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## Ecto-ADPase Activity in the Rat Renal Brush-Border Membranes

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Brush-border membrane vesicles purified from rat kidney cortex exhibit an ectoenzyme activity responsible for the hydrolysis of both ATP and ADP, as well as of other nucleoside tri- and diphosphates. In the presence of  $\text{Ca}^{2+}$  ions, ADP hydrolysis follows the simple Michaelis-Menten kinetics assuming a single catalytic site. The real substrate for ADPase is a divalent cation conjugated ADP. The pH optimum for the hydrolysis is between 7.2 and 8.6.

ADP and ATP hydrolysis show similar heat denaturation curves, and are both resistant to limited proteolysis and to inhibitors of other known ATPases. The enzyme activity is inhibited by: diethyl pyrocarbonate, dithiothreitol, high concentrations of both *N*-ethylmaleimide and azide. The diethyl pyrocarbonate inhibition could be reversed by hydroxylamine, indicating the involvement of histidine and/or tyrosine residues in the reaction. It is proposed that both ADPase and ATPase activities reside within the same enzyme protein.

### INTRODUCTION

Ecto-ATPases are present in many tissues including pancreatic, kidney and liver plasma membranes, vascular endothelial and smooth muscle cells, transverse tubules of skeletal muscles, lymphocytes, neuroblastoma and glioma cells, human granulocytes, Erlich ascites carcinoma cells, human blood platelets, intestinal membranes, as well as in several animal cell

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lines.<sup>1-7</sup> The wide-spread distribution of ecto-ATPase in various tissues suggests an important role of these enzymes in the cellular metabolism.

The exact physiological function is currently unknown, although substrates and products of the reaction, *i.e.* ATP and ADP, are known to influence many biological processes, such as platelet aggregation, vascular tone, neurotransmission, cardiac function and muscle contraction.<sup>8</sup> It has been suggested that ecto-ATPase may be a proton pump, a calcium pump, or it may be involved in maintaining the local concentration of ATP.<sup>3</sup> There is no convincing evidence in favour of any one of the considered functions.<sup>9,10</sup> However, Picher *et al.*<sup>11</sup> have recently demonstrated the existence of three distinct forms of ecto-ATP diphosphohydrolases (EC 3.6.1.5.). They were able to discriminate between the enzyme from pancreas (Type I), enzyme from aorta (Type II) and enzyme from bovine lungs (Type III). Distinct forms of ATPDases were confirmed on the basis of different physical and chemical properties, like migration patterns after PAGE under nondenaturing conditions and differences in denaturation curves following heat and <sup>60</sup>Co irradiation treatment, as well as dissimilar kinetic properties.<sup>12</sup>

The only ecto-ATPase so far isolated, cloned and expressed in cultured mammalian cells is the enzyme from the liver plasma membrane.<sup>13</sup> The deduced amino acid sequence consists of 519 amino acids building a highly complex structure. Structural studies of the amino acid sequence have revealed that liver ecto-ATPase shares substantial homology with several members of the immunoglobulin superfamily of proteins.<sup>14</sup> Rat hepatocyte cell adhesion molecule cell CAM 105, carcinoembryonic antigen, nonspecific cross-reacting antigen and pregnancy specific glycoprotein appeared to be closely related and very similar to the rat liver ecto-ATPase.<sup>15-18</sup> A high degree of homology based on the sequence analysis of tryptic peptides has been established for ecto-ATPase and human biliary glycoprotein 1, and liver-specific glycoprotein pp 120/HA4.<sup>19-20</sup> Indirect immunofluorescence assays and immunogold labeling showed that antibodies against cell CAM 105, which reacted with liver ecto-ATPase, recognized the particular protein in various segments of the renal proximal tubules, plasma membrane of endothelial cells, brush-border membrane vesicles and basolateral membrane vesicles that were prepared from renal cortex.<sup>2</sup> Similarity between cell CAM 105 and rat liver ecto-ATPase has been recently questioned, but no definite conclusion has been reached so far.<sup>21</sup> Katz *et al.* showed that the Ca<sup>2+</sup>Mg<sup>2+</sup> ATPase activity could be localized along the entire nephron with the highest activity measured in proximal and collecting tubules.<sup>22</sup>

Extracellular nucleotides found in the glomerular filtrate are principal substrates for the ecto-enzymes present in the proximal kidney tubule. Adenine nucleotides and their metabolites are involved in the regulation of nu-

merous physiological processes in the kidney, such as blood flow, glomerular filtration rate, urine flow, sodium and potassium excretion and the renin-angiotensin system.<sup>23-25</sup> Karthe *et al.*, showed that exogenous adenosine diphosphate acts as the most potent mitogen for nontransformed African monkey kidney epithelial cells.<sup>26</sup>

ADP, produced through the action of ecto-ATPase, undergoes a further hydrolytic reaction catalyzed by an ecto-ADPase. This activity was found in the aorta, cultured arterial smooth muscle cells, kidney and in erythrocytes.<sup>27-31</sup>

A question may be raised whether the same catalytic molecule is responsible for the hydrolysis of both ATP and ADP in the proximal kidney tubule. The purpose of this work was to characterize the enzyme responsible for the hydrolysis of ADP in brush border membrane vesicles of the proximal kidney tubule as compared to the previously characterized ATP hydrolyzing activity.<sup>30</sup> The effects of different substrates, activators, inhibitors and proteolytic enzymes have been studied and similar kinetic behaviour of both ATPase and ADPase was found.

## MATERIAL AND METHODS

Materials: ADP, potassium salt; IDP, UDP, CPD and ATP, all disodium salts; ATP( $\gamma$ S), App(CH<sub>2</sub>), DEPC, levamisole, polidocanol, papain and lectin from *Arachis Hypogaea* were purchased from Sigma(St. Louis, MO, USA); oligomycin, App(NH)p, NEM, EGTA, Hepes, MES, dithiothreitol, ouabain and trypsin from Fluka (Buchs, Switzerland); concanavalin A from Difco Laboratories (Detroit, USA), and CEA-EIA monoclonal one step test from Abbot (USA). All other chemicals were of analytical grade.

