ISOLATION AND PROPERTIES OF A NATURAL INHIBITOR OF THE CHLOROPLAST ADENOSINE TRIPHOSPHATASE

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1. Introduction

The membranous reversible H^+-ATPase complex of mitochondria, bacteria and chloroplasts has been shown to be the functional component in generating ATP [1]. Structural and functional properties of this ATPase from different sources were found to be similar in many respects [2,3]. The mechanism by which this proton-pumping ATPase functions is still unknown. Control of the system in mitochondria by an endogenous protein inhibitor of the ATPase was first described in [4]. This protein is an easily dissociated subunit of the ATPase complex which specifically and effectively inhibits the ATPase activity of mitochondria [5]. Further studies have underlined the regulatory function of this protein in oxidative phosphorylation as well as in the back flow of energy from ATP [5].

Here, we demonstrate that a similar chloroplast ATPase protein inhibitor could be solubilized by heat treatment of the chloroform-released 7 subunit enzyme or any of the preparations containing the 10 000 M_r subunit, C_2 [6]. The properties of this inhibitor were studied. It was sensitive to trypsin. It inhibited membrane-bound chloroplast Mg^{2+}-ATPase and soluble CF_1-ATPase. This inhibition was pH-dependent and was stimulated by Mg^{2+} and ATP. It did not inhibit the particulate or soluble mitochondrial ATPase activity from bovine heart or insect flight muscles.

Abbreviations: CF_1, Chloroplast coupling factor 1; chl, chlorophyll; EDTA, ethylene diamine tetraacetic acid; Tris, *N*-[Tris(hydroxymethyl)]amino methane; DDT, dithiothreitol; PMS, *N*-methyl-phenazonium methosulphate

2. Materials and methods

Spinach was purchased from a local market. All preparations were made exactly as in [6]. Assay of Ca^{2+}-ATPase activity of CF_1 and that of Mg^{2+}-ATPase of chloroplasts were as in [7]. Mitochondria from bovine heart were isolated according to [8] and from thoracic muscles of *Spodoptera littoralis* moths according to [9]. F_1 was isolated from these mitochondria as in [10]. ATPase activity of mitochondria and that of F_1 was assayed as in [9].

2.1. *Release of the ATPase inhibitor protein by heat*

The 7 subunit ATPase preparation or either of the prep. (6), (9) or (10) in [6] at 4–5 mg protein/ml in distilled water were heated in a water bath at 75°C for 15 min. The very turbid mixtures were centrifuged at (15 000 × *g*) for 20 min and the protein in the clear supernatants was determined and tested for the inhibition of CF_1-ATPase activity.

2.2. *Assay of the inhibitor activity*

Supernatant containing the inhibitor was diluted with water to 20 μg protein/ml. Heat-activated CF_1 was prepared to contain 10 ATPase units/ml (0.5 mg protein/ml). An aliquot (10 μl) of this solution (containing 200 nmol Tris–HCl (pH 7.8), 200 nmol ATP and 50 nmol DTT) was added to 190 μl distilled water containing different concentrations of the inhibitor. After 15 min at room temperature, 0.8 ml Ca^{2+}-ATPase reaction mixture was added and the ATPase activity was assayed. For the assay of the inhibitor against Mg^{2+}-ATPase activity of chloroplasts, 30 μl chloroplast suspension (in 0.3 M sucrose, 20 mM Tris–HCl (pH 7.8), 10 mM NaCl and 15 mM DTT) at 0.7 mg chl/ml, were added to 170 μl 1 mM Tris–HCl

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(pH 8) containing different amounts of the inhibitor and the assay was completed as above except that 0.8 ml Mg^{2+}-ATPase reaction mixture was added instead of the Ca^{2+}-ATPase reaction mixture. Under these conditions incubation of the ATPase in the absence of the inhibitor had no effect on its activity.

