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PARTIAL PURIFICATION AND CHARACTERIZATION OF RABBIT-KIDNEY BRUSH-BORDER (Ca^{2+} or Mg^{2+})-DEPENDENT ADENOSINE TRIPHOSPHATASE

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The ATPase activity of rabbit-kidney brush border can be activated almost equally well by Ca^{2+} and Mg^{2+} and, therefore, should be called (Ca^{2+} or Mg^{2+})-ATPase. This enzyme was solubilized and enriched 14-fold by the following steps: pretreatment with papain removed 69% of alkaline phosphatase without attacking a significant portion of the ATPase activity. Addition of 1% cholate removed 65% of the protein but no ATPase activity. The combination of cholate (0.5%) and deoxycholate (0.4%) solubilized most of the ATPase activity and most of the remaining protein. A column chromatography of the extract on Sepharose CL-2B resulted in an 6.5-fold increase of specific ATPase activity. A precipitation by ammonium sulfate (40% saturation) produced an additional 1.9-fold increase. The yield of this partial purification was 16%. Towards the nucleotides UTP and GTP the enzyme showed an activity slightly higher, and towards ITP and CTP an activity slightly lower than that with ATP. ADP was split about half as fast as ATP. AMP was not accepted by the enzyme. Replacing $MgCl_2$ by $CaCl_2$ resulted in an ATPase activity of 92% of that with $MgCl_2$. Using calcium- and magnesium-ATP as substrates, apparent K_m values of 0.22 and 0.33 mM, respectively, were obtained. The gel electrophoresis revealed the enrichment of a protein with an apparent M_r of 95000 and also that of microvillus actin.

Introduction

The Mg^{2+}-ATPase of the brush-border membrane of the kidney has been described in a series of publications [2–6], but so far a purification has not been attempted. This paper reports on a procedure of extraction from the brush border and a 14-fold enrichment of the enzyme. In addition, it gives some information about its substrate and cation specificity and proposes a renaming of the enzyme to (Ca^{2+} or Mg^{2+})-ATPase [7,8], because it can be activated almost equally well both by Ca^{2+} and Mg^{2+}.

A preliminary report of this work has been presented at the meeting of the Deutsche Physiologische Gesellschaft in September 1982 at Giessen, F.R.G. [1].

Materials

Male rabbits, 1500–2000 g, were purchased from a local dealer. Controlled pore glass beads of 80–120 mesh and 2000 Å pore size, ATP, GTP, UTP, ITP, CTP, D-glucose 6-phosphate, all as the sodium salts, iodine nitroblue-tetrazolium chloride, cholic acid, Tween 80, digitonin, sodium dodecyl-sulfate, and DCCD were bought from Serva Biochemica, Heidelberg (F.R.G.). Sodium

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; FSBA, 5'-*p*-fluorosulfonylbenzoyladenosine.

deoxycholate, ouabain, and dithioerythritol were purchased from Merck AG, Darmstadt (F.R.G.), 1-*O*-*n*-octyl-β-D-glucopyranoside, *n*-decyl-β-D-glucopyranoside, Nonidet P-40, papain (17 units/mg, 25 mg/ml) and FSBA were from Sigma Chemicals Co., München (F.R.G.); Zwittergent TM 3-14 from Calbiochem-Behring, La Jolla (U.S.A.); pyruvate kinase (rabbit muscle) and tricyclohexylammonium phospho*enol*pyruvate from Boehringer Mannheim Co., Mannheim (F.R.G.); Sepharose CL 2B from Pharmacia Fine Chemicals AB, Uppsala, Sweden; L- and D-*p*-bromotetramisole oxalate from Aldrich Europe, Nettetal (F.R.G.). All other reagents were of the highest purity available.

Methods

Preparation of brush border

Cell disruption and differential centrifugation. Briefly, isolated tubule segments [9] were resuspended in a 10-fold volume of 220 mM mannitol, 70 mM sucrose, 0.5 mM dithioerythritol and 2 mM Tris-Hepes (pH 7.4) (isolation buffer), disintegrated by nitrogen decompression (150 pounds per square inch for 15 min at 4°C) in a cell disruption bomb (Parr Instruments Co., Moline, IL 61265) and subjected to differential centrifugation in a modified version of the method described by Schnaitman and Greenawalt [10]. After the first centrifugation at $560 \times g$ for 15 min, which gave the nuclear sediment, the supernatant was centrifuged again under identical conditions. The resulting pellet was added to the nuclear sediment and the supernatant was separated further by centrifugation at $9300 \times g$ for 15 min, sedimenting the mitochondrial fraction. This pellet was suspended in half of the previous volume of isolation buffer and recentrifuged under identical conditions, the resulting pellet being the final mitochondrial fraction. The two supernatants obtained were combined and separated by centrifugation at $12000 \times g$ for 15 min into an intermediate fraction containing mitochondria as well as microsomes, and a supernatant containing most of the microsomes and the cytoplasm. Again, the intermediate fraction was resuspended in half of the previous volume of isolation buffer and washed under identical conditions, the resulting pellet being the final intermediate fraction. The supernatant of this wash was combined with the supernatant containing the microsomes and the cytoplasm and subjected to centrifugation at $50000 \times g$ for 30 min, which sedimented the heavy microsomes (crude brush border) and left the light microsomes and the cytoplasm in the supernatant. The heavy microsomal fraction contained 19.2% of the alkaline phosphatase activity, 8.3% of the $(Na^+ + K^+)$-ATPase activity, 9.3% of the glucose-6-phosphatase activity and 1% of the succinate dehydrogenase activity in 5.1% of the protein of the initial homogenate, indicating a 3.8-fold enrichment of alkaline phosphatase activity as compared to isolated tubule segments, or a 7.6-fold increase in specific activity as compared to that in a homogenate of total kidney [11].

