Demonstration of a Novel Type of ATP-diphosphohydrolase (EC 3.6.1.5) in the Bovine Lung*

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A novel type of ATP-diphosphohydrolase (ATPDase) is demonstrated in bovine lung. The enzyme has an optimum pH of 7.5 and catalyzes the hydrolysis of the β- and γ-phosphate residues from diphospho- and triphosphonucleosides. It requires Ca** or Mg** and is insensitive to ouabain, an inhibitor of Na+/K+-ATPase, P1,P2-di(adenosine 5')-pentaphosphate, an inhibitor of adenylate kinase, and tetramisole, an inhibitor of alkaline phosphatase. In contrast, sodium azide (10 mM), a known inhibitor of ATPDases and mitochondrial ATPases, as well as mercuric chloride (10 μM) and gossypol (2,2'-bis[8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene]) (35 μM) are powerful inhibitors of this enzyme. The same inhibition profile obtained with ATP or ADP as substrate, thereby supporting the concept of a common catalytic site for these substrates. This is further confirmed by enzyme localization after polyacrylamide gel electrophoresis under nondenaturing conditions and by kinetic properties, namely pH dependence profiles, heat inactivation, and 60Co irradiation-inactivation curves. The native molecular mass of the enzyme calculated from 60Co γ-irradiation-inactivation curves is estimated at 70 ± 3 kDa, whereas KM,app and Vmax,app of the ATPDase are evaluated at 7.2 ± 2 μM and 1.1 ± 0.3 mmol of Pi/min/mg protein, respectively. A comparison of the kinetic properties of this ATPDase with those of pig pancreas (Type I) and bovine aorta (Type II) lead us to believe that this enzyme is an hitherto undescribed type of ATPDase. By reference to the previously described ATPDase, we propose to identify this enzyme as ATPDase Type III (EC 3.6.1.5).

ATP-diphosphohydrolase (ATPDase: EC 3.6.1.5)* is a general designation for enzymes that hydrolyze β- and γ-bonds of diphospho- and triphosphonucleosides (1). ATPDases were initially found as soluble and insoluble enzymes in several plant tissues (2-10), and in bacteria and molds (11,12). They have also been described in blood-feeding insects such as ticks (13), bugs (14-16), and mosquitoes (17-19).

Several years ago, we described the first mammalian ATPDase in the pig pancreas (Type I) (20, 21). Since then, mammalian ATPDases have been found in rat pancreas (22), mouse liver and brain, dog kidney, human tumors (23), human term placenta (24), and myometrium (25). An ATPDase was also localized in bovine aorta smooth muscles and endothelial cells (26-28). We have recently demonstrated that the properties of the aorta ATPDase (Type II) are different from those of the pancreas ATPDase (Type I) (29-31). Heat denaturation curves, 60Co γ-irradiation-inactivation curves, and migration patterns on polyacrylamide gel electrophoresis under nondenaturing conditions are the parameters on which we reached our conclusion.

The importance of extracellular nucleotides in blood coagulation is well established (32-34). ADP stimulates platelet aggregation, ATP antagonizes the effects of ADP on platelets, and adenosine inhibits the action of platelet stimulants by their binding on A2 receptors (35). The threshold of platelet activation by ADP is reached between 2 and 5 μM (36). Results from our laboratory showed that platelet aggregation induced by 2 μM ADP can be prevented or even reversed by adding a purified ATPDase fraction from bovine aorta smooth muscle cells (30).

Lung is a highly vascularized tissue where circulating platelets and microemboli become easily trapped in the capillary bed (37). Nevertheless, diffusion studies demonstrate that ATP is rapidly metabolized into adenosine on a single passage through the pulmonary capillary bed (38, 39). Using an indicator dilution technique in rat perfused lungs, Smith and Ryan (40) observed that the mean transit times of ATP, AMP, and an intravascular marker (blue dextran) were identical, suggesting that the enzyme activities responsible for degrading ATP and AMP were confined to the vasculature. Subsequently, a series of elegant experiments utilizing electron microscopic cytochemistry have demonstrated that enzymes responsible for the degradation of ATP and AMP in rat lung were localized to the luminal surface of the plasma membrane of capillary endothelial cells (40, 41).