3. Results and discussion

3.1. *Release of the inhibitor from the ATPase*

In mitochondria, the ATPase inhibitor protein could be released following heat denaturation of F_1 preparations [4,11,12]. Considering the similar properties of the system in both mitochondria and chloroplasts, we attempted to isolate the inhibitor by the same technique. Ammonium sulphate suspensions of either the 7 subunit ATPase or prep. (6), (9) or (10) from [6] were centrifuged down and the pellets were suspended in water at 5 mg/ml in 1 or 2 ml final vol. Those samples were heated as above. After the highly turbid precipitates were removed by centrifugation, the supernatant fractions were assayed for the inhibition of CF_1-ATPase activity as in section 2. The average yield of the inhibitor was 20–25 μg protein/mg CF_1.

3.2. *Properties of the inhibitor*

The inhibitor is completely soluble in water and buffer solutions over pH 6–9. It can be precipitated by 5% trichloroacetic acid and readily redissolved without loss of inhibitory activity. Inhibition of chloroplast Mg^{2+}-ATPase activity by the inhibitor was linear (fig.1). Extrapolation to 100% inhibition gives a stoichiometry of 1.25 μg inhibitor protein/10 μg CF_1 protein (based on a total of 20 μg chl/assay and an average yield of 0.5 mg CF_1 protein/mg chl [13,14]). Assuming that the inhibitor has M_r 10 000 [6], and M_r 325 000 for CF_1 [15], this gives a molar ratio of 4 mol inhibitor/1 mol CF_1 at 100% inhibition. In fig.2 we treated the linear part of the inhibition data of CF_1 Ca^{2+}-ATPase activity shown in fig.1 according to [16,17] to obtain the inhibitor constant K_i and the number of its binding sites, N, on CF_1. This treatment assumes that 1 molecule of inhibitor reacts with 1 site on CF_1 according to a simple equilibrium and that the activity of the enzyme is proportional to the amount of free enzyme. Under these conditions, a plot of (inhibitor)/i *vs* $1/1-i$, should be a straight line representing the equation

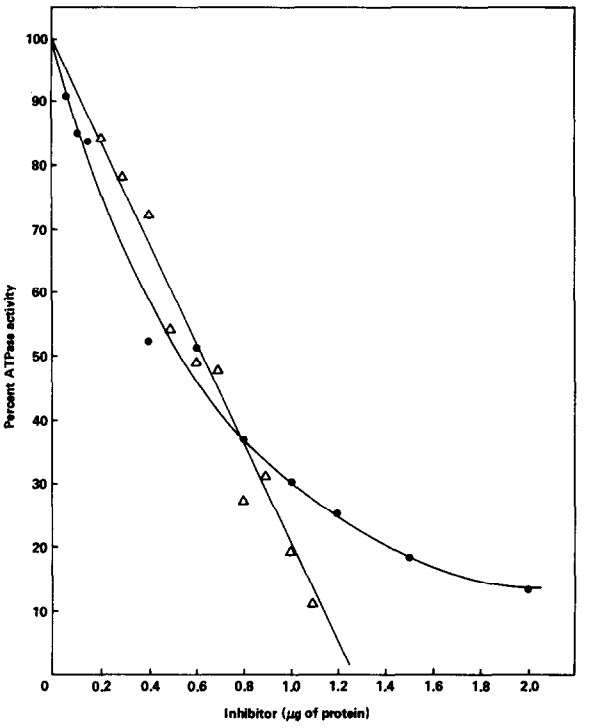

Fig.1. Titration of Ca^{2+}-ATPase activity of CF_1 and the Mg^{2+}-ATPase activity of chloroplasts by the inhibitor. Heat-activated CF_1 (5 μg) or DDT-activated chloroplasts (equiv. 20 μg chl) was incubated with the indicated amounts of the inhibitor in a final volume of 0.2 ml water, in the case of CF_1 or of 5 mM Tris–HCl (pH 8) in the case of chloroplasts. After 15 min at room temperature, 0.8 ml of Ca^{2+} or Mg^{2+}-ATPase reaction mixture was added and the activity was assayed as in section 2. (●——●) CF_1-ATPase activity; (△——△) chloroplast Mg^{2+}-ATPase activity.