### *Preparation of brush-border membrane vesicle.*

Brush-border membrane vesicles were isolated from the kidney cortex of Wistar male rats (150–200 g) according to the Mg<sup>2+</sup>/EGTA precipitation method of Biber *et al.*<sup>32</sup> Briefly, rat kidney cortex slices were homogenized with Ultra-turax homogenizer in an isoosmotic medium containing 5 mmol/L EGTA. BBMV were purified by two successive precipitations with 12 mmol/L MgCl<sub>2</sub> and a differential centrifugation. Applying this method, a high yield of BBMV was obtained. The vesicles thus obtained were largely oriented with the right side out.<sup>32,33</sup> The sidedness and sealedness of the brush-border membrane vesicles were tested mainly as described by Turini *et al.*<sup>9</sup> Isolated and purified vesicle preparations were resuspended in 5 mM Hepes-Tris buffer (pH 7.0), 100 mM KCl and 300 mM mannitol to the pro-



tein concentration of 10–20 mg/mL. Vesicles were used for enzyme activity determination immediately following isolation, or were kept in liquid nitrogen until further use. Purity of brush-border membrane vesicles was checked by measuring the activity of specific marker enzymes: alkaline phosphatase (EC 3.1.3.1.), according to King and Armstrong<sup>34</sup>, leucine arylamidase was measured by using the commercial kit (Boehringer, Mannheim, B.R.D.). The alkaline phosphatase was enriched, as compared to homogenate  $8.9 \pm 3.5$  ( $n = 17$ ) fold and leucine arylamidase  $10.4 \pm 2.6$  ( $n = 17$ ) fold, respectively. Contamination with basal lateral membranes was negligible as measured by the  $\text{Na}^+/\text{K}^+$  ATPase (EC 3.6.1.3.) activity, which was enriched  $0.48 \pm 0.05$  fold.<sup>35</sup>

Protein concentration was measured by the method of Bradford<sup>36</sup> using bovine serum albumin as standard.

#### *Determination of ecto-ADPase and ecto-ATPase activities*

The enzyme activities were assayed at 37 °C in 50 mM Hepes-Tris buffer (pH 7.4) containing 100 mM KCl, 5 mM  $\text{CaCl}_2$ , 50 mM mannitol and in the presence of various inhibitors: 1 mM levamisole to inhibit alkaline phosphatase, 1 mM ouabain to inhibit  $\text{Na}^+/\text{K}^+$  ATPase, 5 mg/L oligomycin to inhibit mitochondrial  $\text{H}^+$  ATPase, 1 mM NEM to inhibit NEM-sensitive endo-ATPase and 56 mg/L concanavalin A to inhibit 5'-nucleotidase with 0.035 mg of vesicle protein. The final incubation volume was 1.060 ml. The reaction started by addition of substrates: 3 mM ATP for ecto-ATPase assay or 1.5 mM ADP for ecto-ADPase assay, respectively. The reaction was stopped after 15 min by addition of 0.1 ml of cold 1.12 M trichloroacetic acid. The amount of inorganic phosphate released was measured by the method of Fiske and Subbarow.<sup>37</sup> IDP, UDP and CDP in 3 mM concentration were used for the study of substrate specificity. Nonhydrolyzable adenosine nucleotide analogues: App(NH)p, Ap(CH<sub>2</sub>)p, App(CH<sub>2</sub>)p and ATP( $\gamma$ S) were used at a concentration of 0.22 mM. The concentration of the regular substrates ADP or ATP was 0.25 mM.

#### *Selective proteolysis.*

Proteolysis with *trypsin* was performed by incubation of BBMV (4.5 mg/ml) with activated trypsin solution (0.25%) containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 2 mM  $\text{CaCl}_2$  and 1 mM dithiothreitol. *Papain* treatment was essentially the same, except the activating solution for *papain* contained 2.5 mM MES (pH 6.2) and 5 mM cysteine. Controls were incubated for exactly the same time with the buffer, but without the protease added.

### *Treatment with lectins.*

*Concanvalin A* and *lectin* from *Arachis Hypogaea*. Lectins were dissolved in the sample buffer and incubated with BBMV for 20 min. The final concentration of the respective lectin was as indicated where appropriate.

### *Reaction with anti-CEA monoclonal antibodies.*

Monoclonal anti-CEA antibodies, directed towards two different epitopes were incubated with BBMV for 60 minutes. Secondary antibodies conjugated with peroxidase were used for identification of the complex. The sensitivity level in detection of CEA was 0.5 ng/mL.

### *Chemical modification of rat kidney brush-border ecto-ADPase and ecto-ATPase*

Brush-border membrane vesicles BBMV (0.03 mg of proteins) were incubated for 30 min with specified agent added from water ( $\text{NaN}_3$ , NEM, ouabain, oxidized glutathione, dithiothreitol) or ethanol stock solutions (diethyl pyrocarbonate). The final concentration of the respective inhibitor is indicated where appropriate.

Statistical analysis of the data was performed using the Student *t*-test at the level of significance  $P < 0.01$ .

## RESULTS

### **Localization of ADPase and ATPase activities**

#### *Lack of inhibition of ADPase and ATPase by limited proteolysis*

Extracellular localization of the nucleotide hydrolyzing site distinguishes ectonucleotidases from other nucleotide degrading enzymes originating from within the cell. Therefore, it could be expected that limited proteolysis of the outward oriented part of the protein might destroy the enzyme activity. Data obtained following the treatment with trypsin and papain revealed no pronounced effects of proteases. An inhibition of about 28% was observed for the ATP and ADP hydrolysis after 3 hours of treatment with trypsin, and almost no effect when papain was applied. It is important to point out that both reactions, *i.e.* ATP and ADP hydrolysis, were affected to the same extent.

### *Solubilization of ecto-ATPase and ecto-ADPase*

Various treatments have been applied to increase the specific activity of the enzyme. CHAPS, the zwitterionic detergent was chosen because it proved to be very efficient in solubilizing other membrane proteins<sup>40</sup> and polidocanol, the nonionic detergent, was successfully used for solubilization of  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$  ATPase from rat liver plasma membranes.<sup>41</sup> Detergents were completely ineffective in solubilizing ATPase and ADPase, but they exhibited strong inhibitory effects. At a CHAPS concentration of 0.11% only 16% of ATPase activity and 10% of ADPase activity were solubilized. Increased detergent concentration (0.75%) produced additional inhibition of both enzyme activities. Polidocanol was slightly better than CHAPS, but we did not succeed in solubilizing more than 28% of the hydrolyzing activity present in the crude BBMV preparation. Enzyme activities were predominantly found in the pelleted fraction. Total protein recovery, both in the supernatant and in the pelleted fraction, ranged between 95 and 100%. However, the recovered nucleotide hydrolyzing activities never exceeded 70%. The level of inhibition was comparable with either ATP or ADP as substrate.

### *Effects of lectins*

Various lectins are reported to modulate the activity of plasma membrane ATPase, most probably because of the binding to the oligosaccharide moiety of the enzyme molecule.<sup>42</sup> According to our measurements, the effect of two lectins, concanavalin A and that from *Arachis Hypogaea*, on the ADPase and ATPase activity was negligible. A moderate inhibition of 19% was induced when the concentration ratio of lectin from *Arachis Hypogaea* to protein was 10:1 and no inhibition was detected when the lectin to protein ratio was increased to 25:1. Experiments with concanavalin A showed that neither of the enzyme activities was affected by this lectin. 56 mg/l of Con A caused no inhibition at all, while a ten times higher concentration inhibited both activities by only 5–10%.