Until recently, it was generally accepted that extracellular nucleotides would be degraded by three different enzymes: an ATPase, an ADPase, and a 5'-nucleotidase (39, 42-48). Although ADPase activities have been reported in the microvasculature of the lung (38-41, 49), it seems reasonable to propose that a similar endothelial ectonucleotidase system would be present in large and small blood vessels. Since ATPDases have been described in large vessels (26-31), the purpose of this work was to verify the presence of an ATPDase.
in the lung and to compare its properties with those of the pancreas and bovine aorta ATPases. Our results demonstrate the existence of an hitherto undescribed type of ATPDase which we propose to identify as Type III.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tetramisole (2,3,5,6-tetrahydro-6-phenylimidazol[2,1-b]thiazole), Tri-HCl, imidazole, glycocerin (1,2,3-propenitratom), sodium chloride (NaCl), sucrose (β-D-fructofuranosyl-α-D-glucopyranoside), Triton X-100, sodium bicarbonate (NaHCO₃), sodium deoxycholate, phenylmethylsulfonyl fluoride, sodium azide (NaN₃), mercuric chloride (HgCl₂), loaded on a 35% (w/v) sucrose, glycerophosphate, glycerol, (2,2′-biphenyl-1,6,7-trihydroxy-5-isopropyl-3-methylphthalene), ApA (diadenosine pentaphosphate), ATP, CTP, UTP, GTP, IDP, UDP, CDP, GMP, UMP, were obtained from Sigma. ADP and AMP were obtained from Boehringer Mannheim, Laval, Québec, Canada. Magnesium chloride (MgCl₂), calcium chloride (CaCl₂), and trichloroacetic acid were obtained from Fisher Scientific Co. Ouabain (3-(6-deoxy-α-L-mannopyranosyl)oxyl-1,5,11a,14,19-pentahydraycyclohept-22(22)-enolide) was purchased from Calbiochem.

Protein assay kit used to measure protein concentration with bovine serum albumin being as a standard and polycrylicamide were obtained from Bio-Rad, Missisaug, Ontario, Canada. [γ-32P]ATP with a specific activity of 15 mCi/mmol was obtained from Amersham Canada. Polyacrylamide gel electrophoresis under nondenaturing conditions along with the bovine aorta ATPDase preparation isolated according to Côté et al. (29). As shown in Fig. 2, a single band is responsible for the hydrolysis of ATP and ADP in the lung preparation, as for the bovine aorta ATPDase preparation, suggesting that the same enzyme might be hydrolyzing both substrates in bovine lungs. Moreover, the aorta enzyme moved at a locus different from that of the bovine lung preparation. This result supports the view that a distinct isoform of ATPDase is present in lungs.

The presence of an ATPDase activity in bovine lungs was further assessed by testing substrate specificity. As shown in Table II, it was found that the enzyme preparation hydrolyses triphosphonucleosides at comparable rates and diphosphonucleoside monophosphate, P₃-glycerophosphate, and p-nitrophenyl phosphate. The biochemical properties of the lung ATPDase were further defined with various inhibitors (Table III). Significant inhibitions were obtained with gossypol (35 μM), an antifertility agent known to reduce ATP content of spermatozoa (54), sodium azide (10 mM), mercuric chloride (10 μM), and tetramisole (5 mM), an alkaline phosphatase inhibitor (55), whereas no significant inhibition was observed with ApA (100 μM), an inhibitor of adenylate kinase (56) and ouabain (3 mM), an inhibitor of Na⁺/K⁺-ATPase (57). The level of inhibition were comparable with either ATP or ADP as substrates, further supporting the concept that a common catalytic site is involved in the hydrolysis of these nucleotides.

This possibility was confirmed by comparing the inactivation profiles of ADPase and ATPase activities after heat and 60Co γ-irradiation-inactivation. As shown in Fig. 3, superimposable curves of heat inactivation were obtained with ADP and ATP, with a temperature of 60 °C corresponding to 50% residual activity. Radiation-inactivation with 60Co also produced identical curves with ADP and ATP as substrates (Fig. 4). These curves allowed an estimation of the molecular mass of the enzyme on its native form, as described by Kepner and Mace (52) and Kempner and Schleorel (53). The latter was evaluated at 70 ± 3 kDa (mean ± S.D.) for ATP and 66 ± 3 kDa (mean ± S.D.) for ADP as substrates.

Kₘ,app and Vₘₐₓ,app of the lung ATPDase were estimated with labeled [γ-32P]ATP from the Woolf Augustinson-Hofsee plots with four different preparations of enzymes (Fig. 5). Reaction rates were measured over very short periods of time to minimize variations of substrate concentrations (less
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TABLE I
ATPase and ADPase activities in fractions isolated from bovine lungs
Homogenate (HG), supernatant I (SI), pellet I (PI), supernatant II (SII), pellet II (PII), microsomal fraction (MF), and purification factor (PF). Results are the mean ± S.E. of eight distinct experiments.

| Fractions | Substrate (2 mM) | Protein | Activity |
|-----------|-----------------|---------|----------|
|           | mg              |         | Total    | Specific |
| HG        | ATP             | 2861 ± 97 | 353 ± 28 | 0.12 ± 0.01 |
|           | ADP             | 2861 ± 97 | 198 ± 16 | 0.07 ± 0.01 |
| SI        | ATP             | 2368 ± 71 | 311 ± 19 | 0.13 ± 0.01 |
|           | ADP             | 2368 ± 71 | 170 ± 12 | 0.07 ± 0.01 |
| PI        | ATP             | 251 ± 25  | 27 ± 6   | 0.11 ± 0.01 |
|           | ADP             | 251 ± 25  | 20 ± 7   | 0.07 ± 0.01 |
| SII       | ATP             | 1995 ± 58 | 46 ± 15  | 0.02    |
|           | ADP             | 1995 ± 58 | 23 ± 8   | 0.01    |
| PII       | ATP             | 376 ± 40  | 210 ± 21 | 0.56 ± 0.01 |
|           | ADP             | 376 ± 40  | 109 ± 18 | 0.29 ± 0.01 |
| MF        | ATP             | 16 ± 5    | 22 ± 5   | 1.37 ± 0.30 |
|           | ADP             | 16 ± 5    | 15 ± 3   | 0.95 ± 0.30 |

