The Calcium-binding ATPase Inhibitor Protein from Bovine Heart Mitochondria

PURIFICATION AND PROPERTIES*

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Two ATPase inhibitor proteins were isolated together from bovine heart mitochondria by a new procedure; each was purified further. The one inhibitor is a Ca**-binding protein. It was found to contain 2 cysteine residues/mol as well as threonine and proline residues, all of which the other inhibitor (first isolated by Pullman and Monroy (Pullman, M. E., and Monroy, G. C. (1963) J. Biol. Chem. 238, 3762-3769) lacks. Its minimal molecular weight was 6390 with 62 amino acid residues/mol, and its isoelectric point was 4.6. Besides differences in size, composition, and response to Ca++, the two inhibitor proteins also differed in response to sulfhydryl compounds, pH, KCl, and cardiolipin. Inhibition by the two inhibitor proteins was additive. Both cross-reacted with mitochondrial ATPase from rat skeletal muscle, Calmodulin, with or without Ca++, had no effect on the activity of either inhibitor protein.

Antibody to the Ca**-binding inhibitor protein did not interact with the Pullman-Monroy inhibitor or have any effect on its activity. The antibody interacted with intact submitochondrial particles that contained both inhibitor proteins but not with particles from which only the Ca**-binding inhibitor had been removed. Clearly, the two inhibitors are distinct immunologically as well as in other properties. The two types of inhibitor protein were also isolated from rat skeletal muscle mitochondria by the new procedure.

Mitochondrial respiration and ATP production supply the energy requirements of the processes for Ca++ influx and efflux in mammalian cells (1, 2). Included are the processes for Ca++ cycling into the mitochondria. It has become clear recently that the kinetic parameters of the Ca++ influx process of mitochondria are such that, contrary to previous suggestions, the organelles do not act as regulators of cytosolic Ca++ levels under normal physiological conditions. However, under pathological conditions of excessive cytosolic Ca++, mitochondria accumulate Ca++, if phosphate is present, and store it in granular deposits as a safeguard for cellular processes (2). It is known that the energy required for Ca++ influx into mitochondria is supplied by both respiration and ATP hydrolysis (3). In the course of investigations on the effect of Ca++ on ATP hydrolysis by the energy-transducing H-ATPase complex of rat skeletal muscle, an ATPase inhibitor protein was isolated (4). It differed from the classical ATPase inhibitor protein first isolated from bovine heart mitochondria (5) in that it bound Ca++ (6). The physiological role of the Ca**-binding inhibitor (CaBI) requires further clarification.

This work shows that bovine heart mitochondria contain two ATPase inhibitor proteins (CaBI and the Pullman-Monroy inhibitor (PMI) (5)). Procedures were developed for the rapid isolation and purification of the two proteins from the same sample of mitochondria. The methods should be applicable to mitochondria of various tissues for they have been applied successfully to rat skeletal muscle mitochondria. Some unique properties of CaBI from bovine heart mitochondria are described, and differences from PMI are outlined.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Isolation of Two ATPase Inhibitor Proteins from Bovine Heart Mitochondria and Purification of Ca++-binding Inhibitor Protein

The procedures are summarized in Table I. Both PMI (9, 12, 14) and CaBI (4, 7) are known to be thermostable to 75 °C. This property allowed the removal of much unwanted protein (Step 2). Gel filtration of the thermostable protein fraction on Sephadex G-75 gave three separate protein peaks (Step 3). Fig. 1A shows the elution profile of the heat-stable protein fraction from rat skeletal muscle mitochondria (for comparison). Fig. 1B shows the elution profile for the comparable fraction from bovine heart. The two profiles are very similar. Peak I of each contained the excluded proteins. Inhibitor protein activity was found in Peaks II and III for both tissues.

Ca++ had no effect on the inhibitory action of Peak II, indicating that it contained PMI, which does not bind Ca++ (19, 20). Peak III was identified as CaBI by the loss of about 96% of the inhibitory activity in the presence of 1 μM Ca++ (7) (see Table IV also). The relative elution positions of the two inhibitor proteins are in line with their relative molecular weights of 9578 for PMI (14) and 6390 for CaBI (see Table III).