## **Biochemical studies**

### *Effect of pH*

A change in hydrogen ion concentration in the tested range of pH 3.62–10.22 produced a bell shaped curve both with ecto-ADPase and ecto-ATPase activity. The pH optimum was established between pH 7.2 and 8.6 for ATP and ADP as substrates (Figure 1.). As the optimum falls into the alkaline pH

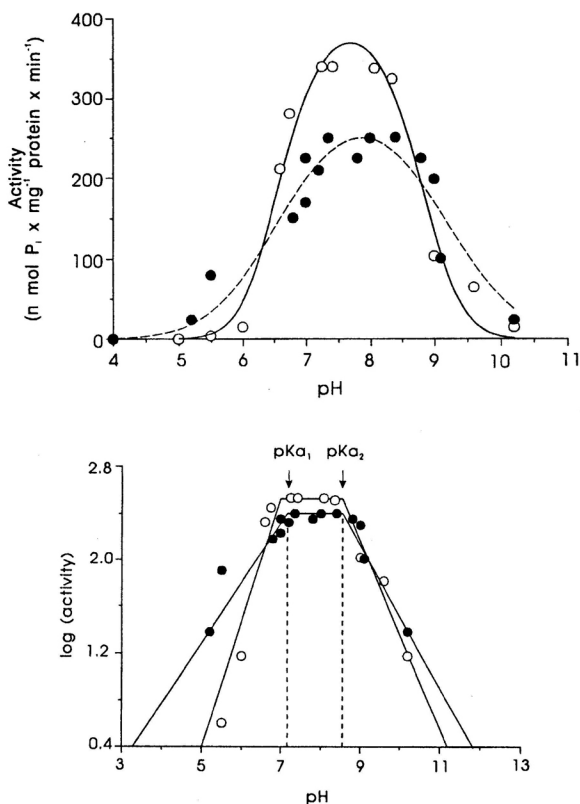


Figure 1. Influence of pH on the reaction rate of ecto-ADPase (closed circles) and ecto-ATPase (open circles). Reaction was followed in 100 mM of the following buffers: sodium citrate pH 3.62 – 5.27; Hepes-Tris pH 6.86 – 7.80; Tris-HCl pH 8.0 – 8.9 and sodium bicarbonate pH 9.1 – 10.22. Calcium concentration was equimolar to substrate concentrations of 1.5 mM ADP and 3 mM ATP, respectively.

region, a possible interference of alkaline phosphatase has to be excluded. Since levamisole 1 mM was routinely included in the reaction medium, its concentration was elevated to 5 mM. The increased levamisole concentration caused only a slight decrease of the measured rate of the ADP hydrolysis, suggesting a negligible contribution of alkaline phosphatase under standard reaction conditions (results not shown). The Dixon plot of the measured reaction rate vs. pH revealed that the biggest change in the reaction rate occurs at pH 7.2 and pH 8.6. Since pH 7.2 corresponds to  $pK_a$  of histidine residues and pH 8.6 to  $pK_a$  of the terminal amino group or sulphhydryle group, it was concluded that these groups play an important role in the active site in both ADP and ATP hydrolyzing activities.

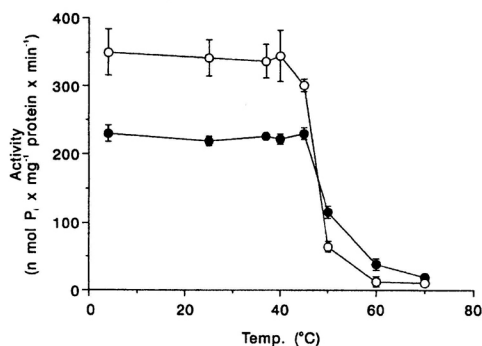


Figure 2. Effect of heat treatment on ecto-ADPase (closed circles) and ecto-ATPase (open circles). Brush-border membrane vesicles were incubated at temperatures varying from 4 to 70 °C for 3 minutes. Enzyme activities were then measured as described under Methods. Results are means  $\pm$  SD from two independent experiments, each done in triplicate.

#### *Temperature inactivation profiles*

Further support to the concept that a common catalytic site might be involved in the hydrolysis of ADP and ATP has been obtained by comparing the inactivation profiles of ADPase and ATPase activities after heat denaturation. As shown in Figure 2, the fraction of the residual activity following denaturation by heat treatment is very similar for both ATP and ADP, with a temperature of 48 °C corresponding to 50% of the remaining activity.

#### *Effect of Ca<sup>2+</sup> and Mg<sup>2+</sup> ions*

In our previous studies we found that ecto-ADPase and ecto-ATPase from BBMV can be activated by either Ca<sup>2+</sup> or Mg<sup>2+</sup> ions. The rate of ADP hydrolysis followed the simple Michaelis-Menten kinetics assuming a single catalytic site, when assayed as a function of Ca-ADP concentration, at a fixed concentration of Ca<sup>2+</sup>.<sup>30</sup> In order to examine the effects of the divalent cation on the rate of nucleotide hydrolysis, we measured the activity in the presence and in the absence of exogenous cations added. As shown in Table I, in the absence of divalent cations, the activity was reduced to less than half of the values obtained when the substrate to Ca<sup>2+</sup> molar ratio was fixed at 1:1. Endogenous Mg<sup>2+</sup> and Ca<sup>2+</sup> contained in the BBMV preparation, as determined by atomic absorption spectrometry, were very low, amounting to 0.036 mM and 0.055 mM, respectively. Therefore, when 1.5 mM EGTA was added to the reaction mixture (in order to chelate trace amounts of Ca<sup>2+</sup> in BBMV preparation), the hydrolysis of ADP and similarly of ATP was further

TABLE I

Effect of detergents on ATPase and ADPase from brush border membrane vesicles.