Controlled pore glass column chromatography. The heavy microsomes (crude brush order) were resuspended in 200 mM mannitol, 10 mM Tris-Hepes (pH 7.5) to an absorbance value of 1.8 at 700 nm wavelength (corresponding approx. to a protein concentration of 10 mg/ml), and purified further by controlled pore glass column chromatography similar to that described by Ohsawa et al. [12]. 4–7 ml of the membrane suspension were mixed with 1/100 volume of 3 mM $MgCl_2$, incubated at 4°C for 20 min, placed onto a glass column with an inner diameter of 1.9 cm, containing 100 ml controlled pore glass beads, which had previously been equilibrated with 200 ml/200 mM mannitol, 0.03 mM $MgCl_2$, 10 mM Tris-Hepes (pH 7.5) and eluted by the same buffer. Under these conditions, only 52% of the alkaline phosphatase activity together with 38% of the protein applied could be eluted from the porous glass, but the column chromatography also reduced the contamination by succinate dehydrogenase in the total eluate to 4%, by glucose-6-phosphatase to 15%, and by $(Na^+ + K^+)$-ATPase to 27% of their previous values, increasing the specific activity of alkaline phosphatase in the total eluate by a factor of 1.4. The last two-thirds of the eluate were half as much contaminated by $(Na^+ + K^+)$-ATPase as the first third and, therefore, taken for further studies. When the specific enzyme activities of this fraction (purified brush border) were compared with those of a homogenate of total kidney, factors of enrichment for alkaline phosphatase, $(Na^+ +$

K^+)-ATPase, glucose-6-phosphatase, succinate dehydrogenase and acid phosphatase of 13.5, 1.44, 0.73, 0.02 and 1.4, respectively, were calculated, which are similar to those of other brush-border preparations.

Treatment with papain

Papain was activated for 15 min in 9 volumes of 5 mM cysteine, 0.03 mM dithioerythritol, 2.5 mM potassium morpholineethanesulfonate (pH 6.2) at 4°C [13]. This solution was added to 9 volumes of purified brush border, which had been resuspended in 200 mM mannitol, 2.5 mM potassium morpholineethanesulfonate, (pH 6.2) to an absorbance of 0.8 at 700 nm wavelength (approx. 2 mg membrane protein/ml). After incubation at room temperature for 30 min, the brush border was sedimented at $50000 \times g$ for 40 min.

Solubilization of brush-border membrane protein

Purified brush border was resuspended in 250 mM sucrose, 10 mM Tris-H Cl (pH 7.4) to an absorbance of 0.4 at 700 nm wavelength (approx. 1 mg protein/ml) and mixed with one-tenth volume of a detergent solution which had been adjusted to pH 7.4 with Tris. The suspension was kept at 4°C for 1 h and sedimented at $50000 \times g$ for another hour. A final concentration of 1% cholate was found to remove about half of the protein into the supernatant without solubilizing or inactivating the (Ca^{2+} or Mg^{2+})-ATPase activity and was therefore taken as the first step of enzyme purification. Following this step, the ATPase in the brush-border remnants was solubilized by resuspension and incubation (1 h at 4°C) in half of the previous volume of 250 mM sucrose, 0.5% cholate, 0.4% sodium deoxycholate and 10 mM Tris-H Cl (pH 7.4).

Column chromatography on Sepharose CL-2B and precipitation by ammonium sulfate

After centrifugation ($50000 \times g$ for 1 h), the supernatant was placed onto a column (16×200 mm) containing Sepharose CL-2B. Equilibration of the column and elution were performed with 250 mM sucrose, 0.5% cholate, 0.05% sodium deoxycholate, 10 mM Tris-H Cl (pH 7.4). Addition of solid ammonium sulfate to the first fractions of the eluate (40% of the saturating concentration [14]) produced a concentration of ATPase activity and a further increase in specific activity.

SDS-polyacrylamide gel electrophoresis

Samples were precipitated by addition of 3 vol. ethanol and separated on slab gels containing a polyacrylamide gradient from 5 to 15%. The buffers for solubilization of the sample and for the preparation of the gels were as described by Laemmli [15]. After electrophoresis, the gels were stained with Coomassie blue and scanned at 586 nm wavelength. The scanned records of the gels were copied onto typewriting paper and the areas of the different peaks cut out, weighed and calculated as percent of total area. When between 10 and 80 μl of a protein mixture (5–40 μg of brush-border protein) were subjected to electrophoresis, this method of estimation always resulted in the same percentage of total protein for a given peak.

