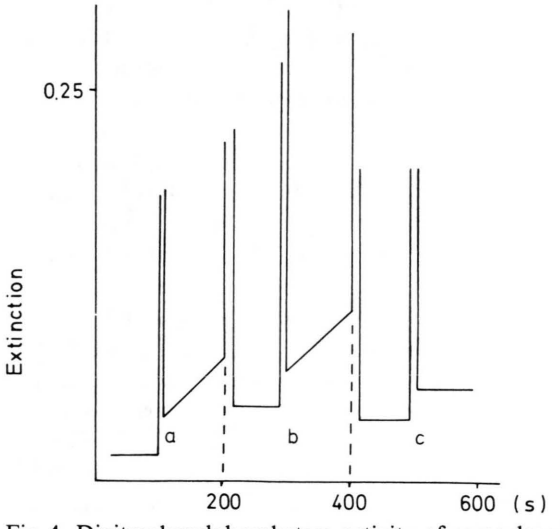


Fig. 4. Dinitrophenylphosphatase activity of sarcoplasmic reticulum preparations determined before and after introducing fluorescamine labelled lipids into the lipid phase of native vesicles. 24 mg of sarcoplasmic reticulum vesicles were suspended in 10 ml 0.1 M KCl pH 8.5. An aliquot of 5 ml was labelled with 20 fluorescamine residues per 10^5 g protein. Subsequently 30 mg sodium cholate was added to both samples which were preserved on ice. The dinitrophenylphosphatase activity was measured spectrophotometrically at 420 nm in 0.1 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 20 mM histidine pH 7.0 at 20 °C and 0.2 mM dinitrophenylphosphate. a) Activity trace of a labelled preparation of 0.1 mg protein · ml^{-1}. b) Activity trace of a mixture of 0.1 mg protein·ml^{-1} unlabelled and 0.1 mg·ml^{-1} labelled sarcoplasmic reticulum vesicular protein. The sample was sonicated briefly after mixing. c) The labelled preparation of 0.1 mg protein·ml^{-1} exhibits no dinitrophenylphosphatase activity. The reactions were started by the addition of substrate where the traces are interrupted at a, b and c.

The possibility that labelling of phosphatidylethanolamine, occurring preferentially to protein labelling, is responsible for the inhibition, was also considered. In three experiments the native membranes were extensively labelled with fluorescamine resulting in a complete inhibition of all functions. The preparations were subsequently solubilized by adding deoxycholate and were mixed with the same quantity of unlabelled deoxycholate solubilized vesicles. It can be assumed that in the solubilized mixture, a complete exchange between labelled and unlabelled lipids takes place [15]. If fluorescamine labelled phosphatidylethanolamine causes the inhibition, one would expect that after deoxycholate has been highly diluted, the ATPase and dinitrophenylphosphatase activities of the mixture will be lower than that of an unlabelled preparation. This, however, is not the case as shown in Fig. 4. The same result was observed when the calcium dependent ATPase was measured after the native lipids of the preparation were exchanged against isolated lipids extensively labelled with fluorescamine. It is very unlikely, therefore, that the substitution of amino residues of the phosphatidylethanolamine moiety is specifically involved in calcium dependent ATP splitting and accompanying calcium transport.

Discussion

The most striking result of this study concerns the finding that the substitution of superficial amino groups of the sarcoplasmic reticulum vesicles by fluorescamine inhibits calcium transport and calcium dependent ATP splitting more effectively than phosphorylation of the protein by ATP or P_i. The amino groups can be considered to residue almost exclusively in the transport protein. Although accessorial proteins present in the membrane are also labelled, they represent about 20% of the membrane protein content. An attempt to correlate enzyme function with the extent of protein labelling is justified only if the substitution of amino lipids is shown not to interfere with the measured activities. At least 50% of the phosphatidylethanolamine moiety can be exchanged by its fluorescamine substituted derivative without affecting the activity of the calcium dependent ATPase. This exchange corresponds to a labelling of 6 residues per 100 000 g protein. At this degree of labelling of native vesicles all functions are extinguished. We, therefore, can assume that labelling of the amino residues of the lipids does not affect the function of the calcium pump. A linear relationship between the degree of substitution of various functionally important residues in the membrane protein and enzyme activity has been observed for various substituting reagents. For instance, in the calcium transport protein, substitution of the thiol residues causes a linear decline in calcium dependent ATPase activity. To inactivate the enzyme completely, the substitution of four residues is sufficient in native vesicles [16, 17], while all thiol residues present in the molecule must be occupied in purified enzyme preparations [18]. Evidently the solubilization procedure increases the number of available thiol groups to a maximum. In the case of amino group substitution by fluorescamine solubilization results only in a twofold increase in the

number of residues which must be blocked for complete inactivation of the ATPase. This means that only 5% of the totally available residues, which can be blocked if the reagents are used in excess, suffice to block the enzyme activities completely [1, 3].

