The oxidation of sulfhydryl groups in mitochondrial F_1-ATPase decreases the rate of its inactivation by the natural protein inhibitor

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The oxidants of the SH groups (*o*-iodozobenzoate, oxidized glutathione, etc.) and the divalent cations of some metals (Zn^{2+} and Cd^{2+}) significantly slow down the rate of inactivation by the protein inhibitor of the isolated F_1-ATPase and ATPase in submitochondrial particles. Modification of SH groups in the ATPase does not change the rate of inactivation but completely prevents the effect of oxidants.

Mitochondrial H^+-ATPase *Natural protein inhibitor* *SH group* *Redox regulation*

1. INTRODUCTION

The regulation of H^+-ATPase in coupling membranes is of great importance to cellular energetics. The decrease in membrane potential ($\Delta\bar{\mu}H^+$) in mitochondria [1], chloroplasts [2] and in some bacteria [3] triggers off the special mechanism of inhibition of ATP hydrolysis by H^+-ATPase. The activity of this enzyme increases many-fold when the membrane is re-energised. This mechanism preserves the intracellular pool of ATP when $\Delta\bar{\mu}H^+$ on the membrane is decreased for some reason. The activation of the latent chloroplast ATPase during illumination is a result of the $\Delta\bar{\mu}H^+$-dependent release of tightly bound ADP and of the reduction of disulphide bound in the enzyme molecule [2,4]. The redox state of the ATPase is probably controlled by a thioredoxin system of the chloroplast which depends in turn on the NADPH concentration and on the activity of a photosynthetic electron-transport chain [4].

The mitochondrial ATPase is regulated by both ADP bound to the enzyme [5] and the natural protein inhibitor [1]. In the presence of ATP this inhibitor blocks the ATPase under de-energized conditions. The energization of the membrane leads to the rapid (~100 ms) activation of the enzyme and the subsequent slow [6] (or rapid [7]) dissociation of the inhibitor.

We show here that the oxidants of the SH groups and the divalent cations of some metals (Zn^{2+} and Cd^{2+}) significantly slow down the rate of inactivation by the protein inhibitor of the isolated F_1-ATPase and ATPase in submitochondrial particles (SMP).

The preliminary results of this work have been published [8].

2. MATERIALS AND METHODS

Mg,Mn-SMP from beef heart mitochondria were isolated according to Hansen and Smith [9]. The particles depleted of the protein inhibitor (S-SMP) were prepared by filtration of Mg,Mn-SMP on a Sephadex G-50 column [10]. The protein inhibitor was isolated according to Pullman and Monroy [1] and purified by DEAE-Sephadex chromatography [11]. F_1-ATPase was isolated from beef heart mitochondria according to Knowles and Penefsky [12]. The following medium was used to measure the ATPase activity of S-SMP: 200 mM sucrose, 50 mM Hepes, 10 mM KCl, 1 mM phospho*enol*pyruvate, 0.3 mM NADH, 5 μM EDTA, 3 mM $MgSO_4$, 2 mM ATP,

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2 U/ml pyruvate kinase, 3 U/ml lactate dehydrogenase, 2 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 3 μM rotenone (or 5 mM KCN), pH 7.0, at 22°C. CCCP, rotenone and sucrose were abolished from the medium when the ATPase activity of F_1-ATPase was measured. Tangents were drawn to determine ATPase rate changes in the course of the ATPase reaction in the presence of the protein inhibitor. The specific activity of the protein inhibitor was assayed by preincubation for 15–20 min with S-SMP in the following medium: 250 mM sucrose, 50 mM Hepes, 10 mM KCl, 2 U/ml pyruvate kinase, 5 mM phospho*enol*pyruvate, 5 mM ATP, 10 mM $MgSO_4$ and 0.1 mM EDTA, pH 6.7, at 22°C. The amount of the protein inhibitor that causes 50% inhibition of 0.2 U ATPase is taken as 1 unit. The preparation of the protein inhibitor used here has a specific activity of 6000 U/mg.

3. RESULTS AND DISCUSSION

Fig.1 shows the kinetics of the inactivation of ATPase in S-SMP by the protein inhibitor. Various reagents, which oxidize vicinal thiols to disulphide bonds, *o*-iodozobenzoate (curve 4), oxidized glutathione (curve 3) and dithiodibutyric acid (not shown), slow down the rate of ATPase inactivation by the protein inhibitor. Modification of SH groups in SMP by DTNB (curve 5) or 2 mM iodoacetate (not shown) does not change the rate of inactivation but completely prevents the action of oxidants. These results indicate that the decrease in inactivation rate by oxidants is due to the appearance of a disulphide bond in a molecule of the enzyme but not simply to modification of the SH groups. The protein inhibitor does not contain SH groups [13], so the target of the attack by the oxidants is ATPase itself.

The effect of oxidants was only observed when they were added directly to the incubation medium in the presence of ATP and the protein inhibitor. Preincubation of SMP with oxidants in both the present and absence of ATP does not change the rate of inhibition after addition of the protein inhibitor. These data indicate that 2 SH groups in the ATPase molecule become closer or become available solely as a result of the interaction between the inhibitor and the enzyme.