Treatment		Solubilized protein (%)	Solubilized ATPase (%)	ATPase specific activity	Solubilized ADPase (%)	ADPase specific activity
None	—	—	—	687	—	416
CHAPS	0.11 %	35	16	314	10	119
	0.75 %	47	5	73	2	18
Polidocanol	0.11 %	45	26	397	28	259
	0.75 %	40	24	413	28	291

Membranes (2 mg prot./mL) were preincubated with indicated concentrations of CHAPS and polidocanol in the Hepes-Tris buffer (pH 7.4) for 30 min at 4 °C. The pellet with the remaining proteins was removed by centrifugation at 33000 × g for 45 min. Activities are expressed as nmol P<sub>i</sub> × mg<sup>-1</sup> prot. × min<sup>-1</sup>. Each point represents the mean value of three independent experiments.

reduced to only 13–19% of the maximal, Ca<sup>2+</sup> stimulated activity. Addition of 1.5 mM EDTA caused even greater reduction of the activity, leaving only 4–8% of the control values. The low activity observed in the presence of 1.5 mM EGTA and 1.5 mM EDTA, when neither Ca<sup>2+</sup> nor Mg<sup>2+</sup> were available for the

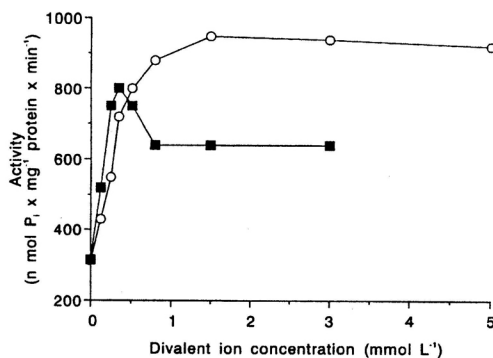


Figure 3. Effect of Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations on the reaction rate of ADP hydrolysis. Ca<sup>2+</sup> ADPase and Mg<sup>2+</sup> ADPase activities were measured in Tris-Hepes buffer pH 7.4, as described in Methods. The reaction was initiated by addition of ADP, final concentration 1.5 mM, to which the indicated concentration of respective cation, in the form of Cl<sup>-</sup> salt, was added. In the experiments where Ca<sup>2+</sup> influence was followed no Mg<sup>2+</sup> was added to the medium and vice versa. Data are means from five different enzyme preparations.

formation of the complex with  $\text{ADP}^{3-}$ , would suggest that the complex cation-ADP is a true substrate for ADPase. Addition of either  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  stimulated the catalytic reaction but  $\text{Ca}^{2+}$  was more effective. As shown in Figure 3 the ADPase activity in the presence of 1.5 mM  $\text{Ca}^{2+}$  was higher than when measured with an analogous  $\text{Mg}^{2+}$  concentration. The addition of  $\text{Mg}^{2+}$  ions in the low range of concentrations (0.1–0.4 mM) caused a considerable increase of ADPase activity. However, further increase of  $\text{Mg}^{2+}$  concentration caused a limited drop in the activity but, in all cases, it was found to be higher than the analogous activity of the reaction not stimulated by cations (Figure 3).

### Substrate specificity

The rate of hydrolysis of various nucleoside diphosphates: IDP, ADP, UDP, and CDP and of adenosine triphosphate was measured in the presence of 3 mM  $\text{Ca}^{2+}$  as activating cation. Table II shows that maximal hydrolysis rates are similar for all substrates tested, with the exception of ATP, from which  $P_i$  was hydrolyzed at a 33% higher rate than from ADP. IDP was shown to be a relatively better substrate than other nucleoside diphosphates.

These results are not conclusive regarding distinction whether ADP hydrolysis occurs at the same active site as the hydrolysis of ATP. This is why  $P_i$  production was monitored in the experiments with a combination of ADP and ATP, both at high (3 mM) and at low (0.5 mM) substrate concentration. No further increase in the rate of phosphate cleavage could be observed following the addition of 3 mM ADP.

TABLE II

Effect of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on the reaction rate of ATP and ADP hydrolysis by brush border membrane vesicles

Addition	Relative activity (%)	
	ADPase	ATPase
None	33 ± 2	41 ± 2
$\text{Ca}^{2+}$ (2mM)	100	100
$\text{Mg}^{2+}$ (2mM)	66 ± 3	74 ± 3
EDTA (1.5 mM)	4 ± 1	8 ± 3
EGTA (1.5mM)	13 ± 6	19 ± 6
EDTA (1.5mM) + EGTA (1.5mM)	4 ± 2	8 ± 2

The rate of phosphate production was determined in 50 mM Hepes-Tris buffer (pH 7.4) containing 100 mM KCl, 50 mM mannitol, 2 mM ADP or 2 mM ATP and divalent cations, EDTA or EGTA as indicated. Data are means ± SD from 3-5 membrane preparations

In order to provide more information concerning the possible existence of distinct and independent ATPase and ADPase catalytic sites, we used different ATP and ADP nonhydrolyzable analogues and compared their effects upon the ATP and ADP hydrolysis, respectively. We, therefore, tested the ability of ATP analogues to block ATP hydrolysis, and that of ADP analogues to block ADP hydrolysis. It could be assumed that ATPase and ADPase co-exist as two separate catalytic sites, with different substrate specificities. In that case, the inhibition of ATP hydrolysis due to the presence of nonhydrolyzable ATP analogues should not have a great influence on ADP hydrolysis and vice versa. Indeed, the addition of App(NH)p exerted strong inhibition of the ATP hydrolysis, while ADP hydrolysis was not affected (Table III). At the 0.22 mM concentration of App(NH)p only 69% of the original ATPase activity remained, while ADP hydrolysis was not inhibited at all. Quite unexpectedly though, Ap(CH<sub>2</sub>)p was more efficient in inhibiting the ATP than ADP hydrolysis. The degree of inhibition was concentration dependent (results not shown). In the case of App(CH<sub>2</sub>)p, neither ATP nor ADP hydrolysis were affected. It has been reported that ATP( $\gamma$  S) could be slowly hydrolyzed by various ATPases.<sup>45</sup> When we tested ATP( $\gamma$  S) as a substrate analogue, we found that it inhibited ADP hydrolysis and ATP hydrolysis to a similar extent.

TABLE III

Substrate specificity of ecto-nucleotidase in brush border membrane vesicles

Substrate	Relative nucleotidase activity (%)
ATP (3 mM)	100
ADP (3 mM)	67.3 $\pm$ 3.1
IDP (3 mM)	82.8 $\pm$ 2.6
UDP (3 mM)	63.4 $\pm$ 3.6
CDP (3 mM)	59.3 $\pm$ 2.8
ATP (3 mM) + ADP (3 mM)	89.4 $\pm$ 5.6
ATP (0.5 mM) + ADP (0.5 mM)	85.2 $\pm$ 3.7*

The rate of phosphate production was determined in 50 mM Hepes-Tris buffer (pH 7.4) containing 100 mM KCl, 3 mM CaCl<sub>2</sub>, 50 mM mannitol as described in Methods. The reaction was initiated with the addition of the substrates.

The rates of 3 mM ATP hydrolysis which was taken as 100 % corresponds to 756  $\pm$  52 nmol P<sub>i</sub> mg<sup>-1</sup> prot.  $\times$  min<sup>-1</sup>.

\* The rate of 0.5 mM ATP hydrolysis which was taken as 100 % corresponds to 574  $\pm$  16 nmol P<sub>i</sub>  $\times$  mg<sup>-1</sup> prot.  $\times$  min<sup>-1</sup>.