Assay of enzymes and of protein

The assay of marker enzymes was described previously [16]. The estimation of (Ca^{2+} or Mg^{2+})-ATPase activity was performed in 75 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.2 mM Na_2EDTA, 1.6 mM ouabain, 5 mM NaN_3, 50 mM NaCl and 5 mM Na_2ATP (adjusted to pH 7.4 with Tris) at 37°C for 15 min in a final volume of 1 ml. Variation of this composition will be presented in the table legends. After incubation, the reaction was stopped by the addition of 0.5 ml 10% sodium dodecyl sulfate [17]. The tubes were then centrifuged at $8000 \times g$ for 5 min and the supernatants taken for the measurement of inorganic phosphate according to Lindberg and Ernster [18]. For the calculation of enzyme activity, the value of an assay containing no added Mg^{2+} was always deducted. Protein was determined according to Lowry et al. [19].

Results and Discussion

Extraction of (Ca^{2+} or Mg^{2+})-ATPase by cholate and deoxycholate

Detergents which had been used successfully in the isolation of other membrane-bound enzymes [20–23] were checked for their ability to extract protein and (Ca^{2+} or Mg^{2+})-ATPase activity. The concentrations used were between 0.03 and 1.0%

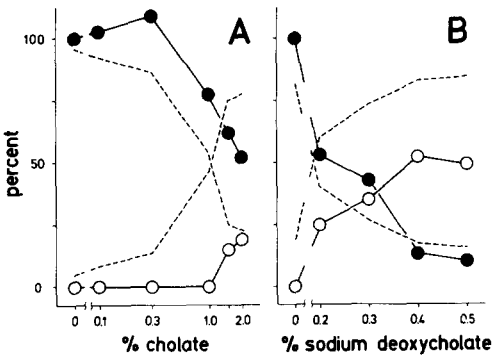

Fig. 1. (A) Extraction of protein and (Ca^{2+} or Mg^{2+})-ATPase activity by various concentration of cholate from the brush border. Incubation and subsequent centrifugation are described under Methods. ●, (Ca^{2+} or Mg^{2+})-ATPase activity in the sediment; ○, (Ca^{2+} or Mg^{2+})-ATPase activity in the supernatant; - - - - -, protein in the sediment and the supernatant. All values depicted are expressed as percent of the value in the membrane suspension before cholate treatment. (B) Extraction of protein and (Ca^{2+} or Mg^{2+})-ATPase activity from the cholate-treated brush-border remnants by a combination of cholate and deoxycholate. The sediment obtained after the treatment of the brush border with 1.0% cholate was resuspended in a buffer containing 0.5% cholate and increasing concentrations of deoxycholate. Incubation and subsequent centrifugation are described under Methods. The symbols are the same as in (A).

for the detergents digitonin, cholate, sodium deoxycholate and octylglucoside, between 0.01 and 0.3% for Tween 80, sodium dodecyl sulfate and Lubrol WX, and between 0.003 and 0.1% (w/v) for Zwittergent TM 3-14, Nonidet P-40 and decylglucoside. In general, low detergent concentrations had only a minor effect upon (Ca^{2+} or Mg^{2+})-ATPase activity, whereas higher concentrations always caused a partial inactivation of the enzyme and an extraction of at least 20% of the ATPase activity and of the protein. Decylglucoside and cholate, however, were exceptions: 0.03% decylglucoside inactivated the ATPase by 60% without extracting any protein; with 1.0% cholate, on the other hand, 46% of the brush-border proteins was brought into the supernatant without a major inactivation or solubilization of the enzyme (Fig. 1A). The latter observation, therefore, offered a way of a 1.5-fold enrichment of (Ca^{2+} or Mg^{2+})-ATPase.

A solubilization, the initial aim of the investigation of the detergent effects, was accomplished by the combination of cholate and deoxycholate. After treatment with 1.0% cholate, the brush-border remnants were resuspended in 0.5% cholate, 250 mM sucrose, 10 mM Tris-H Cl (pH 7.4), which had been supplemented with increasing concentrations of deoxycholate. As shown in Fig. 1B, 0.4% sodium deoxycholate solubilized 50% of the (Ca^{2+} or Mg^{2+})-ATPase activity and left only 13% in the sediment, which means a concomitant decrease of total ATPase activity by 37%. This inactivation, however, was partially reversed during the following steps of enzyme purification, when the concentration of detergent was lowered, because in most experiments, the yield of the ATPase activity was higher in the subsequent purification steps than after this deoxycholate extraction.