(inhibitor)/$i = N + K_i/1-i$, in which i is the degree of inhibition. The inhibitor constant K_i estimated from the slope of the line in fig.2 was found to be 1.2×10^{-7} M. The number of binding sites for the inhibitor was estimated by the extrapolation of the line to the ordinate axis, which gives 4 binding sites which is in agreement with the above calculated number of the stoichiometry between the inhibitor and CF_1 when the chloroplast ATPase was completely inhibited. Using the same plot close to 4 binding sites were calculated when bovine heart F_1-ATPase was titrated with its inhibitor [17]. The inhibitor constant estimated here agrees with [17]. Although in [18] 1 mol inhibitor protein/F_1 was found by direct binding studies, the above results when compared to [17] indicate the similarity between the present inhibitor and that from bovine heart mitochondria.

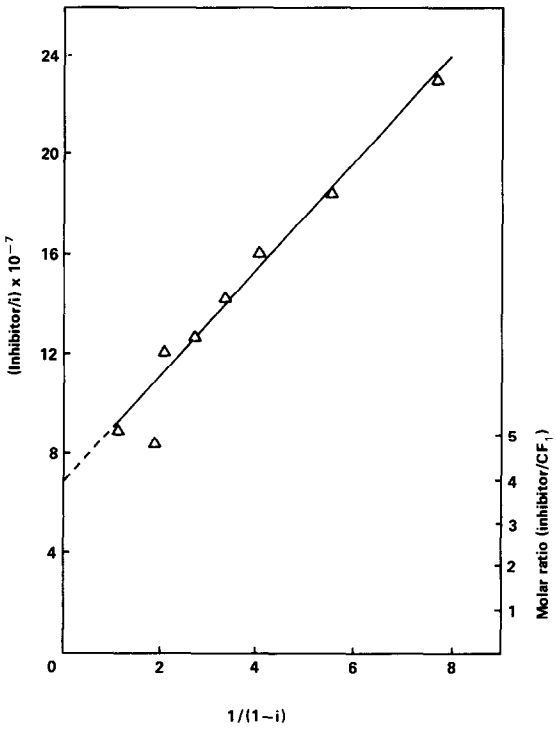

Fig.2. Eassen–Stedman plots of the inhibition of Ca^{2+}-ATPase activity of CF_1 by the inhibitor. The inhibition data shown in fig.1 for CF_1-ATPase were plotted as described in the text. (Inhibitor) is given in μg protein; it represents the percentage inhibition. The ratio (inhibitor)/(CF_1) is in mol/mol.

3.3. *Specificity of the inhibitor*

The inhibitor was specific in its inhibition of chloroplast ATPase, both soluble and membrane-bound. ATPase activity of fragmented mitochondria from bovine heart [8] or from insect muscles [9] and that of their extracted F_1 [10] were not affected by the inhibitor at a level of concentration which inhibited 80% of the chloroplast ATPase activity.

3.4. *Effect of trypsin and pH on the ATPase and the inhibitor*

As shown in table 1, the activity of the inhibitor was destroyed by exposure to trypsin. A striking increase in ATPase activity of the heat-activated CF_1 was seen after treatment with trypsin. The effects of trypsin on CF_1 and the inhibitor were prevented by trypsin inhibitor. Similar effects of trypsin on the ATPase of mitochondria, chloroplast and bacteria have been observed before and were attributed to the destruction of the inhibitor which was known to be highly sensitive to trypsin [4]. These results indicate similar properties of the inhibitor in chloroplasts. The effect of pH on the interaction between the inhibitor and CF_1-ATPase is shown in fig.3. It should be noticed that these pH values refer to the pH at which the inhibitor and the ATPase were incubated before the ATPase assay. The latter was carried out in all cases at pH 8. In this respect the chloroplast ATPase

Table 1
Effect of trypsin on the inhibitor activity

Additions	ATPase activity (μmol P_i . mg protein^{-1} . min^{-1})	Activity (%)
None	16	100
Inhibitor	8	50
Inhibitor + trypsin	24	150
Trypsin inhibitor + inhibitor + trypsin	9.2	57
Trypsin	27.6	176
Trypsin + trypsin inhibitor	17.2	107