*Chemical modification of ecto-ADPase and ecto-ATPase.*

The Dixon plot of the pH dependence curve (Figure 1.) pointed to histidine and cysteine as two amino acids possibly involved in the hydrolysis of ADP. Therefore, we have used specific chemical modifiers directed towards these residues and assayed the enzyme activity in such conditions.

The reaction of diethyl pyrocarbonate (DEPC) with proteins is highly specific to histidine residues when pH is near neutral values.<sup>46</sup> In the weakly alkaline solutions, however, DEPC can react also with lysine, tyrosine and cysteine. Strong inhibition of BBMV ATPase and ADPase was observed after 30 min of incubation with 1mM DEPC (Figure 4). Hydroxylamine

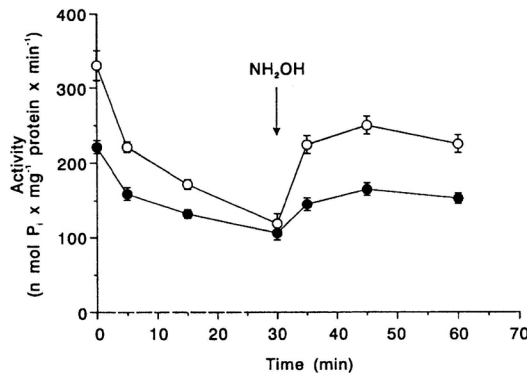


Figure 4. Time dependence of DEPC inhibition of ADP (closed circle) and ATP hydrolysis (open circles). DEPC was added to the 0.250 ml of reaction mixture containing 50 mM Tris-Hepes pH 7.4, 5 mM CaCl<sub>2</sub> and 100 mM KCl, as 0.01 ml of freshly prepared ethanolic solution, and preincubated with the vesicles at 4 °C. Thirty minutes after addition of DEPC (indicated by arrow), hydroxylamine was added to the final concentration of 200 mM. At various time points, aliquots with 0.025 mg of proteins were assayed for ADPase and ATPase activity. Each point represents the mean value ± SD from two different enzyme preparations, each done in triplicate. Data are presented as percent of the activity in the absence of inhibitor. The rate of ADP hydrolysis was 223 nmol Pi × mg<sup>-1</sup> prot. × min<sup>-1</sup> and that of ATP hydrolysis was 330 nmol Pi × mg<sup>-1</sup> prot. × min<sup>-1</sup>.

rapidly reversed most, but not all, of the lost activity. Since hydroxylamine reverses histidine or tyrosine, but not lysine or cysteine modification<sup>5</sup>, the former two appeared to be essential for the enzyme activity.

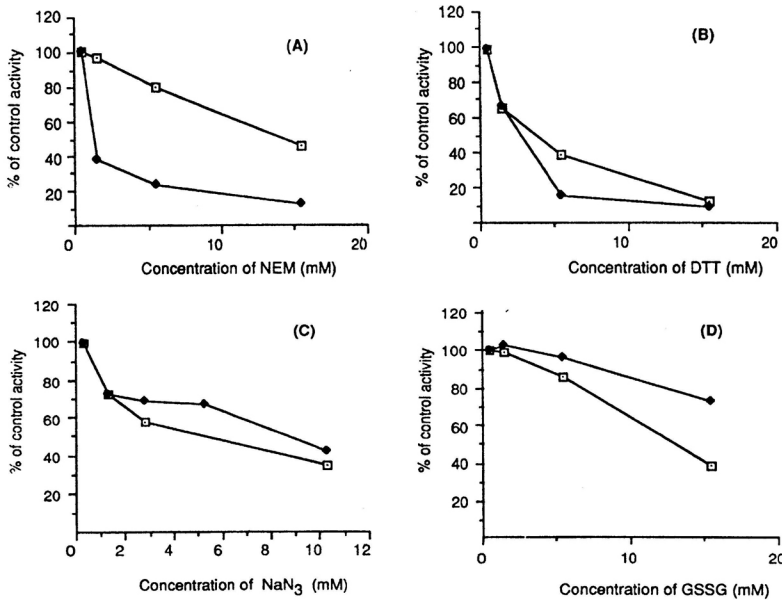


Figure 5. Activity of ecto-ADPase (diamonds) and ecto-ATPase (open squares) from the brush-border membrane vesicles in the presence of various concentrations of four inhibitors. *N*-ethylmaleimide (NEM), panel A; dithiothreitol (DTT), panel B; sodium azide (NaN<sub>3</sub>), panel C; oxidized glutathione (GSSG), panel D. Brush-border membrane vesicles were preincubated with the inhibitor at the concentration indicated for 30 min; except for DTT where preincubation lasted for 3.5 hours. Data are expressed as percentages of the control nucleotidase activity obtained with 1.5 mM of respective substrate.

There has been some ambiguity concerning the role of SH groups in the activity of ecto-nucleotidases. NEM, dithiothreitol and oxidized glutathione were applied to test specific effects on SH groups. Concentrations of the respective inhibitors were in the range of 1–15 mM. The results in Figure 5 showed a noncompetitive inhibition produced by NEM in the case of ADP hydrolysis and a mixed inhibition was observed in the case of ATP hydrolysis. In separate experiments, we determined the constant for NEM inhibition of ADP hydrolysis and found it to be  $K_i=2.0$  mM. The inhibition produced by DTT was much more pronounced. Sodium azide at concentrations higher than 1 mM caused a significant inhibition of both ADP and ATP hydrolysis.

A comparison of the ecto-ADPase and ecto-ATPase properties is shown in Tables IV and V. According to the results obtained, it appears that these two nucleotide hydrolyzing activities share a great deal of similarities.

TABLE IV

Inhibition of ADPase and ATPase by nonhydrolysable analogues of ATP: App(NH)p, App(CH<sub>2</sub>)p, ATP(γS) and of ADP: Ap(CH<sub>2</sub>)p, respectively

Analogue 220 μM	Relative activity (%)	
	ATPase	ADPase
Control	100	100
App(NH)p	69 ± 3	103 ± 1
App(CH <sub>2</sub> )p	108 ± 4	112 ± 7
ATP(γS)	76 ± 3	71 ± 8
Ap(CH <sub>2</sub> )p	57 ± 3	75 ± 3

Brush border membrane vesicles were assayed for ATPase and ADPase activity in the presence of 220 μM of the analogue indicated. Reaction was initiated by the addition of the substrate (ADP or ATP) with the final concentration 250 μM. Vesicles were preincubated with analogue indicated for 30 min at 37 °C. Control activities for the ATPase and ADPase reactions in the absence of analogues added were 206 ± 3 nmol P<sub>i</sub> × mg<sup>-1</sup> prot. × min<sup>-1</sup> and 140 ± 23 nmol P<sub>i</sub> × mg<sup>-1</sup> prot. × min<sup>-1</sup>, respectively. Data presented are percentages (mean ± SD) of the control nucleotidase activity obtained from two or three membrane preparations.