Column chromatography on Sepharose CL-2B, treatment with papain, and ammonium sulfate fractionation

Addison and Scarborough [17] purified a Mg^{2+}-ATPase from a solubilized plasma-membrane fraction of *Neurospora crassa* by column chromatography on Sepharose CL-6B. Similarly, Sepharose CL-2B was found to separate brush-border membrane proteins solubilized by deoxycholate. In both cases, ATPase activity came near the void volume, whereas the elution of most of the other proteins was more retarded by the gel, suggesting either a highly aggregated state of the ATPase or the presence of large complexes between detergent, lipids and the ATPase. As shown in Fig. 1, detergents stimulated and inactivated (Ca^{2+} or Mg^{2+})-ATPase activity depending upon the concentrations applied, and therefore did not allow an exact statement about the effectiveness of a purification step. In order to gain further evidence for the enrichment of the ATPase activity, the decrease of the activity of other brush-border enzymes was also followed. Alkaline phosphatase, γ-glutamyltranspeptidase, trehalase and leucine aminopeptidase behaved rather similarly to (Ca^{2+} or Mg^{2+})-ATPase during the extraction procedure depicted in Fig. 1, except that they were not inactivated by deoxycholate (data not shown). In contrast to the extraction, the column chromatography revealed a striking difference between these enzymes. (Ca^{2+} or Mg^{2+})-ATPase and alkaline phosphatase eluted with the first peak of protein

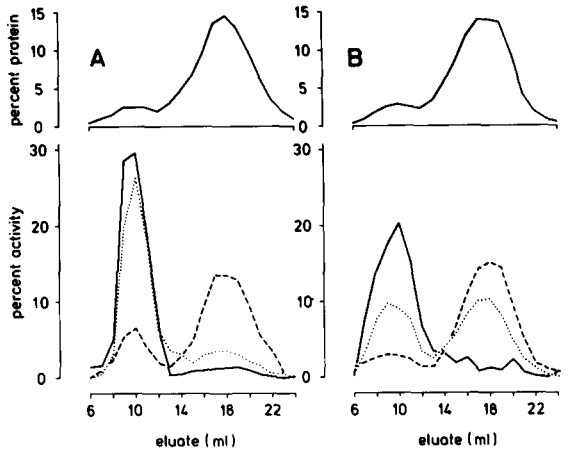

Fig. 2. (A) Column chromatography of the deoxycholate-solubilized brush border (see Fig. 1B and Methods) on Sepharose CL-2B. ———, (Ca^{2+} or Mg^{2+})-ATPase activity: · · · · · ·, alkaline phosphatase activity; - - - - - -, trehalase activity in the fractions of the eluate. All values are expressed as percent of the activity applied to the column. The curves represent the mean of four observations. (B) Column chromatography of the papain-treated and deoxycholate-solubilized brush border (see Fig. 1B, Table I, and Methods). The symbols and the expression of enzyme activity are the same as in (A).

from the column (Fig. 2A), whereas γ-glutamyltranspeptidase and leucine aminopeptidase were eluted much later with the bulk of protein (data not shown). Trehalase activity was present in both peaks. Whether the trehalase activities in the two peaks represent isoenzymes or just two different states of aggregation cannot be decided at present.

Papain is known to remove most of the ectoenzymes from the brush-border membrane [13,24]. Table I shows that papain solubilized 69% of alkaline phosphatase activity but removed only 20 and 7% of (Ca^{2+} or Mg^{2+})-ATPase and of the protein, respectively, into the supernatant. Pretreatment of the brush border with papain, therefore, can be used to separate (Ca^{2+} or Mg^{2+})-ATPase in part from those enzymes which comigrate with it in the column chromatography. Consequently, digestion with papain was included in the procedure of purification as the first step. The extraction by cholate and deoxycholate which followed had not be modified because, regarding the solubilization of enzyme activities, the papain-treated brush border disintegrated in the same way as intact brush border (data not shown). The column chromatography of the deoxycholate extract obtained from the papain-treated brush border reveals a smaller contamination of the first fractions (ATPase peak) by alkaline phosphatase and also by trehalase (Fig. 2B). Precipitation of the first 6 ml of the eluate by ammonium sulfate at a concentration of 40% of saturation resulted in a loss of (Ca^{2+} or Mg^{2+})-ATPase activity – a loss

TABLE I

YIELD OF ENZYME ACTIVITIES AND OF PROTEIN AFTER VARIOUS STEPS OF (Ca^{2+} or Mg^{2+})-ATPase PURIFICATION

Activity and protein are expressed as percent ± S.D. of the value in the starting material (purified brush border). Two ways of purification are depicted, the second involving treatment with papain. The overall recoveries were 71.0 ± 8.8, 85.2 ± 4.2 and 92.2 ± 8.3 for (Ca^{2+} or Mg^{2+})-ATPase, alkaline phosphatase and protein, respectively, in the purification without papain. In the method involving papain, the overall recoveries amounted to 78.2 ± 12.0, 95.4 ± 6.3 and 87.1 ± 18.9, respectively. For details see Methods.