TABLE II
Substrate specificity of ATP diphosphohydrolase activity in bovine lung
Results are the mean of three experiments each in triplicate. The ATP hydrolysis rate, which was taken as 1, corresponds to 1.4 units/mg protein.

| Substrate          | Relative activity |
|--------------------|-------------------|
| ATP, GTP, ITP      | 1.0               |
| CTP, UTP           | 0.9               |
| ADP                | 0.7               |
| IDP                | 0.6               |
| UDP                | 0.5               |
| CDP                | 0.4               |
| AMP, GMP, UMP      | <0.1              |
| ATP + ADP (1 mM)   |                   |
| β-Glycerophosphate | 0.1               |
| p-Nitrophenyl phosphate | 0.0               |

TABLE III
Effect of inhibitors on ATPDase activity
Results are the mean ± S.E. of three experiments each in triplicate. 0.01% CHAPS was added to the incubation medium.

| Inhibitors | Concentration (10^-3 M) | Inhibition |
|------------|-------------------------|------------|
|            |                         | ADP        | ATP        |
| ApuA       | 0.1                     | 3 ± 1      | 5 ± 1      |
| Gossypol   | 0.035                   | 38 ± 3     | 45 ± 5     |
| Na-azide   | 10.0                    | 53 ± 3     | 49 ± 4     |
| Ouabain    | 3.0                     | 3 ± 1      | 3 ± 1      |
| HgCl₂      | 0.01                    | 60 ± 4     | 63 ± 4     |

DISCUSSION

Our laboratory has been involved for many years in the study of ATPDase. As shown by Dixon and Webb (58), this enzyme has the property of catalyzing the hydrolysis of γ-
and β-phosphate residues from triphospho- and diphospho-nucleosides, including both ATP and ADP. The fact that this protein can hydrolyze the latter substrates led to a persistent confusion about the real identity of some ATPase and ADPase activities reported in the literature. We have previously characterized two distinct types of ATPDase in pig pancreas (Type I) (20, 21) and bovine aorta (Type II) (29-31).

In this work, the existence of a third type of ATPDase is demonstrated in bovine lung tissues. This demonstration is based on several indirect and direct evidences. In addition to similar behavior with respect to ATP and ADP hydrolysis as a function of pH and inhibitors, the enzyme responsible for the hydrolysis of ATP has the same locus of migration as the one involved in the hydrolysis of ADP, after polyacrylamide gel electrophoresis under nondenaturing conditions. Direct evidence for the existence of the ATPDase comes from the
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rate of ADP or ATP, and therefore rules out the possibility that phosphate is produced from ADP as a result of the conversion of this substrate to AMP and ATP. Moreover, the fact that our enzyme preparation can hydrolyze pyrimidine as well as pyrimidine diphospho- and triphosphonucleotides corroborates our conclusion.

Biochemical and electrophoretic properties of the lung ATPDase differ from those of the previously described ATPDases from pig pancreas (Type I) (20, 21) and smooth muscle cells of bovine aorta (Type II) (29–31), and so constitute a novel type of ATPDase. The three main facts on which this conclusion was reached are different migration patterns after polyacrylamide gel electrophoresis, and different denaturation curves by heat and 60Co γ-irradiations (29). The latter technique allowed a gross estimation of the native molecular mass of these enzyme. The molecular mass of bovine lung ATPDase (70 ± 3 kDa) is much lower than the values reported for pig pancreas (132 kDa) (29) or bovine aorta (189 kDa) (29).

Several ADPase and ATPase activities have been reported in the lung (38–41). For instance, Dawson et al. (49) described plasma-membrane ATPase and ADPase activities in rat lung microsomal fractions. These activities showed an optimum pH of 7.5 and had comparable sedimentation properties. However, the authors at the time believed that these activities were attributable to separate proteins.

Perfusion studies have demonstrated that ATPase, ADPase, and 5′-nucleotidase activities in lungs are confined to the vascular endothelial cells and that nucleotides do not enter the cells prior to their hydrolysis into adenosine (40). Since ATPDases described in large vessels (26–31) are involved in the control of platelet aggregation and blood coagulation, it seems reasonable to propose that the ATPDase isolated in bovine lung fulfills a similar task in pulmonary capillary bed. Our laboratory has recently reported ATPDase activity in brain capillaries.

Defective hydrolysis of extracellular nucleotides in blood vessels have been associated to various diseases. For instance, pulmonary thrombosis is particularly associated with diabetes (59–61). Perfusion studies performed on diabetic rat lungs demonstrate that ATPase and AMPase activities are significantly reduced, thus increasing risks of thrombosis (62). We are now investigating the localization of this ATPDase in bovine lung and the demonstration of its physiological role in such pathological conditions.

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