THE EFFECT OF ATP UPON THE REACTIVITY OF SH GROUPS IN SARCOPLASMIC RETICULUM MEMBRANES

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1. Introduction

The alkylation of SH groups in sarcoplasmic reticulum membranes by *N*-ethylmaleimide occurs in several kinetically distinct phases [1–4].

Approximately 4 SH groups per 10^5 g protein react relatively fast with *N*-ethylmaleimide accompanied by inhibition of Ca transport and Ca-activated ATP hydrolysis [1]. According to Hasselbach [1] ATP or ADP protects one of these SH groups from reaction with NEM with preservation of Ca transport and ATPase activity.

An additional class of 3 SH groups per 10^5 g protein react 20–25 times slower with NEM and so far they have no clearly established relationship to any sarcoplasmic reticulum function. The remaining 3–4 very fast reacting SH groups probably do not belong to the intrinsic proteins of sarcoplasmic reticulum [1].

It seems well established that most of the SH groups of sarcoplasmic reticulum are associated with the Ca-transport ATPase [2]. The subject of this report is the specificity of the protection exerted by ATP or ADP against the reaction of SH groups with alkylating agents and the implications of this effect upon the mechanism of Ca transport.

The specificity of the protective effect of ATP was investigated by proteolytic cleavage of the sarcoplasmic reticulum proteins after reaction with [^{14}C]NEM in the presence or absence of ATP and analysis of the distribution of radioactivity among the SH-peptides after separation by high voltage electrophoresis.

Abbreviation: NEM, *N*-ethylmaleimide.

2. Experimental

Sarcoplasmic reticulum membranes were isolated as described earlier [5]. The membranes were reacted with [^{14}C]NEM at pH 8.5 under conditions described in the Legends. The labeling reaction was stopped by the addition of 50-fold excess of unlabeled NEM. The proteins were precipitated with cold trichloroacetic acid, collected by centrifugation and washed three times with 2% trichloroacetic acid, once with 90% acetone, once with dry acetone, and twice with ethylether. The dry powders were suspended in 0.06 N HCl and hydrolyzed with pepsin for 2–20 h as described earlier [6]. The peptide mixture was separated by high voltage electrophoresis in pyridine–acetate buffer, pH 3.5 at 70 V/cm voltage gradient for 2 h. The radioactive SH peptides were localised by radioautography.

Microsomes were labeled with [^{32}P]ATP using Ca or Mg and Ca as activators in the presence of 0.1 M KCl and 10 mM histidine, pH 7.3. The ^{32}P-labeled peptides obtained by peptic hydrolysis were separated by high voltage electrophoresis as described earlier [6]. The peptide mixture was separated by high voltage electrophoresis in pyridine–acetate buffer, pH 3.5 at 70 V/cm voltage gradient for 2 h. The radioactive SH peptides were localised by radioautography.

3. Results

Sarcoplasmic reticulum was labeled with [^{14}C] NEM in the presence of 3.2 M guanidine at pH 7.5. After peptic hydrolysis of the labeled membranes

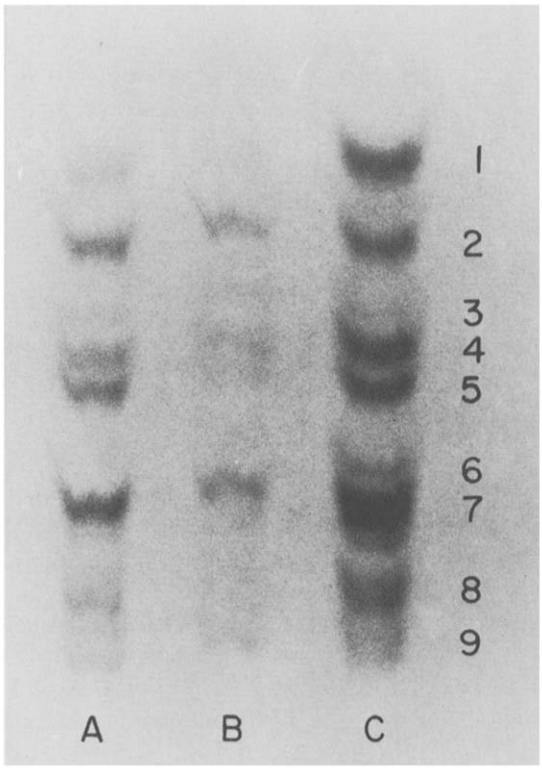

Fig.1. The effect of guanidine and ATP upon the reaction of SH groups in sarcoplasmic reticulum membranes. Microsomes (3.5 mg protein/ml) were reacted with 0.33 mM [^{14}C]NEM in a solution of 16 mM Tris, pH 8.5, and 2.5 mM $CaCl_2$ for 15 min at 25°C (sample A). To sample B and C 1.6 mM ATP and to sample C 3.2 M guanidine were also added. The labeling reaction was stopped with 14 mM unlabeled NEM. The proteins were precipitated with TCA, washed, hydrolysed with pepsin and subjected to high voltage electrophoresis as described under Experimental. The peptide bands visualized by autoradiography are labeled 1–9 in decreasing order of mobility.

the resulting peptide mixture was resolved by high voltage electrophoresis into nine radioactive bands which presumably correspond to distinct SH peptides (fig.1C). Essentially identical peptide patterns were obtained after peptic digestion of 2 to 20 h duration. The higher level of radioactivity in band 7 may be due to preferential labeling or may imply the presence of more than one SH group. The number of moles of NEM incorporated in the presence of guanidine ranged between 9–12 mol per 10^5 protein.