Different substances indicated in the table were added in the order mentioned with concentrations as follows: CF_1 inhibitor, 1 μg protein; trypsin, 2 μg protein; trypsin inhibitor, 10 μg protein in 190 μl final vol. After 10 min at room temperature CF_1 (10 μg protein in 10 μl) was added and the mixtures were left for another 10 min before the assay of the ATPase activity. Other conditions were exactly the same as in fig.1

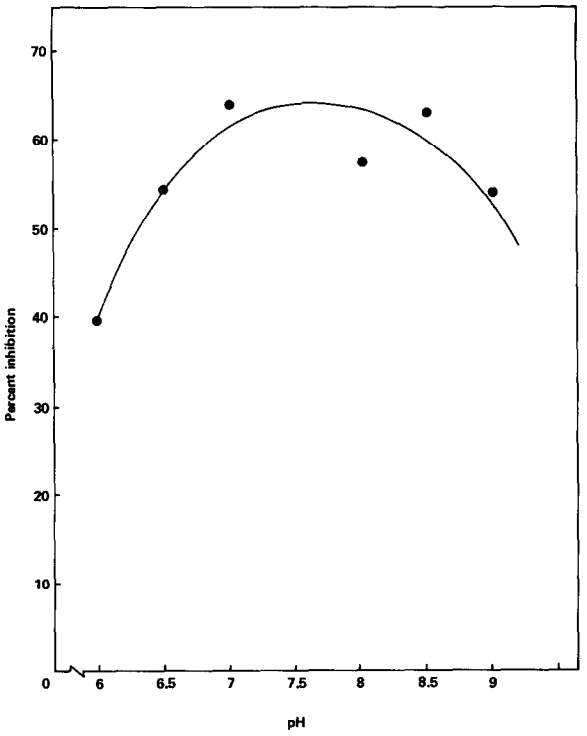

Fig.3. Effect of pH on the activity of the inhibitor. The inhibitor (1 μg protein) was incubated for 15 min at room temperature with 5 μg heat-activated CF_1 and 5 mM Tris–HCl at the pH-values indicated in 0.2 ml final vol. ATPase activity was measured afterwards as in fig.1.

inhibitor is different from that of mitochondria, where it was found that a sharp decline in its interaction with the ATPase was occurred above pH 7.

3.5. *Requirement for Mg^{2+} and ATP for inhibition of ATPase activity*

As shown in [22] the inhibition of mitochondrial ATPase activity by its specific inhibitor requires incubation of the protein with the ATPase in the presence of Mg^{2+} and ATP. Under the experimental conditions in table 2 a pronounced inhibition of chloroplast ATPase by the inhibitor was noticed without addition of either Mg^{2+} or ATP, however, addition of one or the other at 0.5 mM markedly increased the degree of the inhibition. These results suggest a role for Mg^{2+} and ATP in the interaction between the inhibitor and the ATPase in chloroplast.

3.6. *Relation of the C_2 subunit of the 7 subunit ATPase to the inhibitor*

In [6] it was shown that subunit C_2 appeared to have an inhibitory effect on CF_1-ATPase activity. Therefore, we have used preparations containing this subunit to prepare the inhibitor described here. However, all attempts to show the identity of C_2 with the inhibitory protein released from these preparations by heat have failed because of the tendency of the water-soluble protein inhibitor to polymerize. Many of the properties of the C_2-containing ATPase resemble those of the mitochondrial ATPase inhibitor complex [5,6]. We are investigating these similarities in greater detail.

Table 2
Effect of $MgCl_2$ and ATP on the interaction between the inhibitor and the enzyme

Addition	Mg^{2+}-ATPase activity (μmol P_i . min^{-1} . mg chl^{-1})	Activity (%)
None	15	100
Inhibitor, 0.7 μg	6.3	42
$MgCl_2$, 100 nmol (0.5 mM)	17.2	115
Inhibitor, 0.7 μg + $MgCl_2$, 100 nmol	4.8	32
ATP, 100 nmol	9.6	64
Inhibitor, 0.7 μg + ATP, 100 nmol	3.4	23
$MgCl_2$, 100 nmol + ATP, 100 nmol	10.6	71
Inhibitor, 0.7 μg + $MgCl_2$, 100 nmol + ATP, 100 nmol	3.6	24

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