There was more than a 3-fold increase in recovery of inhibitor protein units after Step 2 (Table I). This could have been due in part to the inclusion of 2-mercaptoethanol in the

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1 The abbreviations used are: CaBI, the Ca**-binding ATPase inhibitor protein; PMI, the Pullman-Monroy ATPase inhibitor protein; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; CaM, calmodulin; PBS, phosphate-buffered saline; FPLC, fast protein liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography.

2 "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Protein from rat skeletal muscle mitochondria was applied to a buffer of Step 2; it was found, for example, that the specific activity of the starting fraction was 3.1 mg/100 units after storage for 3 weeks at -70°C could be restored by incubation for 1 h at 0°C in 0.25 M sucrose containing 5 mM 2-mercaptoethanol. Thereafter, 2-mercaptoethanol or di-thiothreitol was included in the solutions. In contrast, sulfhydryl compounds had no effect on the activity of PMI. The main reason for the increase in total units after Step 3 was, however, the removal of an inhibitory protein into Peak I. 0.5 µg of protein of Peak I (concentrated and dialyzed) reversed the inhibitory action of 0.1 µg of Peak III by 70%, but had no effect on the activity of Peak II. The protein of Peak I is being investigated.

CaBI was then purified from Peak III. Unlike PMI (11, 12), CaBI was not retained on a DE52 column at an alkaline pH (Step 4), indicating that the two inhibitors differed in ionic charge at this pH. This step still served to remove some impurities. A 1.4-fold purification was then achieved by chromatofocusing over a pH range of 4-6 (Step 5). Further purification was found after chromatofocusing over a narrower pH range (Step 6). The pI of CaBI of bovine heart was estimated to be 4.6 from this step. The specific activity remained the same after gel filtration on Superose (Step 7). Electrophoresis of the final fraction of CaBI by the method of Laemmli (13) revealed one band of protein with a very small shadow just above.

**Purification of PMI**

The procedure was that of Pullman (11), except that the alkaline extraction step was omitted (Table II). PMI emerged in the effluent in Step 1, the main purpose of which was to remove cytochrome c from the sample. Unlike CaBI, PMI bound to DE52 at pH 8.5 and was eluted with a salt gradient in a single peak (Step 2). The greatest purification was achieved by Step 3; the final specific activity was slightly higher than that found by others (11). Electrophoresis (13) of the final fraction revealed one major band with an impurity similar to the putative "dimer" described by Pullman (11).

**Amino Acid Composition of CaBI**

The ratio of acidic to basic residues in CaBI from bovine heart mitochondria (Table III) is in line with its acidic pI of 4.6 (Table I, Step 6). Two cysteine residues/mol and no methionine or histidine were found. In contrast, PMI has no cysteine, proline, or threonine residues, possesses 5 histidines, and has a molecular weight of 9678 (14); PMI has been sequenced (12, 14). The pI of PMI was estimated to be from 7.6 to 10.5 (see Ref. 14); our estimate of 8.1 by isoelectric focusing in gels in the absence of urea (21) is comparable to the value of 7.7 determined by a different method for a sample of comparable purity (14). Clearly, CaBI from bovine heart is

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**Table I**

<table>
<thead>
<tr>
<th>Step and fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mitochondria</td>
<td>2,870.0</td>
<td>11,200.0</td>
<td>8.9 x 10^4</td>
<td>7.355</td>
<td>100</td>
</tr>
<tr>
<td>2. Heat-stable proteins</td>
<td>24.5</td>
<td>585.0</td>
<td>4.30</td>
<td>7,355</td>
<td>100</td>
</tr>
<tr>
<td>3. Sephadex G-75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak II</td>
<td>6.0</td>
<td>19.4</td>
<td>1.73</td>
<td>89,286</td>
<td>40.2</td>
</tr>
<tr>
<td>Peak III</td>
<td>6.0</td>
<td>123.3</td>
<td>12.04</td>
<td>97,656</td>
<td>280.0</td>
</tr>
<tr>
<td>4. DE52 effluent (Peak III)</td>
<td>2.0</td>
<td>66.5</td>
<td>6.95</td>
<td>104,170</td>
<td>99.7</td>
</tr>
<tr>
<td>5. Chromatofocusing (pH 6-4)</td>
<td>2.9</td>
<td>44.8</td>
<td>6.51</td>
<td>145,349</td>
<td>93.7</td>
</tr>
<tr>
<td>6. Chromatofocusing (pH 5-4)</td>
<td>2.3</td>
<td>42.7</td>
<td>6.67</td>
<td>156,250</td>
<td>96.0</td>
</tr>
<tr>
<td>7. Superose*</td>
<td>1.0</td>
<td>3.58</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Only 3.74 mg of the fraction from Step 6 was applied.*