TABLE V

Comparison of the ecto-ADPase and ecto-ATPase activities

Enzyme property	ADPase	ATPase
Number of catalytic sites	1	2*
K <sub>m</sub> (mM)	0.38	0.160*
Substrate specificity	IDP > ADP > UDP > CDP	9.0*
Sodium azide (1-15 mM)	inhibition	ITP > ATP > UTP > CTP*
NEM (1-15 mM)	noncompetitive inhibition (K <sub>i</sub> = 2mM)	inhibition
Glutathione oxidized (1-15 mM)	slight inhibition	mixed inhibition slight inhibition
Dithiothreitol	mixed inhibition	mixed inhibition
Ouabain	no influence	no influence
Diethyl pirocarbonate	inhibition reactivation with NH <sub>2</sub> OH	inhibition reactivation with NH <sub>2</sub> OH
App(NH)p	no influence	inhibition
Ap(CH <sub>2</sub> )p	inhibition	inhibition
App(CH <sub>2</sub> )p	no influence	no influence
ATP(γS)	inhibition	inhibition
Trypsin	no influence	no influence
Papain	no influence	no influence
CHAPS	inhibition	inhibition
Polidocanol	inhibition	inhibition
Thermal denaturation	T <sub>1/2</sub> 48 °C	T <sub>1/2</sub> 48 °C

\*Ref 30

## DISCUSSION

The results presented provide evidence that brush-border membrane vesicles, purified from homogenates of rat kidney cortex, possess an ecto-enzyme activity responsible for the hydrolysis of both ATP and ADP. The enzyme catalyzes hydrolysis of gamma and beta groups from tri- and diphospho nucleosides, yielding nucleoside monophosphate and inorganic phosphate as products. We assumed that the enzyme described in this paper belongs to the ecto-ATPase family because the ATP and ADP hydrolysis measured resulted from the action of an enzyme appearing in the BBMV that are impermeable to ATP and are prepared in such a way that the orientation is largely with the right side out.<sup>32, 33</sup> Moreover, this ATPase exhibits a broad specificity towards nucleoside di- and tri-phosphates similar to that of ecto-ATPases in other mammalian cells and tissues. Kinetic studies of the hydrolytic reaction of ATP and ADP, performed in the presence of different inhibitors and activators, revealed a great similarity between these two catalytic processes.

The hydrolysis rate of ATP and ADP, respectively, was not affected by the presence of various inhibitors known to inhibit other ATPases. The results showed that ecto-ADPase from BBMV was insensitive to inhibitors of P-type ATPases, such as ouabain and vanadate, inhibitors of V-type ATPases (NEM in concentrations lower than 1 mM) and inhibitors of F-type ATPases (oligomycin and azide in concentrations lower than 1 mM).<sup>7, 30</sup>

The enzyme described in this paper is apparently resistant to limited proteolysis, as well. Trypsin and papain caused only a slight, but equal, drop of both the ATPase and ADPase activities. Though unexpected, this high resistance towards proteolysis may be understood as a result of extensive glycosylation of the enzyme molecule. Kidney ecto-ATPase is known to be highly glycosylated. Glycosylation could indeed protect the enzyme against the extracellular proteases.<sup>2,13</sup> The role of the carbohydrate part of the enzyme molecule was additionally tested in the presence of various lectins. Riordan *et al.* have reported that Con A and wheat germ agglutinin produced a marked stimulation of the liver plasma membrane  $Mg^{2+}$  ATPase.<sup>4</sup> However, our experiments with kidney BBMV did not demonstrate any stimulation of the ADPase activity with lectins from *Arachis Hypogaea* and concanavalin A, respectively. More information about the structural features of the enzyme could be obtained using purified protein molecules only. Unfortunately, despite several attempts, conditions could not be provided to ensure effective solubilization of the enzyme with a simultaneous retention of ecto-ADPase activity. Polidocanol and CHAPS caused either partial or complete loss of the ATP and ADP hydrolyzing activity, respectively.

Kinetic studies of the kidney ecto-ADPase revealed that ADP hydrolysis in the presence of  $Ca^{2+}$  followed the simple Michaelis-Menten kinetics with a single catalytic site and an apparent  $K_m = 0.38 \pm 0.06$  mM.<sup>30</sup>

The rate of hydrolysis was substantially reduced when no cation was added to the reaction medium and even higher reduction was detected when 1.5 mM EGTA and 1.5 mM EDTA were added in order to chelate trace amounts of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  present in the preparation. Addition of 1.5 mM EGTA, *i.e.* the absence of  $\text{Ca}^{2+}$ , was less inhibitory than the absence of  $\text{Mg}^{2+}$  produced by the addition of 1.5 mM EDTA. It seems that a small amount of divalent ions bound to the membrane is needed to establish full activity. According to atomic absorption measurements the internal concentrations of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  ions introduced with the membrane preparations were 0.036 mM and 0.055 mM, respectively. These values represent the total concentration of the cations found in the BBMV, but this does not necessarily prove that these ions were bound to the enzyme studied (Table I and Figure 3). It has been shown, however, that the substrate for the majority of ADPases is a  $(\text{Ca}^{2+}/\text{Mg}^{2+})$ -ADP complex, rather than free  $\text{ADP}^{3-}$ .  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  do not activate the rate of hydrolysis in exactly the same manner. When the reaction rate is plotted as a function of  $\text{Ca}^{2+}$  concentration at a fixed ADP concentration, the rate increases steadily up to 1.5 mmol of  $\text{Ca}^{2+}$  when the equimolar ratio of substrate to cation is reached. No significant changes occur by further  $\text{Ca}^{2+}$  increase. This is in contrast to the results obtained with  $\text{Mg}^{2+}$  where a sharp increase of activity is attained up to 0.4 mM  $\text{Mg}^{2+}$  while a further increase in the  $\text{Mg}^{2+}$  concentration caused a relative drop in the activity (Figure 3). These results can be rationalized only if different binding sites for the  $\text{Mg}$  - ADP complex and for the  $\text{Mg}^{2+}$  ions on the enzyme molecule are considered. Occupancy of the  $\text{Mg}^{2+}$  site inhibits rather than activates the enzyme. Similar observations with the purified porcine pancreatic ATP diphosphohydrolase were described by Laliberte *et al.*<sup>48</sup> They assigned the  $\text{Mg}^{2+}$  site as a low affinity site.

