A POTENT INHIBITORY PROTEIN OF CHLOROPLAST OR MITOCHONDRIAL ATPase FOUND IN APROTININ PREPARATION

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Received 19 December 1979

1. Introduction

During the course of investigations of the mechanism of energy-transducing system in living cells, various ATPase inhibitors have been found in the ATPase molecule; chloroplast ATPase inhibitor (CF_1-I) [1], mitochondrial ATPase inhibitor (F_1-I) [2], bacterial ATPase inhibitor (BF_1-I) [3] and one of the troponin components in muscle (TN-I) [4]. They were found as one component of the subunits in each ATPase molecule and play an important role in regulating the ATPase activity. It has been shown in our laboratory that a troponin component, TN-I, inhibits non-competitively mitochondrial, chloroplast or bacterial ATPase, and that the F_1-inhibitor inhibits strongly actomyosin or chloroplast ATPase [5–9].

These experiments deal with the isolation of a protein from a commercial aprotinin preparation, which dramatically inhibits chloroplast and mitochondrial ATPase.

2. Materials and methods

An aprotinin preparation, Trasylol (lot no. 4313p, 500 000 KIE/70–100 mg) [10,11], was supplied from Bayer AG. The chloroplast coupling factor 1 (CF_1) was prepared from spinach by the method in [12] and was activated by heat at 60°C for 4 min. The mitochondrial coupling factor 1(F_1), AS-particles, was obtained from heavy layer beef-heart mitochondria as in [13]. Chloroplast ATPase activity was determined as follows; To a solution (0.5 ml) containing 40 mM Tricine–NaOH buffer (pH 8.0), 10 mM $CaCl_2$, 8 mM ATP was added activated-CF_1 (2.0 μg) in the presence and the absence of an ATPase inhibitor. After incubating the reaction mixture at 37°C for 10 min, the reaction was stopped by adding 2.0 ml 3% trichloroacetic acid. The amount of P_i liberated from ATP was measured as in [14]. Measurement of mitochondrial ATPase activity was carried out as follows: An AS-particle solution containing 0.5 mM $MgSO_4$, 0.5 mM ATP, 25 mM KCl and 15 mM Tris–Hepes (pH 6.7) was preincubated with an ATPase inhibitor for 15 min at 37°C. After the incubation, 25 μl sample solution was taken out and was added to 0.5 ml solution containing 15 mM Tris–sulfate buffer (pH 7.2), 5 mM $MgSO_4$, 4 mM ATP, 5 mM phosphoenol pyruvate and 17 μg pyruvate kinase. The reaction was continued for 10 min at 37°C and stopped by the addition of 1.5 ml 7.5% trichloroacetic acid. The amount of P_i was measured. The aprotinin preparation was subjected to chromatography with Sephadex G-75 equilibrated with 5 mM Tricine–NaOH (pH 7.0) and with Sepharose 6B equilibrated with the same buffer solution. The protein concentration was determined as in [15], using bovine serum albumin as a standard. An inhibitory protein found in aprotinin preparation was digested with chymotrypsin (1:25 by wt) at 37°C for 1 h and the digestion was stopped with tosylphenylalanine chloromethyl ketone (TPCK) [16].

3. Results and discussion

The aprotinin preparation was chromatographed on Sephadex G-75, which is shown in fig.1. There exist a minor band eluted in tubes no. 13–21 and a major band eluted in tubes no. 25–45. The latter

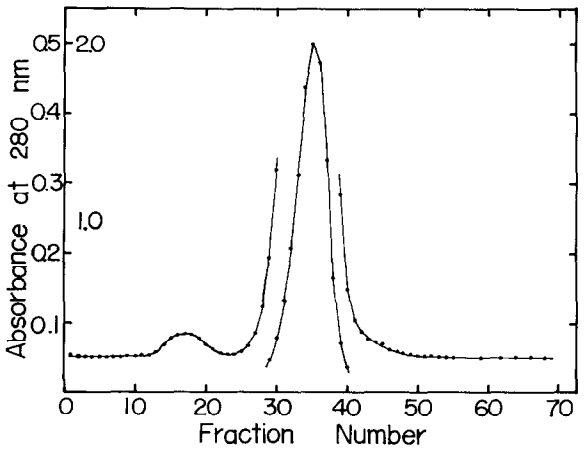

Fig.1. Elution pattern of the aprotinin preparation by chromatography with Sephadex G-75 equilibrated with 5 mM Tricine–NaOH (pH 7.0). The aprotinin preparation (25 mg) was dissolved with 0.5 ml 5 mM Tricine–NaOH (pH 7.0) and was applied to Sephadex G-75 column (1 × 50 cm). Elution was performed with the same buffer solution as above and 1.0 ml protein solution was taken in each test tube. The first band; an inhibitory protein with mol. wt 100 000. The second band; aprotinin with mol. wt 6512.