At high concentrations of the protein inhibitor the ATPase inhibition follows first-order kinetics. A hyperbolic curve is observed when k_{app} of the inhibition is plotted vs the inhibitor concentration (the concentration of the enzyme remains constant) (fig.2A). It is apparent from these results that the inhibition of the ATPase by the protein inhibitor is a two-step process:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EI^*$$

The rapid reversible binding [1], which does not lead to inactivation is followed by slower and practically irreversible interconversion [2], resulting in complete inhibition of the ATPase activity. A linear plot of $1/k_{app}$ vs inhibitor concentration (fig.2B) gives both the $K_s = k_{-1}/k_{+1} \simeq 2 \times 10^{-7}$ M (if $k_2 \ll k_{-1}$) and $k_2 = 1.3$ min^{-1}.

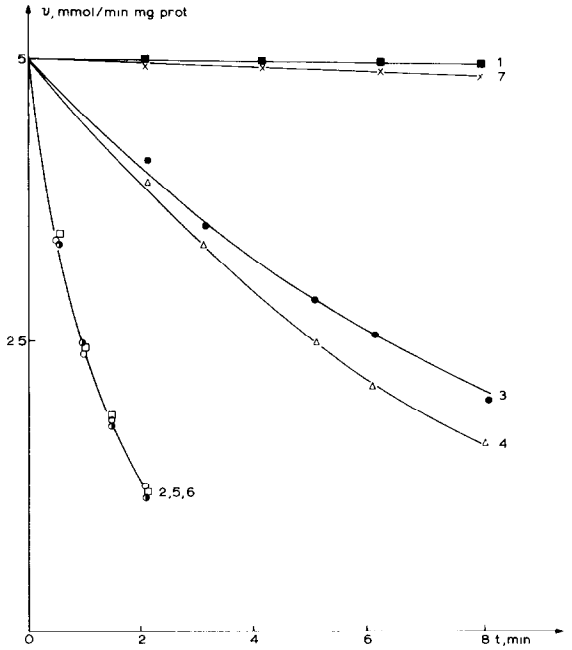

Fig.1. The inhibition of ATPase in S-SMP by the protein inhibitor. ATPase activity of S-SMP was measured as described in section 2. 10 μg/ml of protein of S-SMP (curves 1–7) and 1.25 μg/ml of the protein inhibitor (curves 2–7) were added to the reaction medium. The medium additionally contained: 2 mM *o*-iodozobenzoate (curve 4), 10 mM oxidized glutathione (curves 3,5), 20 μM $CdSO_4$ and 20 mM cysteine (curve 6), 20 μM $CdSO_4$ (curve 7). In the case of curve 5, S-SMP were preincubated with 50 μM DTNB, pH 7.0, for 5 min.

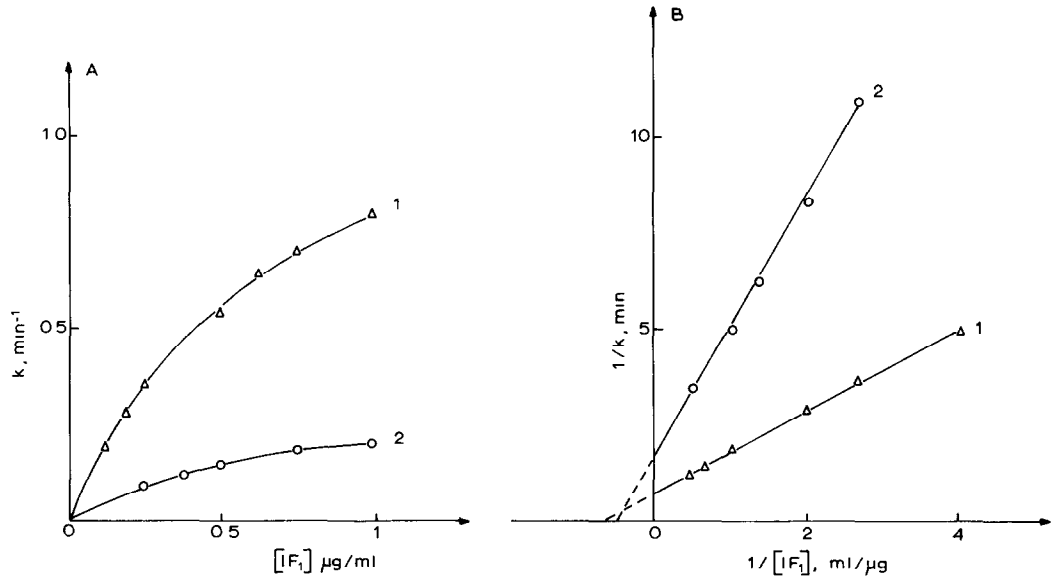

Fig.2. The dependence of the rate constant (k) of the inhibition of ATPase in S-SMP on the concentration of the protein inhibitor. The values of first-order constants (k) were determined from semi-logarithmic plots of inhibition under the conditions described in fig.1. Curves: 1 (A,B), no additions, 2 (A,B) with 10 mM oxidized glutathione.