	(Ca^{2+} or Mg^{2+})-ATPase		Alkaline phosphatase		Protein	
	without papain	with papain	without papain	with papain	without papain	with papain
Purified brush border	100	100	100	100	100	100
Sediment after papain treatment	–	80.4 ± 4.9	–	30.6 ± 7.6	–	92.7 ± 3.0
Cholate sediment	66.3 ± 12.6	50.9 ± 14.0	74.8 ± 15.3	15.4 ± 3.6	48.6 ± 8.6	27.6 ± 7.9
Deoxycholate supernatant	31.2 ± 9.7	22.6 ± 8.0	55.5 ± 17.3	12.7 ± 2.8	35.5 ± 12.3	20.9 ± 5.0
Fractions 6–12 of column eluate	26.8 ± 8.9	20.5 ± 2.0	29.9 ± 9.9	5.9 ± 3.2	4.3 ± 1.3	2.9 ± 1.1
Ammonium sulfate precipitate	11.9 ± 4.8	16.4 ± 6.1	9.4 ± 4.7	2.5 ± 1.7	1.5 ± 1.3	1.2 ± 0.4

less severe in the papain-treated material – but also in an increase of specific activity by a factor of 1.3 and 1.9, respectively (Table I).

In Table I, the yield of (Ca^{2+} or Mg^{2+})-ATPase activity and of protein after the various steps of purification are listed. Alkaline phosphatase, the brush-border enzyme most difficult to remove, was also included in Table I. It shows that papain treatment not only brought about a major improvement regarding the enrichment of (Ca^{2+} or Mg^{2+})-ATPase – a higher yield of activity in a smaller amount of protein as compared to the procedure without papain – but also reduced alkaline phosphatase activity quite effectively to less than one-third in the final material. Because alkaline phosphatase interferes with the assay (Ca^{2+} or Mg^{2+})-ATPase under certain conditions [5], the latter observation indicates some advantage when a characterization of the enzyme is intended (see below).

Additional information about the specific activities of (Ca^{2+} or Mg^{2+})-ATPase and alkaline phosphatase after the various purification steps is given in a separate table (Table II). As can be seen, the ATPase activity increased 13.7-fold from 123 to 1681 nmol/min per mg protein, whereas alkaline phosphatase activity showed only a 2-fold increase from 1280 to 2667 nmol/min per mg. When stored over a period of 3 months in 0.5% cholate, 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) at 4°C, the partially purified ATPase (purification involving papain) lost only 17 ± 26% (S.D.) of its activity.

TABLE II

SPECIFIC ACTIVITIES OF (Ca^{2+} or Mg^{2+})-ATase AND ALKALINE PHOSPHATASE AFTER VARIOUS STEPS OF (Ca^{2+} or Mg^{2+})-ATPase PURIFICATION INVOLVING PAPAIN

Specific activities are expressed as nmol/min per mg protein and were calculated from the data obtained in the experiments depicted in Table I.

Fraction	Activity	
	(Ca^{2+} or Mg^{2+})-ATPase	Alkaline phosphatase
Purified brush border	123	1280
Sediment after papain treatment	107	423
Cholate sediment	227	714
Deoxycholate supernatant	133	778
Fractions 6–12 of column eluate	869	2604
Ammonium sulfate precipitate	1681	2667

Properties of the solubilized and partially purified (Ca^{2+} or Mg^{2+})-ATPase

Effects of inhibitors Table III describes the effects of some inhibitors upon the partially purified (Ca^{2+} or Mg^{2+})-ATPase and compares them with those inflicted on the enzyme of the intact brush border. Ouabain is usually included in the reaction mixture to prevent the participation of ($Na^+ + K^+$)-ATPase [5]. Omission of ouabain increased

TABLE III

ACTIVITY OF (Ca^{2+} or Mg^{2+})-ATPase AFTER THE OMISSION OF OUABAIN AND AZIDE, AFTER THE ADDITION OF L- AND B-BROMOTETRAMISOLE AND THEOPHYLLINE TO THE REACTION MEDIUM AND AFTER PREINCUBATION WITH DCCD AND FSBA

The ATPase assay was conducted in the purified brush border and in the partially purified enzyme (purification involving papain). Activity is expressed as percent of control ± S.D. DCCD was added in 5 µl ethanol to 1 ml reaction mixture, FSBA in 2 µl dimethylsulfoxide.

Treatment	Relative activity	
	Purified brush border	Partially purified (Ca^{2+} or Mg^{2+})-ATPase
Control	100	100
Omission of ouabain	105 ± 15	100 ± 11
Omission of azide	127 ± 25	100 ± 5
Addition of L-bromotetramisole (0.2 mM)	89 ± 20	97 ± 10
Addition of D-bromotetramisole (0.2 mM)	92 ± 15	98 ± 9
Addition of theophylline (2.5 mM)	79 ± 7	96 ± 15
15 min preincubation with:		
0.1 mM DCCD	68 ± 12	99 ± 13
1.0 mM DCCD	57 ± 14	105 ± 10
1 h preincubation with:		
0.1 mM FSBA	63 ± 16	50 ± 11
1.0 mM FSBA	31 ± 14	19 ± 10