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**Fig. 1.** Gel filtration of heat-stable protein fraction from mitochondria on Sephadex G-75. A, an aliquot of 66.5 mg of protein from rat skeletal muscle mitochondria was applied to a column (2.6 x 57 cm) previously equilibrated with 0.25 M sucrose containing 5 mM 2-mercaptoethanol, 5 mM Tris/Cl buffer (pH 8.0). Three-ml fractions were eluted with this buffer at a flow rate of 2 ml/min. Protein (and specific activity) of peak fractions were: Peak II, 3.1 mg (92,600), and Peak III, 26.5 mg (111,111). The specific activity of the starting fraction was 7,143. B, an aliquot of 33.6 mg of protein from Step 2 (Table I) from bovine heart mitochondria was applied and eluted as described for A. Protein recovered was 0.68 mg from Peak II and 21.5 mg from Peak III.
not a proteolytic fragment of PMI, nor is it apparently derived from it.

The comparable CaBI from rat skeletal muscle mitochondria was purified. It showed a minimal molecular weight of 6200, at least 60 residues/mol, and a very similar amino acid composition to that of CaBI of bovine heart. Both CaBI proteins contained blocked N-terminal residues.

### Ca$^{2+}$ and Activity of Inhibitor Proteins

The experiments were conducted for CaBI as described before (7); a similar biphasic curve was obtained. Briefly, the preincubation medium for the assay of inhibitor protein activity contained 0.5 mM Mg$^{2+}$ (see “Experimental Procedures”). The addition of 10^{-4}, 10^{-4}, 10^{-7}, and 10^{-6} M Ca$^{2+}$ to the preincubation medium dropped inhibition by CaBI from an initial high of 94% to 81.7, 65.5, 35.3, and 3.3%, respectively. Inhibition increased again to 51.0, 74.4, and 91.7% with the preincubation medium dropped inhibition by CaBI from an initial high of 94% to 81.7, 65.5, 35.3, and 3.3%, respectively. EGTA reversed the effects of Ca$^{2+}$. In contrast, Ca$^{2+}$ had no effect on the activity of PMI, as others have also found (19, 20).

### Effect of Calmodulin

The report that calmodulin (CaM) and polyglutamate reversed the inhibitory action of an ATPase inhibitor protein from rat liver mitochondria (15) prompted these experiments. Like rat liver mitochondria, bovine heart mitochondria also contain CaM (16, 17). CaM was dialyzed in 0.25 M sucrose, 10 mM Tris sulfate buffer (pH 7.0) at 4°C prior to use. 5 μg CaM and 80 μM Ca$^{2+}$, the concentrations used by others (15), were added to the preincubation medium for the assay of inhibitor protein activity. Controls were preincubated without CaB, PMI, CaM, or Ca$^{2+}$, as appropriate. CaM alone inhibited ATPase activity by 5% in the absence of Ca$^{2+}$ and by 0.3% in its presence. CaM, with or without Ca$^{2+}$, had no effect on the activity of 0.1 μg of CaBI, which gave 51.1% inhibition of ATPase activity. However, as expected (see above), inhibition by CaBI was reduced to 21.1% by 80 μM Ca$^{2+}$ alone. Thus, it is apparent that CaM is not the inhibitory substance of Peak I (Table I, Step 3). Polyglutamate and oncomodulin (25) were also without effect on the activity of CaBI. In addition, CaM did not affect the activity of PMI from either bovine heart or rat skeletal muscle, with or without Ca$^{2+}$. The reasons for these differences from the rat liver system (15) are not known.

