Properties of ATP-ase of the microvillus membrane isolated from epithelial cells of rabbit small intestine

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Properties of ATP-ase of the microvillus membrane isolated from epithelial cells of rabbit small intestine*

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Abstract

For the purpose to investigate the physiological functions of microvillus ATPase, general properties of the enzyme were studied on the microvillus membranes isolated from rabbit intestinal epithelial cells. 1) ATPase of the microvillus membranes was activated with Mg2+. Mg.ATP complex was thought to be a substrate of the enzyme. The Michaelis constant for ATP of the ATPase was a value of 0.8 to 1.0 mM. 2) The microvillus ATPase was also activated with Ca2+, but the affinity was lower than a half of that of Mg2+. 3) The optimum pH of the ATPase was about 7.8. 4) Activity of the microvillus ATPase was markedly inhibited by treating with deoxycholate (DOC), and the activity inhibited was partially restored by washing the microvillus membrane with distilled water. The structure of the membranes destroyed by treating with DOC was also partially restored by the same procedure. 5) Ultrasonic treatment also markedly destroyed the microvillus membrane and inhibited ATPase activity. Damaged ultrastructure and ATPase activity both were partially restored by treating with phospholipid, EPL. 6) Simultaneous presence of Na+ and K+ stimulated scarcely the ATPase of purified microvillus membranes. 7) The microvillus ATPase was slightly activated in the presence of n-glucose. Phloridin gave little effect on the activity of the microvillus ATPase.
The studies on the absorption mechanisms of sugars, amino acids and others in the small intestine have advanced extensively by using everted-sac system (1). Wilson et al. (2-4) and Crane et al. (5-7) proved by using the everted-sac system that many kinds of sugars are actively transported through the small intestinal wall. They found that the absorption of D-glucose depends on sodium ion (Na+) inside the membrane, suggesting that the sugar-transport might be coupled with energy-dependent Na+ pumping (5-7).

On the other hand, Miller and Crane (8) established the preparative method of brush borders of intestinal epithelial cells. They suggested that terminal digesting enzymes of sugar and peptide localized in the brush borders. Oda et al. (9, 10) purified microvillus membranes from the isolated microvillus borders by the method of ultrasonication and glycerol density gradient centrifugation, and found repeating particles (elementary particles), measuring approximately 60 Å in diameter, on the surface of microvillus membranes. They partially purified the elementary particles after solubilization with papain from the microvillus membranes, and suggested that the elementary particles coincide with or include an enzyme molecule such as disaccharidase and peptidase, which carries out terminal hydrolytic digestion of carbohydrates and proteins, respectively. They also indicated that Mg2+-dependent ATPase and alkaline phosphatase localized in the microvillus membranes in relatively high concentrations were recovered in the papain-insoluble membraneous residue.

The present communication describes general properties of ATPase
in microvillus membranes and its correlation with sugar transporting system.

MATERIALS AND METHOD

Microvillus borders of small intestinal epithelial cells were isolated from rabbits essentially according to the method of Miller and Crane (8, 10). Microvillus membranes were purified from the microvillus borders according to the method of Oda et al. (9, 10). Namely, the microvillus borders were disrupted by ultrasonic oscillation at 20 Kc for 5 min (Ultrasonifier of Kajo Dempa Co., Ltd., 7ψ tip), and layered on the discontinuous glycerol density gradient of 20, 40, 60, 80 and 100 per cent, and centrifuged at 160,000 × g for 60 min. The fraction between 40 to 60 per cent glycerol layers was used for assay as purified microvillus membranes. The sample was stocked in 40% glycerol at -5～-10°. ATPase activity was assayed mainly in the medium containing 2 mM ATP, 5 mM MgCl₂, 0.1M NaCl (or KCl), 40 mM Tris-Cl (pH 7.4), and microvilli in various concentrations. The reaction mixture was incubated at 25° and the reaction was stopped by addition of 5% trichloroacetic acid (TCA) at appropriate times after the start of the reaction. Inorganic phosphate released was estimated by the method of Martin and Doty (11). Protein concentration was determined by the biuret method of Gornall et al. (12).