ATPase activity only in the intact brush border, whereas no significant change of ATPase activity was seen in the partially purified material, indicating that contamination by $(Na^+ + K^+)$-ATPase was negligible. Azide, an indispensable requisite for the assay of brush order (Ca^{2+} or Mg^{2+})-ATPase in previous work [5] and in the intact brush border, was apparently no longer necessary for the assay of partially purified (Ca^{2+} or Mg^{2+})ATPase. Omission of azide did not lead to a significant change in activity. This indicates a brush border (Ca^{2+} or Mg^{2+})-ATPase essentially free of mitochondrial ATPase. L-bromotetramisole, a specific inhibitor of alkaline phosphatase [25], decreased the activity in the enriched material and its source, the purified brush border, by 3 and 11%, respectively, indicating a reduction in the participation of alkaline phosphatase in the assay. For comparison, the D-isomer of bromotetramisole was also tried and found not to inhibit the activity significantly in either fraction. Theophylline, another potent inhibitor of alkaline phosphatase [26], inhibited the reaction significantly – as could be expected – only in the purified brush border by 21%.

The mitochondrial Mg^{2+}-ATPase [27] and a number of plasma-membrane-bound Mg^{2+}-dependent ATPases [28–30] including the brush border (Ca^{2+} or Mg^{2+})-ATPase [5] can be inhibited by DCCD. As shown in Table III, after solubilization and enrichment, the brush border (Ca^{2+} or Mg^{2+})-ATPase no longer displays sensitivity to DCCD. This particular feature has also been observed in the mitochondrial ATPase, in which DCCD sensitivity and catalytic activity are located on different subunits of a rather complex enzyme [31,32]. Whether, in analogy, the brush border (Ca^{2+} or Mg^{2+})-ATPase has also lost a DCCD-sensitivity-conferring subunit during its purification can only be confirmed by an experiment in which the DCCD sensitivity is reconstituted as was done with the mitochondrial enzyme [31,32]. The possibility of two kinds of (Ca^{2+} or Mg^{2+})-ATPases in the brush-border membrane – one being DCCD-sensitive and the other not, and the latter being enriched in the partially purified enzyme preparation – can be excluded, because the (Ca^{2+} or Mg^{2+})-ATPase activity eluted from the column (see Table I) could be inhibited by DCCD to the same extent as the brush-border-membrane-bound (Ca^{2+} or Mg^{2+})-ATPase (data not shown). Only the final step of ammonium sulfate precipitation, in which less than 20% of the activity was lost (Table I), abolished the sensitivity to DCCD. An alternative explanation to the loss of a subunit could be a conformational change, which might have been introduced in the enzyme during the precipitation by ammonium sulfate. Treatment with papain certainly did not inflict this loss of DCCD sensitivity, because the enzyme enriched by the procedure without papain treatment displayed the same feature (data not shown). Finally, the inhibition by FSBA is shown in Table III. Contrary to the reaction between FSBA and the pancreatic plasma membrane (Ca^{2+} or Mg^{2+})-ATPase, which requires a day for completion [29], the kidney brush border (Ca^{2+} or Mg^{2+})-ATPase is almost totally inhibited after an incubation of 1 h.

Specificity of (Ca^{2+} or Mg^{2+})-ATPase towards nucleotides and divalent cations. Tables IV and V demonstrate that the brush-border membrane-bound and the partially purified enzyme has the same requirements for substrates and divalent cations. Towards UTP and GTP, the enzyme showed an activity slightly higher and towards ITP and CTP an activity slightly lower than that with ATP. This sequence of hydrolytic activities does not agree with the results of a previous study, in which the highest rate was obtained with ITP [5]. The discrepancy can be explained by the different methods used for the stopping of the enzyme reaction. In the previous study, the reaction was terminated by boiling for 2 min, which hydrolyses the different metal-ion complexed nucleotides to an extent which is always small but differing [33]. In this study, the reaction was stopped in a milder way by the addition of sodium dodecyl sulfate and, therefore, a more correct description is given. In addition, ADP and AMP were checked as possible substrates of the enzyme. When contaminating alkaline phosphatase was blocked by theophylline, (Ca^{2+} or Mg^{2+})-ATPase showed no activity with AMP. ADP, however, was split at a rate of 44% of that with ATP under identical conditions. Similar rates with ADP of 15 and 20–25% were obtained with the (Ca^{2+} or Mg^{2+})-ATPases from the T-tubular membrane of skeletal muscle [8] and

TABLE IV

ACTIVITY OF Mg^{2+}-DEPENDENT HYDROLYSIS OF NUCLEOTIDES AND OF ADP AND AMP IN THE PURIFIED BRUSH BORDER AND IN THE ENZYME PREPARATION PARTIALLY PURIFIED BY THE METHOD INVOLVING PAPAIN

Under (1), activity was estimated with the use of a nucleotide-regenerating system as described previously [5]. Under (2), the usual reaction mixture was employed (see Methods). The concentration of theophylline used in some experiments was 2.5 mM. Activity is expressed as percent of the hydrolytic activity with ATP. The data represent the means of eight observations.