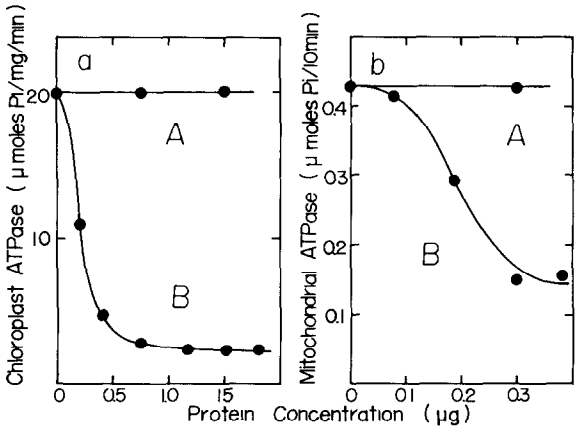

Fig.2. Inhibition of the enzymic activity of chloroplast or mitochondrial ATPase with the inhibitory protein obtained from the first band of the chromatogram and with the purified aprotinin obtained from tube no. 36. (a) Change of the chloroplast ATPase activity with the purified aprotinin (curve A) and the inhibitory protein (curve B). (b) Change of the mitochondrial ATPase activity with the purified aprotinin (curve A) and the inhibitory protein (curve B).

band was identified as aprotinin with mol. wt 6512 [11]. From the chromatogram, it can be said that the aprotinin preparation contains a very small amount of an unknown substance as impurity which has a higher molecular weight than aprotinin itself. Approximate molecular weight of the contaminant was estimated to be 100 000 by chromatography with Sepharose 6B.

Figure 2a represents the inhibitory effect of the contaminant (tubes no. 13–21) and purified aprotinin (tube no. 36) on the enzymic activity of chloroplast ATPase. The enzymic activity was almost completely lost with 0.6 μg contaminant (curve B). Chloroplast ATPase activity inhibition of 50% ocurred with 0.175 μg of the contaminant. On the other hand, the purified aprotinin did not inhibit the chloroplast ATPase activity at all (curve A).

The enzymic activity of mitochondrial ATPase was also inhibited with the contaminant which is shown by curve B in fig.2b. The enzymic activity was decreased with increasing the contaminant concentration and kept at a constant level (34% of the original activity) with >0.3 μg. The reason for not causing a complete inhibition is unclear but a similar phenomenon had been observed when bacterial ATPase was inhibited with one of the troponin components, TN-I [17]. The amount of the contaminant with which 50% of the maximum inhibition of the mitochondrial ATPase took place was 0.175 μg. No inhibition was observed by the purified aprotinin (curve A).

The nature of the contaminant was studied by the following experiments. The inhibitory protein was digested with chymotrypsin and its remaining inhibitory activity on chloroplast ATPase was tested. Only 30% of the inhibitory activity remained after digestion. Inhibitory action of the contaminant on the chloroplast ATPase did not decrease after digestion with ribonuclease. The inhibitory protein did not inhibit the activities of other enzymes such as ribonuclease or asparaginase.

In fig.2a, almost complete loss of the chloroplast ATPase activity took place at 0.6 μg inhibitory protein when 2.0 μg chloroplast ATPase was used in the reaction system. Assuming the chloroplast ATPase and the inhibitory protein to be mol. wt 325 000 and 100 000, respectively, stoichiometric inhibition occurs in the interaction between the ATPase molecule and the inhibitory protein molecule. On the other hand, in the study on the reconstitution of the chloroplast ATPase inhibitor (ε-subunit) and the chloroplast ATPase, ~10-fold molar excess of the ε subunit was necessary to inhibit 50% of the ATPase activity [1].

It is surprising that the protein inhibits more effectively than the authentic inhibitory component and that a potent inhibitory protein of chloroplast and mitochondrial ATPases exists in the aprotinin preparation although the purification procedure of aprotinin mainly removes macromolecules such as protein and nucleic acid from bovine lung.

The aprotinin preparation commercially available (transylol, lot no. 4313p) inhibited 50% of the enzymic activity of the chloroplast ATPase with 6.25 μg, which is ~36-times greater than the 0.175 μg obtained for the inhibitory protein. Therefore, the amount of the inhibitory protein is 2.8% (1/36 × 100) of the aprotinin preparation. The amount of the inhibitory protein in the preparation was, however, ~1%, as estimated from the area of each band in the chromatogram. The difference may be attributed to the presence of other inhibitory proteins. In fact, a protein obtained from tubes no. 25–33 had an inhibitory activity to chloroplast ATPase.

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