### Inhibitor Protein Content of Mitochondrial Fractions from Bovine Heart

The PMI sample shown in Fig. 2 was prepared by the conventional method of alkaline extraction (9, 11). PMI prepared by our new method gave similar migration. Crude alkaline extract, the first fraction in the isolation of PMI by the conventional method of alkaline extraction (9, 11), contained no detectable CaBI (Fig. 2). Thus, the PMI samples that were isolated by this method and studied by others would not be expected to contain CaBI. PMI (9, 12, 14) and CaBI (7) are known to be heat-stable proteins, and both were identified in this fraction from mitochondria. It is also apparent from Fig. 2 that A-particles contain both CaBI and PMI and that stripping of A-particles with 1–5 mM Ca$^{2+}$ (7) removed CaBI, but not PMI.

The distribution of the two inhibitors in the various mitochondrial fractions was verified by subjecting the extracts to isoelectric focusing in gels, followed by elution of the proteins (21) and assaying for inhibitory activity. The pl of CaBI from bovine heart was estimated to be 4.9 by this method. The pl of PMI was increased from 8.1 to 10.7 when urea was present in the buffers (see also Ref. 22). That urea causes an alkaline shift in pl, particularly for proteins with values greater than 7, is known (23). The pl of PMI from rat skeletal muscle mitochondria was also close to 8.0 in the absence of urea.

### Immunological Studies

**Antibody and Inhibitor Protein Activity**—Fig. 3A shows that antibody to CaBI prevented inhibition by CaBI; maximal effect was obtained at 25 ng of antibody/200 ng of CaBI, at which point inhibition by CaBI became negligible. The antibody, at the same concentrations, had no effect on the inhibitory activity of PMI. Fig. 3B shows that the antibody interacted with intact A-particles; the A-particle-antibody complex was bound to Pansorbin and was removed by centrifugation. The resulting supernatant fractions showed decreasing ATPase activity as the amount of antibody was increased. When the A-particles were stripped of CaBI, but not PMI...
proteins were precipitated in 70% ethanol (Table Lane 2, Brilliant Blue R-250 in 9.2% acetic acid and 45.5% methanol. De-
phosphatase conjugate, was diluted 1:3000. Interaction be-
j3-lactoglobulin (18.4), lysozyme and cytochrome c (14.3 and 12.3),
In  contrast, no interaction was observed with our PMI from
(dns). In 0.25 M sucrose. The mixture was added to the standard preincubation medium for the assay of inhibitor protein activity; incubation was continued for a further 20 min at 25 °C. Aliquots were then removed for assay of ATPase activity. Controls contained no antibody and inhibitor protein, no antibody, or no inhibitor protein. The specific ATPase activity of control samples was 8.82. E, antibody, in the amounts specified, was preincubated at 4 °C for 2.5 h with 1 mg of intact A-particles (C) or 1 mg of Ca2+-stripped A-particles (○) from bovine heart. The respective specific ATPase activities of the particles were 1.13 and 8.82. Pan-
row for 2.5 h with 1 mg of intact A-particles (C) or 1 mg of Ca2+-stripped A-particles (○) from bovine heart. The respective specific ATPase activities of the particles were 1.13 and 8.82. Pan-
sorbentin added to a final concentration of 2%; incubation was
 addition of 1 
(see Fig. 2), interaction with antibody was insignificant (Fig. 3B).
Dot-blot Immunoassays—The method used is described un-
der "Experimental Procedures." Antibody to CaBI was diluted 1:500; the second antibody, goat anti-rabbit IgG alkaline phos-
hosphate conjugate, was diluted 1:3000. Interaction between the antibody and CaBI from bovine heart was detectable
from 100 to 0.63 ng of antigen. The antibody interacted
with antibody was insignificant (Fig. 3B).

Inhibition of ATPase Activity by CaBI and PMI Together—
Table IV shows the additive inhibition of mitochondrial

ATPase by CaBI and PMI from bovine heart. As before, addition of 1 μM Ca2+ to the preincubation medium reduced inhibition by CaBI, but not that by PMI. The resulting Ca2+-bound, inactive CaBI did not interfere with inhibition by PMI when both were present together. Either inactive CaBI was
bound at a separate site on the ATPase complex apart from the PMI-binding site or inactive CaBI no longer bound to the
ATPase complex. The latter view is favored by the finding
that CaBI is released from A-particles by 1-5 μM Ca2+ (7).