Substrate	Relative activity	
	Purified brush border	Partially purified (Ca^{2+} or Mg^{2+})-ATPase
(1) Nucleotide-regenerating system present		
ATP	100	100
UTP	126	117
GTP	110	105
ITP	79	98
CTP	89	86
(2) Usual reaction mixture		
ATP	100	100
ATP plus theophylline	79	96
ADP	36	52
ADP plus theophylline	29	42
AMP	10	9
AMP plus theophylline	1	0

TABLE V

ACTIVITY OF THE ATPase IN THE PRESENCE OF VARIOUS DIVALENT CATIONS

Activity was estimated in the purified brush border and in the partially purified enzyme (purification involving papain) and is expressed as percent of control ± S.D. (5 mM $MgCl_2$, 0.2 mM EDTA). In the first part of the experiments, 0.2 mM EDTA was present as usual, in the second part of the table, EDTA was omitted.

Additions (mM)	Relative activity	
	Brush-border preparation	Partially purified enzyme
(1) 0.2 mM EDTA present		
5 mM $MgCl_2$	100	100
1 mM $CaCl_2$	68 ± 12	76 ± 6
2 mM $CaCl_2$	82 ± 14	75 ± 11
5 mM $CaCl_2$	102 ± 14	92 ± 12
10 mM $CaCl_2$	81 ± 27	89 ± 17
5 mM $MnCl_2$	57 ± 8	59 ± 22
5 mM $CoCl_2$	58 ± 4	55 ± 19
5 mM $ZnCl_2$	41 ± 14	52 ± 5
5 mM $FeCl_2$	37 ± 21	45 ± 24
5 mM $SrCl_2$	17 ± 16	22 ± 9
5 mM $BaCl_2$	11 ± 10	6 ± 10
5 mM $CuCl_2$	7 ± 6	6 ± 6
(2) Without EDTA		
5 mM $MgCl_2$		88 ± 12
5 mM $MgCl_2$ + 5 μM $CaCl_2$		92 ± 7
5 mM $MgCl_2$ + 50 μM $CaCl_2$		94 ± 11
5 mM $MgCl_2$ + 500 μM $CaCl_2$		95 ± 15

the plasma membrane of pancreatic acinar cells [7], respectively.

Replacing 5 mM $MgCl_2$ by equimolar concentrations of $CaCl_2$ did not change ATPase activity significantly. All other divalent cations tested activated the enzyme to a minor extent than Mg^{2+}. With manganese- and cobalt chloride, more than half of the activity of that with $MgCl_2$ was gained. But zinc-, iron-, and strontium chloride also activated the enzyme between 52 and 17%. Only the replacement by barium- and copper chloride resulted in an activity of 11% or less (Table V).

A high-affinity (Ca^{2+} + Mg^{2+})-ATPase has been described in the basal-lateral plasma membrane of the proximal tubule cell [34]. In order to check for a possible activation by low concentrations of Ca^{2+}, a combination of Mg^{2+} plus Ca^{2+} was also tried. In these experiments, the actual concentrations of free Ca^{2+} were not calculated, but since a rather broad range of $CaCl_2$ additions was made, a stimulation by Ca^{2+}, if it takes place, should have been seen. However, the combination of 5 mM $MgCl_2$ with 5–500 μM $CaCl_2$ resulted only in a negligible increase of activity between 5 and 9% (Table IV).

The low specificity of the enzyme towards the nucleotides and the divalent cations resembles most the plasma-membrane-bound (Ca^{2+} or Mg^{2+})-ATPase described in pancreatic acinar cells [7], liver cells [35] and skeletal muscle [8] which in part are believed to be ectoenzymes.

Kinetic analysis Fig. 3 shows the activity of the brush-border-bound and the partially purified enzyme with dependence on the concentration of MgATP and CaATP in a double-reciprocal plot. Using this figure, apparent K_m values of 0.40 and

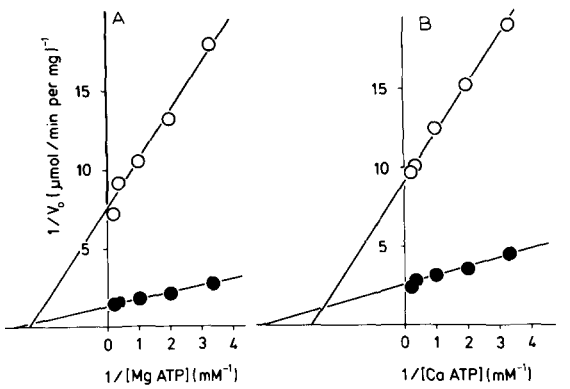

Fig. 3. Activity of (Ca^{2+} or Mg^{2+})-ATPase with dependence on the concentration of MgATP (A) and CaATP (B) in a double-reciprocal plot. ○, Activity in the purified brush border; ●, activity of the enzyme partially purified by the procedure involving papain. EDTA was omitted from the reaction mixture. Reaction time was 5 min. Each point represents the mean of four separate experiments.