Several experiments were designed in order to define the number of distinct catalytic sites responsible for the hydrolysis of ADP and ATP. The results obtained with nonhydrolyzable ATP and ADP analogues  $\text{App}(\text{CH}_2)_p$ ,  $\text{ATP}(\gamma \text{S})$ ,  $\text{Ap}(\text{CH}_2)_p$  demonstrated a similar competition with both ATP and ADP hydrolysis (Table III), thus indicating a great deal of parallelism between these two processes. Some difference in the response to the presence of a particular analogue during the catalytic reaction could only be seen in terms of the degree of inhibition produced. The ATP hydrolysis was more sensitive to the presence of  $\text{App}(\text{NH})_p$  than the corresponding phosphate cleavage from ADP. Although the rate of ATP hydrolysis was considerably reduced, the rate of ADP hydrolysis was apparently unchanged.

The fact that ADP and ATP were hydrolyzed at a comparable rate in the presence of substrate analogues and that the combination of ATP and ADP in the low (0.5 mM) and in the high (3 mM) concentration range does not appear to be additive calls for further elucidation (Table II). In our previous work, we tried to pick up the reaction products of the ATP hydrolysis. Identification of the reaction products by thin layer chromatography showed that

adenosine and inorganic phosphate were the only reaction products, without any accumulation of ADP as an intermediate.<sup>30</sup> The apparent  $K_m$  values for both ATP and ADP are of the same order of magnitude. Therefore, the ADP would initially accumulate if it was released from the enzyme during ATP hydrolysis, as a consequence of the significantly higher initial concentration of ATP. The rates of hydrolysis in the mixed-substrate reactions were approximately 10% larger than the arithmetic mean of the rates found in the presence of individual substrates only. This is very different from what one would expect if the activities were additive and independent. Thus, the results obtained support the scheme of sequential hydrolysis of ATP through the stepwise reactions ATP – ADP – AMP occurring after the single enzyme – substrate interaction. This is in accordance with the findings of Yagi *et al.*<sup>49</sup> who found similar results in the vascular endothelial and smooth muscle cells, respectively. However, these findings are in contrast with the results of Gordon *et al.*<sup>50</sup> who found that the hydrolysis of extracellular ATP and ADP is catalyzed by two separate enzymes in cultured endothelial cells from pig aorta. In addition, our experiments confirm that ecto-ADPase from BBMV exerts a nucleotidase activity with a broad substrate specificity. Various nucleoside diphosphates were degraded at comparable rates (Table II). These results point to an interesting analogy with the substrate specificity measured for ecto-ATPase.<sup>30</sup> Namely, the relative affinity for nucleoside triphosphates was very similar: ITP>ATP>GTP>UTP>CTP. As demonstrated, the ecto-ADPase from BBMV does not distinguish between the different purine and pyrimidine bases in the nucleotide substrates. These findings are in accordance with our previous findings for ecto-ATPase, only when assayed at a low (0.1 mM) concentration of substrates.<sup>30</sup> At saturating conditions (5mM), purine nucleoside triphosphates proved to be better substrates than pyrimidine nucleotides. The broad specificity observed agrees well with the findings for ecto-nucleotidase from liver<sup>3</sup>, from sarcoma plasma membranes<sup>7</sup> and from bovine lung<sup>11</sup>. It argues for the name of the enzyme to be ecto-nucleotidase rather than ecto-ATPase or ecto-ADPase.

In addition, numerous biochemical properties of the kidney brush-border ecto-nucleotidase suggest that this enzyme may belong to the recently described ATPDase Type III (EC 3.6.1.5.) that was found in the bovine lung.<sup>11</sup> Authors have found a single band in the PAGE under nondenaturing conditions that was responsible for the hydrolysis of ATP and ADP. These results are in good agreement with a great deal of similarities that we found between ecto-ADPase and ecto-ATPase in BBMV. Similar behaviour with chemical modification agents, inactivation of histidine residues by pretreatment with DEPC and subsequent reactivation with  $\text{NH}_2\text{OH}$ , insensitivity to proteases, incompatibility with the detergents, insensitivity to ouabain, levamisole, sensitivity to 10 mM sodium azide, similar kinetic properties, pH dependence profiles, heat inactivation both with ADP and ATP as substrates support this assumption.

However, the enzyme described has some striking biochemical differences from the  $\text{Ca}^{2+}$  sensitive ATP diphosphohydrolase from pig pancreas (Type I), including a different pH optimum and much higher  $K_m$  values for ATP and ADP.<sup>43, 48</sup>

Studies involving chemical modifiers (Figures 4, 5), detergents and proteases demonstrate the hardy nature of this enzyme, similar to the liver ecto-ATPase<sup>41</sup> and  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ATPase from transverse tubule.<sup>46</sup>

However, it appears that SH groups play an important role in the active enzyme conformation as suggested from the Dixon plot of the pH rate dependence. The results obtained with dithiothreitol and with high concentrations of NEM strongly support this assumption. Both ATP and ADP hydrolysis were affected although, at low substrate concentrations, ADP hydrolysis was influenced more effectively.

Data presented in Table IV compare the results obtained with the ATP and ADP hydrolyzing activity. There is no doubt that most of the agents used produce analogous effects regardless of whether they affect the enzyme structure, substrate or compete in the reaction. This is why it can be concluded that we may cope with a single enzyme able to hydrolyze ADP, as well as ATP, in a stepwise manner. Additional data are needed to support quantitatively the detailed mechanism.

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## REFERENCES

1. M. Lambert, and J. Christophe, *Biochem. J.* **91** (1978) 485.
2. I. Sabolić, O. Čulić, S. H. Lin, and D. Brown, *Am. J. Phys.* **262** (1992) F217.
3. S. H. Lin, *J. Biol. Chem.* **260** (1985) 10976.
4. E. L. Gordon, J. D. Dearson, E. S. Dickinson, D. Moreau, and L. L. Slakey, *J. Biol. Chem.* **264** (1989) 18986.
5. T. L. Kirley, *J. Biol. Chem.* **263** (1988) 12682.
6. A. Filippini, R. E. Taffs, T. Agui, and H. Sitkovsky, *J. Biol. Chem.* **265** (1990) 334.
7. A. F. Knowles, R. E. Isler, and J. F. Reece, *Biochim. Biophys. Acta* **731** (1983) 88.
8. J. L. Gordon, *Biochem. J.* **233** (1986) 309.
9. F. Turrini, I. Sabolić, Z. Zimolo, B. Moewes, and G. Burckhard, *J. Membr. Biol.* **107** (1989) 1.
10. S. H. Lin, *J. Biol. Chem.* **260** (1985) 7850.
11. M. Picher, Y. P. Cote, R. Beliveau, M. Potier, and A. R. Beaudoin, *J. Biol. Chem.* **268** (1993) 4699.
12. Y. P. Cote, R. Beliveau, M. Potier, and A. R. Beaudoin, *Biochim. Biophys. Acta* **1078** (1991) 187.
13. S. H. Lin, and G. Guidotti, *J. Biol. Chem.* **264** (1989) 14408.
14. S. H. Lin, O. Čulić, D. Flanagan, and D. C. Hixson, *Biochem. J.* **278** (1991) 155.