Cross-reactivity of Inhibitor Proteins with Heterologous ATPase—CaBI from bovine heart inhibited the activity of
mitochondrial ATPase from heart and skeletal muscle to the
same degree (0.1 μg gave 56.3% inhibition under the standard assay conditions). Similarly, CaBI (0.1 μg) from rat skeletal
muscle inhibited ATPase activity from heart or muscle by

Other Properties of CaBI

Inhibition of ATPase Activity by CaBI and PMI Together—
Table IV shows the additive inhibition of mitochondrial

FIG. 3. Interaction of antibody to CaBI from bovine heart with inhibitor proteins from bovine heart. Antibody was pre-
pared as described under "Experimental Procedures." A, antibody, in the amounts specified, was incubated for 1 h at 0 °C with 200 ng of
CaBI (○) or 200 ng of PMI (■) in 0.25 M sucrose. The mixture was added to the standard preincubation medium for the assay of inhibitor protein activity; incubation was continued for a further 20 min at 25 °C. Aliquots were then removed for assay of ATPase activity. Controls contained no antibody and inhibitor protein, no antibody, or no inhibitor protein. The specific ATPase activity of control samples was 8.82. E, antibody, in the amounts specified, was preincubated at 4 °C for 2.5 h with 1 mg of intact A-particles (C) or 1 mg of Ca2+-stripped A-particles (○) from bovine heart. The respective specific ATPase activities of the particles were 1.13 and 8.82. Pan-

TABLE IV

Inhibition of ATPase activity by CaBI and PMI from bovine heart

<table>
<thead>
<tr>
<th>Additions to preincubation medium</th>
<th>ATPase activity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaBI - PMI - Ca2+</td>
<td>units/mg</td>
<td>%</td>
</tr>
<tr>
<td>µg     µg     µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0      0      0</td>
<td>8.80</td>
<td>0</td>
</tr>
<tr>
<td>0.05   0      0</td>
<td>6.39</td>
<td>27.4</td>
</tr>
<tr>
<td>0      0.05   0</td>
<td>6.43</td>
<td>26.9</td>
</tr>
<tr>
<td>0.05   0.05  0</td>
<td>4.01</td>
<td>54.5</td>
</tr>
<tr>
<td>0.2    0      0</td>
<td>0.44</td>
<td>95.0</td>
</tr>
<tr>
<td>0.2    0      1.0</td>
<td>8.30</td>
<td>5.7</td>
</tr>
<tr>
<td>0      0.2    0</td>
<td>0.41</td>
<td>95.3</td>
</tr>
<tr>
<td>0.2    0      1.0</td>
<td>0.43</td>
<td>95.2</td>
</tr>
<tr>
<td>0.2    0.2   1.0</td>
<td>0.07</td>
<td>99.2</td>
</tr>
</tbody>
</table>
about 56.4%. PMI of the two tissues, however, showed a preference for the homologous ATPase complex. PMI from bovine heart (0.1 μg) inhibited ATPase activity from heart by 66.9%, but inhibited that from muscle by 46.2%. Similarly, PMI from rat skeletal muscle (0.1 μg) inhibited muscle ATPase activity by 52.7%, but inhibited heart ATPase activity by 42.5%. CaBI may be a more highly conserved protein.

Effect of Cardiolipin, KCl, and pH on Inhibitor Protein Activity—Table V shows that inhibition by PMI fell from the physiological roles of the two inhibitors. Also to be considered are their relative abundance, sites of binding on the isoelectric points, were in the responses to Ca2+, KCl, pH, and ATPase by PMI. In contrast, no effect of cardiolipin on the activity of CaBI was found. Table V also shows that inhibition by CaBI remained essentially unchanged when as much as 50 mM KCl was added to the preincubation medium. In contrast, as others have also found (9), PMI was very sensitive to KCl; 20 mM reduced inhibition from 56.7 to 35.2%, with a further reduction to 12.5% at 50 mM. Probably the KCl prevented the binding of PMI to the ATPase complex because KCl has been used to facilitate the removal of PMI from A-particles in the preparation of inhibitor-depleted AS-particles (28). Cardiolipin may also prevent the binding of PMI to the ATPase (see Ref. 10).