The data presented in fig.2 (curve 2) indicate that the addition of oxidized glutathione lowers the value of k_2, but K_s does not change. Similar results were obtained when *o*-iodozobenzoate (2 mM) was used instead of glutathione (not shown). It is obvious, therefore, that the oxidants affect the slow interconversion of the enzyme-inhibitor complex. This structural rearrangement is probably a rate-limiting step of protein inhibitor action in mitochondria [6].

Fig.3 shows the kinetics of inactivation of isolated F_1-ATPase by the protein inhibitor in the presence or absence of oxidants. It is evident from these data that the redox state of SH groups in F_1-ATPase controls the rate of inhibition by the protein inhibitor.

Ions of various divalent metals similar to oxidants of SH groups significantly lower the rate of inactivation of the ATPase by the protein inhibitor. The rate of inhibition falls 10–20-fold when EDTA is omitted from the incubation medium. Addition of 5 μM EDTA results in the complete restoration of the maximal rate of inhibition. Cd^{2+} and Zn^{2+} are most potent in lowering the inactivation rate: addition of 20 μM of these ions (in the presence of 5 μM EDTA) results in an almost 20-fold decrease in the inhibitor activity

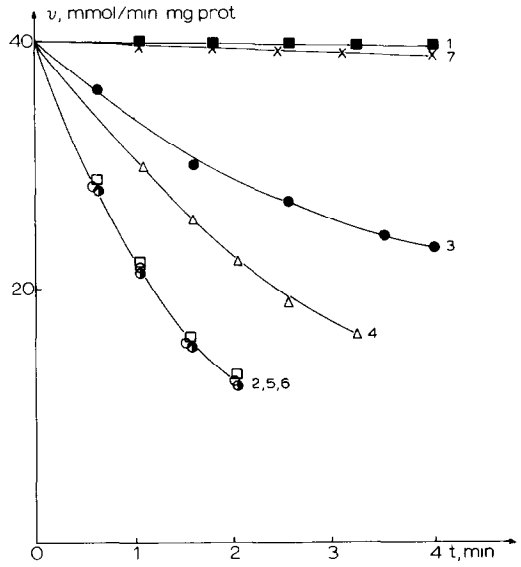

Fig.3. The inhibition of isolated F_1-ATPase by the protein inhibitor. The ATPase activity of F_1-ATPase was measured as described in section 2. 1 μg/ml of protein of F_1-ATPase (curves 1–7) and 1.25 μg/ml of the protein inhibitor (curves 2–7) were added to the reaction medium. The medium additionally contained: 10 mM oxidized glutathione (curves 3,6), 0.25 mM $K_3Fe(CN)_6$ (curve 4), 20 μM $CdSO_4$ (curve 7). In the case of curves 5 and 6 F_1-ATPase was preincubated with 50 μM DTNB, pH 7.0, for 5 min.

(fig.1, curve 7; fig.3, curve 7). Co^{2+} and Ni^{2+} (20 μM) and Mn^{2+} (100 μM) (also in the presence of 5 μM EDTA) lower the rate of inactivation 2- or 3-fold. It cannot be excluded that these ions replace other metal ions initially present in the incubation medium (i.e. Cd^{2+} or Zn^{2+}) from their complex with EDTA. The participation of Fe^{2+} or Fe^{3+} in regulating the action of the protein inhibitor seems unlikely because tartrate (100 μM) and salicylate (100 μM) do not affect the rate of the protein inhibitor's action.

The mechanism of the metals' action is not completely clear. Cd^{2+} and Zn^{2+}, which are most effective in slowing down the inactivation, can form tight complexes with vicinal SH groups. So, these metals can act in a similar manner to the oxidants linking the vicinal SH-groups. On the other hand, the effect of Cd^{2+} can be reversed by monothiols such as cysteine (fig.1, curve 6) or reduced glucathione (not shown) as well as by dithiothreitol. These properties are not characteristic of the binding of Cd^{2+} to vicinal SH groups, from which it can be displaced only by dithiol compounds [14]. In contrast to the oxidants, the effect of the metal cannot be prevented by the modification of ATPase by iodoacetate or DTNB (not shown). These data indicate that the action of metals is more likely to be the result of binding with a single SH group of the enzyme.

The effect of oxidants and metal ions on the rate of the ATPase inactivation by the protein inhibitor may reflect the existence of the special regulatory mechanism in mitochondria. This mechanism may prevent the hydrolysis of ATP under conditions of hypoxia, when the respiratory chain fails to generate a high $\Delta\bar{\mu}H^+$ on the membrane. The decrease in respiration rate results in a more reduced state of the matrix components (glutathione, for example, or the hypothetical thioredoxin-like protein) and under these conditions protein inhibitor effectively blocks ATP hydrolysis.

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