0.32 mM for the membrane-bound and the partially purified enzyme could be calculated, respectively, when Mg^{2+} was used for activation. With CaATP as the substrate, similar apparent K_m values of 0.33 and 0.22 mM, respectively, were obtained. On the (Ca^{2+} or Mg^{2+})-ATPases from the T-tubular membrane of skeletal muscle [8] and the plasma membrane of pancreatic acinar cells [7], similar K_m values between 0.17 and 0.34 mM were observed.

The calculation of a V_{max} of an enzyme of unknown purity has only little meaning.

Gel electrophoresis. Fig. 4 shows that a 14-fold enrichment is still far from an isolation of the enzyme. After the partially purified (Ca^{2+} or Mg^{2+})-ATPase (purification involving papain) was separated into its protein constituents by gel electrophoresis, between three and five peaks could be seen. Only two of them, however, were constantly present and contained a higher percentage of the total protein than their counterparts in the brush border. A peak with an apparent molecular weight of 95 000 ± 5 000 (S.D.) was enriched from 1.8 ± 0.3% (S.D.) in the brush border to 8.3 ± 0.8% in the partially purified enzyme preparation. Another peak with an apparent molecular weight of 43 500 ± 1 100 was enriched from 7.0 ± 1.3 to 11.7 ± 3.8%. The latter could contain the brush-border actin, because it contributes a similar percentage

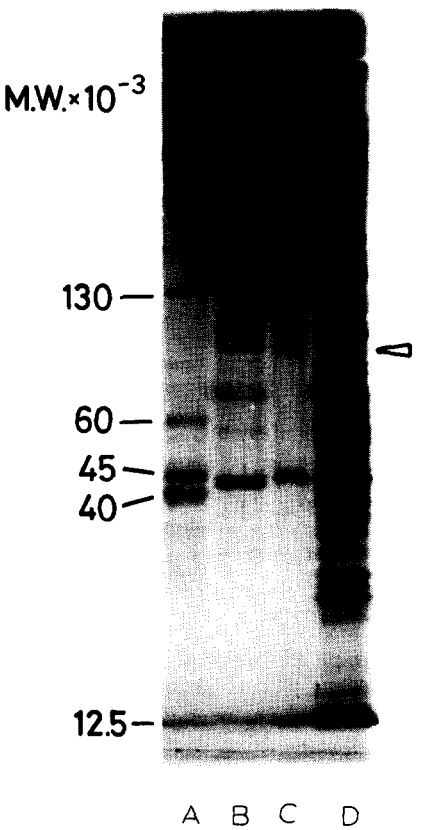

Fig. 4. SDS-polyacrylamide gel electrophoresis of the proteins of the purified brush border (lane D) and the (Ca^{2+} or Mg^{2+})-ATPase partially purified by the procedure without and with the use of papain, respectively (lanes B and C, respectively). The arrow points to a protein band of an apparent M_r of 95 000, which is enriched in lane C (purification involving papain) and to a smaller extent in lane B (purification without papain). Proteins of known molecular weight were run in parallel (lane A): β-galactosidase, subunit M_r 130 000; catalase, subunit M_r 60 000; ovalbumin, M_r 45 000; aldolase, subunit M_r 40 000; cytochrome *c*, M_r 12 500 [37].

to the brush-border protein and has the same molecular weight as the previously identified microvillus actin [36].

Thus, only the 95 kDa band is left as a candidate for the (Ca^{2+} or Mg^{2+})-ATPase or possibly a catalytically active subunit of this enzyme. The 3-fold increase in protein percentage of this band during the ATPase purification without papain (Fig. 4B) and its 4–5-fold increase when papain was included (Fig. 4C), does not correlate very well with the 7- and 14-fold increase of specific ATPase activity, respectively.

But the contribution of 1.8% to the brush-border protein by the 95 kDa peak might be too high to be true, because a small band framed by two large bands (Fig. 4D) is always overestimated by the 'cut and weigh' method (see Methods). Therefore, the increase of the 95 kDa protein by the enzyme purification can be expected to be higher than estimated and might come close to the enrichment of (Ca^{2+} or Mg^{2+})-ATPase activity.

Conclusions

In conclusion, this paper demonstrates a method for the enrichment of brush-border (Ca^{2+} or Mg^{2+})-ATPase. This partial purification offered the opportunity of characterizing the enzyme under conditions in which interfering enzyme activities such as the ($Na^+ + K^+$)-ATPase, the mitochondrial Mg^{2+}-ATPase, and alkaline phosphatase were absent or reduced and also made it possible to determine a tentative molecular weight of brush-border (Ca^{2+} or Mg^{2+})-ATPase.

In Introduction, it has been implied that the Mg^{2+}-dependent ATPases so far characterized in the kidney brush border are (Ca^{2+} or Mg^{2+})-ATPases. This might be true for some species like the rabbit. In other species, the presence of other or additional kinds of ATPases could be conceivable. Whether the (Ca^{2+} or Mg^{2+})-ATPase is an ectoenzyme like that in the plasma membrane of pancreatic acinar cells [7] has to be elucidated in future studies.

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