The same peptides were labeled to a slightly lesser extent in the absence of guanidine, leading to the incorporation of 4–5 moles of [^{14}C]NEM per 10^5 g protein (fig.1A).

Addition of ATP in a final concentration of 1.6 mM sharply inhibited the incorporation of [^{14}C]NEM into seven SH peptides with relatively minor change in the labeling of peptides 2 and 7 (fig.1B). The total amount of NEM incorporated into microsomal proteins in the presence of ATP was only 30–50% of that observed in ATP-free control samples.

These observations suggest that the effect of ATP is not confined to SH groups in the immediate vicinity of the active site but it is presumably mediated by a conformational change induced by ATP in the Ca transport ATPase or could result from protein–protein interactions.

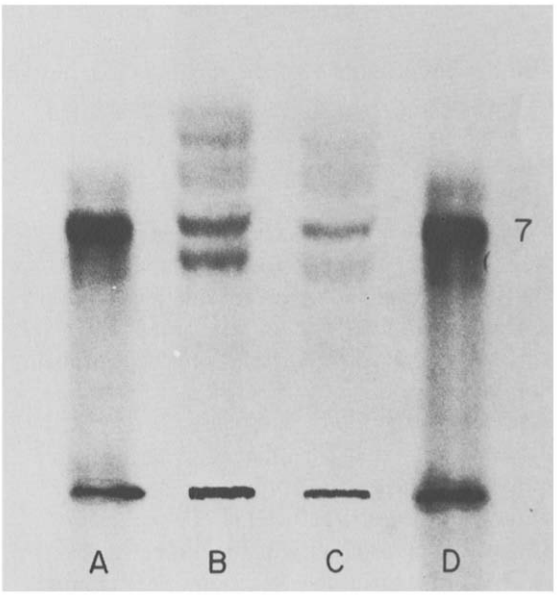

Fig.2. Comparison of the mobility of SH peptides with that of the phosphopeptide obtained after labeling the active site with [^{32}P]ATP and [^{32}P]acetylphosphate. The phosphorylation of enzyme with [^{32}P]ATP (A) or [^{32}P]acetylphosphate (D) was performed in a medium of 0.1 M KCl, 10 mM histidine pH 7.3, 5 mM $CaCl_2$ and 0.5 mM [^{32}P]ATP or [^{32}P]acetylphosphate at 2°C for 20 sec. The peptide mixture was prepared for electrophoresis as described earlier [6].

Reaction with [^{14}C]NEM (0.66 mM) was carried out in the presence of 33 mM Tris buffer pH 8.5, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ (B) for 15 min at 25°C. To sample C in addition to the components of B 3.3 mM ATP was also added.

The mobility of alkylated SH-peptides was compared with that of the phosphopeptide obtained by specifically labeling the active site of Ca transport ATPase with [^{32}P]ATP or [^{32}P]acetylphosphate.

The reaction of [^{32}P]ATP or [^{32}P]acetylphosphate with the Ca transport ATPase leads to the formation of aspartylphosphate intermediate. The portion of the molecule which contains the acylphosphate is released as a distinct [^{32}P]phosphopeptide upon peptic hydrolysis of the labeled membrane [6] and can be readily separated by high voltage electrophoresis.

The electrophoretic mobility of the phosphopeptide is similar to one of the SH peptides (peptide 7) under several conditions of electrophoresis, raising the possibility that the SH peptide originates from the active site (fig.2). Interestingly the reactivity of this SH peptide with NEM is among the least sensitive to inhibition by ATP.

4. Discussion

Differential labeling in the presence of substrate frequently permits the identification of functional groups at the active site of an enzyme. While under certain conditions this may also be true for the Ca^{2+}-transport ATPase of sarcoplasmic reticulum, our observations so far indicate a rather broad inhibition by ATP of the reactivity at least seven SH groups of the membrane with NEM. The protective effect of ATP appears less pronounced on two additional SH peptides, one of which has similar electrophoretic mobility to the phosphopeptide derived from the active site of the Ca transport ATPase.

The influence of ATP upon the reactivity of so many SH groups in the membrane may be explained by clustering of SH groups in the vicinity of ATP binding site [7,8], conformational change in the ATPase molecule during the ATPase cycle [9], or by a shift in the equilibrium between ATPase monomers and oligomers in the membranes [10,11] in favor of associated structures.

In view of these observations, protection by ATP may be a difficult approach for selective identification of SH groups involved in the catalytic activity of the Ca transport ATPase.

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