As pointed out by Asai and Morales [19], a linear relationship between the decline in enzyme activity and the extent of substitution means that only one specific residue of several is essential for the enzymatic reaction. Since our preparations were substituted under the same conditions, we must assume that the same total number of amino residues were equally available for substitution and the same amino groups were labelled. Therefore, the different number of substituted amino residues resulting in complete inhibition of the different functions poses an interesting problem; for phosphorylation of the enzyme by ATP one amino residue out of four would be essential, while for the calcium dependent ATPase of native vesicles only one out of two residues would be required for activity. The fact that phosphorylation by ATP is reduced by 50% by the occupation of two residues out of a set of four, while at the same degree of substitution the calcium dependent ATPase activity is completely abolished appears to be compatible with at least two different mechanisms. 1. Although for the phosphoryl transfer reaction only one out of four residues is needed, calcium dependent ATPase activity requires the interaction of two out of four distinct amino groups. If this is the case, calcium dependent ATPase activity should decline more steeply than the phosphoryl transfer reaction and the activity should decline nonlinearly with the substitution of three residues required for total blockage. The experimental graph is not precise enough to exclude this possibility. 2. In the native preparations, calcium transport and the calcium dependent ATPase result from a cooperation of several enzyme molecules each having four equivalent amino groups of which one is essential. The occupation of such a group in one molecule would lead to the inhibition of the total cooperative unit. Fig. 5 shows how the activity would decline assuming dimeric, trimeric or tetrameric structures for the calcium transporting unit. The graphs are nonlinear and resemble the decline of phosphoprotein formation from inorganic phosphate and that of calcium transport. The theoretical graph which best fits the experimental points for calcium transport is that of a trimeric structure, while the inhibition of phosphoprotein formation by inorganic phosphate is best described by a dimeric structure. The concept that several ATPase molecules have to cooperate for calcium transport in ATPase activity in native membranes gains independent support from the observation that the relation between the degree of amino group substitution and the remaining enzymatic activity is different for different substrates of the enzyme and can be modified by the addition of ethyleneglycol. It was shown by the results depicted in Fig. 2 that at the same extent of substitution of three amino residues per 100 000 g nitrophenylphosphatase activity of the enzyme is inhibited by 70% in the presence of ethyleneglycol, while in its absence the activity is reduced only by 20%. This indicates that the presence of ethyleneglycol renders the enzyme more sensitive to amino group substitution. This enhancement in sensitivity is brought about by ethyleneglycol only at 37 °C. The most plausible mechanism for this effect is likely the coupling of initially independent enzyme molecules to one functional unit by the presence of ethyleneglycol. The nonlinear decline of the nitrophenylphosphatase activity additionally supports the assumption of a cooperativity between an assembly of the ATPase molecules in the membrane. An oligomeric structure of the calcium transport system has recently been

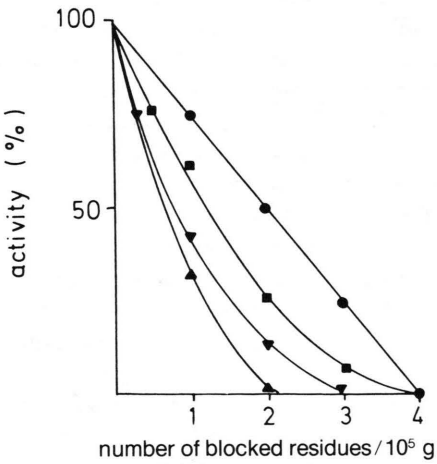


Fig. 5. The decline of the activity of monomeric and oligomeric enzymes varying with the extent of labelling of their functional relevant residues. It was assumed that from four residues available for substitution only one was essential for function. ● the enzyme is monomeric; ■, ▼ and ▲ represent the activity decline when the enzyme operates as a dimeric, trimeric or tetrameric structure, respectively.

proposed by Pick and Racker [20] on account of the analysis of the inhibiting effect of DCCD on the calcium activated ATPase. Yet, as pointed out by the authors, only part of the ^{14}C labelled DCCD that might have reacted with the protein remained covalently bound. Therefore, the relationship between the extent of labelling and the inhibition appears ambiguous in these experiments.

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