15. O. Čulić, Q. H. Huang, D. Flanagan, D. C. Hixson, and S.-H. Lin, *Biochem. J.* **285** (1992) 47.
16. P. H. Cheung, N. L. Thompson, O. Čulić, D. C. Hixson, and S.-H. Lin, *J. Biol. Chem.* **268** (1993) 13015.
17. S. Benchimol, A. Fuks, S. Jothy, N. Beauchemin, K. Shiota, and C. P. Stanners, *Cell* **57** (1989) 327.
18. A. M. Mahrenholz, C. H. Yeh, J. E. Shively, and S. A. Hefta, *J. Biol. Chem.* **268** (1993) 13015.
19. Y. Hinoda, M. Neumaier, S. A. Hefta, S. Drzeniek, C. Wagner, L. Shively, L. J. F. Hefta, J. E. Shively, and R. J. Paxton, *Proc. Natl. Acad. Sci. USA* **85** (1988) 6959.
20. A. F. Knowles, *Biochem Biophys. Res. Commun.* **207** (1995) 535.
21. S. M. Najjar, D. Accili, P. Neubert, J. Lernberg, R. Margolis, and S. I. Taylor, *J. Biol. Chem.* **268** (1993) 1201.
22. A. I. Katz, and A. Doucet, *Int. J. Biochem* **12** (1980) 125.
23. J.-B. Gouyon, and J.-P. Guignard, *Dev. Pharmacol. Ther.* **13** (1989) 113.
24. L. J. Arend, G. L. Bakris, J. C. Burnett Jr, and C. Megerian, *J. Lab. Clin. Med.* **110** (1987) 406.
25. M. G. Collis, G. S. Baxter, and J. R. Keddie, *J. Pharm. Pharmacol.* **38** (1986) 850.
26. S. Kartha, B. Atkin, T. E. Martin, and G. Toback, *Exp. Cell Res* **200** (1992) 219.
27. K. Yagi, Y. Arai, N. Kato, K. Hirota, and Y. Miura, *Eur. J. Biochem.* **180** (1989) 509.
28. J. D. Pearson, J. S. Charleton, and J. L. Gordon, *Biochem. J.* **190** (1980) 421.
29. J. D. Pearson, J. S. Charleton, and J. L. Gordon, *Biochem. J.* **190** (1980) 421.
30. O. Čulić, I. Sabolić, and T. Žanić-Grubišić, *Biochim. Biophys. Acta* **1030** (1990) 143.
31. J. Luthje, A. Schomburg, and A. Ogilvie A. *Eur. J. Biochem.* **175** (1983) 285.
32. J. Biber, B. Steger, and W. Haase, *Biochim. Biophys. Acta* **647** (1981) 169.
33. C. Evers, W. Haase, H. Murer, and R. Kinne, *Membr. Biochem. I* (1978) 203.
34. E. J. King, and A. R. Armstrong, *A. R. Can. Med. Assoc. J.* **31** (1934) 376.
35. W. Berner, and R. Kinne, *Eur. J. Physiol. (PflugersArch.)* **361** (1976) 269.
36. M. M. Bradford, *M. M. Anal. Biochem.* **72** (1976) 248.
37. C. N. Fiske, and Y. SubbaRow, *J. Biol. Chem.* **66** (1925) 375.
38. S. H. Lin, and W. E. Russell, *J. Biol. Chem.* **263** (1988) 12253.
39. J. E. Smolen, and G. Weismann, *Biochim. Biophys. Acta* **512** (1978) 525.
40. L. M. Hjelmeland, *Proc. Natl. Acad. Sci. USA* **77** (1980) 6368.
41. S.-H. Lin, and J. N. Fain, *J. Biol. Chem.* **259** (1984) 3016.
42. J. R. Riordan, M. Slavik, and N. Kartner *J. Biol. Chem.* **252** (1977) 5449.
43. D. LeBel, G. G. Poirier, St-Jean, P. Phaneuf, J. F. Laliberte, and A. R. Beaudoin, *J. Biol. Chem.* **255** (1980) 1227.
44. J. J. Sarkis, J. A. Guimaraes, and J. M. C. Ribeiro, *Biochem. J.* **233** (1986) 885.
45. D. J. Montague, T. J. Peters, and H. Baum, *Eur. J. Biochem.* **139** (1984) 529.
46. A. Saborido, G. Moro, and A. Megias, *J. Biol. Chem.* **266** (1991) 23490.
47. R. Hori, H. Macgawa, M. Kato, T. Katsura, and K-i. Inui, K-i. *J. Biol. Chem.* **264** (1989) 12232.
48. J. F. Laliberte, and A. R. Beaudoin *J. Biol. Chem.* **25** (1982) 3869.
49. K. Yagi, M. Shinbo, M. Hashizume, L. S. Shimba, S. Kurimura, and Y. Miura, *Biochem. Biophys. Res. Commun.* **180** (1991) 1200.
50. E. L. Gordon, J. D. Pearson, and L. L. Slakey, *J. Biol. Chem.* **261** (1986) 15496.



**SAŽETAK****Aktivnost ekto-ADPaze u membranama četkaste prevlake  
bubrega štakora**

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U radu je opisana aktivnost ekto-enzima odgovornog za hidrolizu ATP, ADP, te di- i trifosfata drugih nukleozida u vezikulama izoliranim iz membrana četkaste prevlake bubrega štakora. U prisutnosti iona  $\text{Ca}^{2+}$  hidroliza ADP slijedi jednostavnu Michaelis-Menten-ovu kinetiku, uz pretpostavku jednoga katalitičkog mjesta. Pravi supstrat za ADPazu jest kompleks ADP i dvovalentnog kationa. Optimum hidrolitičke aktivnosti jest između pH 7,2 i pH 8,6.

Hidroliza ATP i ADP pokazuje sličnu dinamiku denaturacije toplinom, kao i sličnu neosjetljivost na ograničenu proteolizu i na inhibitore drugih poznatih ATPaza. Enzimska aktivnost može se inhibirati dietil-pirokarbonatom, ditiotreitolum i visokim koncentracijama *N*-etilmaleimida i azida. Inhibicija dietil-pirokarbonatom može se ukloniti hidroksilaminom, što upućuje na ulogu histidilnih i/ili tirozilnih ostataka u enzimskoj reakciji. Pretpostavljeno je da se obje enzimске aktivnosti, i ATPazna i ADPazna, nalaze na istomu enzimskom proteinu.