Inhibition by 0.2 μg of CaBI from bovine heart was 60% at pH 5.5 (data not shown), increased to 95% at pH 6.5, remained constant to pH 7.5, and then decreased slowly thereafter. A criticism of PMI has been that its pH optimum for inhibition of mitochondrial ATPase is significantly below physiological pH (18). We found that dialysis in 0.25 M sucrose, 0.25 mM EDTA, 10 mM Tris sulfate buffer (pH 8.0) of a PMI fraction with pH optimum below 6.0 (9) resulted in a shift in optimum to a pH range of 5.5–7.5. The reason for this shift is not clear at present.

Differences between CaBI and PMI

Significant differences between the two inhibitor proteins, apart from disparities in size, amino acid composition, and isoelectric points, were in the responses to Ca2+, KCl, pH, and cardiolipin. Presumably it is these differences that determine the physiological roles of the two inhibitors. Also to be considered are their relative abundance, sites of binding on the ATPase complex, and the effect of changes in the sulfhydryl titer of the membranes.

At resting levels of Ca2+, estimated to be 0.1–0.5 μM in heart mitochondria (2, 26), and normal Mg2+ levels (~1 mM), the active form of CaBI would predominate. As the mitochondrial Ca2+ level is increased to about 1 μM, CaBI would become progressively inactive. Possibly the ATP hydrolysis that ensues during this inactive CaBI period provides energy for Ca2+ uptake to maintain the micromolar Ca2+ levels in the mitochondrial matrix at which the dehydrogenases of the Krebs cycle are activated (2, 32). PMI is not affected by Ca2+, but it may be in less abundance than CaBI (see Fig. 1); also, the reversal of PMI activity by as little as 20–50 mM KCl must be considered. In this regard, K+ is the major monovalent cation of mitochondria; it was estimated that normal liver mitochondria contain from 100 mM (30) to as much as 170 mM (31) KCl.

Two stabilizing protein factors that promoted the more rapid and stable binding of an ATPase inhibitor protein to mitochondrial ATPase were isolated from yeast and bovine heart mitochondria (27). Neither inhibited ATPase activity nor contained a blocked N terminus (27). It is not likely that CaBI is one of these proteins. However, a connection between these factors and the putative inhibitory protein of Peak I (Fig. 1) cannot be ruled out.

Acknowledgment—We are indebted to Dr. Francis Jay (Department of Medical Microbiology of this University) for his advice in the preparation of polyclonal antibody to CaBI from bovine heart.

REFERENCES

EXPERIMENTAL PROTOCOL

1. **Extraction of ATPase Inhibitor Protein**
   - **Materials**: ATPase inhibitor protein from mitochondria, buffer (50 mM Tris-HCl, pH 7.0), and extraction buffer (50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM DTT).
   - **Procedure**:
     1. Homogenize the mitochondria in buffer to obtain a mitochondrial homogenate.
     2. Centrifuge at 100,000 g for 1 hour at 4°C to remove insoluble material.
     3. Extract the ATPase inhibitor protein from the supernatant using extraction buffer.
     4. Centrifuge again at 100,000 g for 1 hour at 4°C to obtain a clear supernatant.
     5. The protein concentration is determined using a protein assay kit.

2. **Purification of ATPase Inhibitor Protein**
   - **Materials**: ATPase inhibitor protein, ammonium sulfate, DEAE-Sepharose column, and dialysis against buffer.
   - **Procedure**:
     1. Dissolve the protein in buffer containing 10% (w/v) ammonium sulfate.
     2. Apply the solution to a DEAE-Sepharose column pre-equilibrated with buffer.
     3. Elute the protein with a gradient of buffer containing 0-1 M ammonium sulfate.
     4. Collect the fractions containing the protein.
     5. Dialyze the collected fractions against buffer.

3. **Characterization of ATPase Inhibitor Protein**
   - **Materials**: ATPase inhibitor protein, ATPase, and assay buffer.
   - **Procedure**:
     1. Measure the ATPase activity in the presence of the inhibitor.
     2. Determine the inhibitory constant (Ki).
     3. Calculate the IC50 value.

4. **In vivo Experiments**
   - **Materials**: ATPase inhibitor protein, animal model, and relevant materials.
   - **Procedure**:
     1. Administer the inhibitor to the animal model.
     2. Measure the changes in the ATPase activity.
     3. Evaluate the therapeutic effects.

5. **Conclusion**
   - Summarize the results and discuss the implications of the findings.
   - Future research directions are suggested.

**References**