Electron microscope observation was conducted as described in the previous paper (9).

RESULTS

Electron microscope observation of the isolated microvillus membranes: The isolated microvilli negatively stained with phosphotungstate (PTA) present a vesicular or tubular form (Fig. 1). The vesicles are larger than 2,000 Å in diameter and the size is enormously larger than that of the microvilli in sectioned specimens. The result probably indicates that the microvilli were swollen in the course of preparation. Isolated microvilli negatively stained with PTA showed repeating particles (elementary particles) of 50 to 60 Å in diameter on the surface of the membranes.
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Kinetic properties of the microvillus ATPase: Effects of glycerol on microvillus-ATPase activity were examined at the following conditions; 2 mM ATP, 5 mM MgCl₂, 0.1 M KCl, 40 mM Tris-Cl (pH 7.4), 0.325 mg microvillus protein per ml, at 25° (Fig. 2). Microvillus-ATPase activity decreased linearly as the concentration of glycerol increased. And it was almost completely inhibited with 40% glycerol. Therefore, the microvillus preparations were washed with sufficient volume of distilled water to remove glycerol, and used for the following assays.

![Graph showing the effect of glycerol on microvillus ATPase activity.](http://escholarship.lib.okayama-u.ac.jp/amo/vol25/iss1/2)

The effect of ATP on microvillus-ATPase activity in the reaction mixture containing 5 mM MgCl₂, 0.1 M KCl, 40 mM Tris-Cl buffer (pH 7.4) at 25° is illustrated in Fig. 3. The Lineweaver-Burk plot is linear within the range of the ATP-concentrations examined in the experiment, and the $K_m$ and $V_{max}$ values calculated from the plot are $0.9 \times 10^{-3} M$ and $0.3$ to $0.5 \mu$ mole per mg protein per minute, respectively.

The dependency of microvillus-ATPase activity on the concentration of MgCl₂ at various concentrations of ATP is shown in Fig. 4. Without adding Mg²⁺ to the reaction medium, ATPase activity was negligible in any concentration of ATP. As illustrated in Fig. 4, ATPase activity reached maximum at almost the same concentration of MgCl₂ as that of ATP and kept the maximum activity in the higher concentration of MgCl₂. The results suggest that Mg-ATP complex is a substrate of microvillus-ATPase. In the following experiments, magnesium ion concentration...
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Fig. 3 Lineweaver-Burk plot of the Mg²⁺-dependent microvillus ATPase. ATPase activity was measured in 5 mM MgCl₂, 0.1 M KCl, 0.04 M Tris-Cl (pH 7.4), and 0.125 mg microvillus protein/ml at 25°.

Fig. 4 Effect of Mg²⁺ on microvillus ATPase. ATPase activity was measured in 0.5-2.0 mM ATP, 0.1 M KCl and 0.04 M Tris-Cl (pH 7.4) at 25°. Concentration of ATP; -○-, 2 mM; -□-, 1 mM; -△-, 0.5 mM.

was usually fixed at 5 mM. In Fig. 5 Mg²⁺-dependency of microvillus-ATPase is compared with its Ca²⁺-dependency. The microvillus-ATPase was also activated with Ca²⁺, but the affinity to Ca²⁺ was very low comparing with that to Mg²⁺.
Fig. 5 Effect of Mg$^{2+}$ and Ca$^{2+}$ on microvillus ATPase. ATPase activity was measured in 2 mM ATP, 0.1 M KCl and 0.04 M Tris-Cl (pH 7.4) at 25°.
-○-, MgCl$_2$; -○-, CaCl$_2$.

Fig. 6 Arrhenius plot of microvillus ATPase. ATP was used at a concentration of 2 mM. Other experimental conditions were similar to those described in Fig. 3.

Temperature-dependency of microvillus ATPase was examined nearly at the $V_{\text{max}}$. As illustrated in Fig. 6, the Arrhenius plot gives two straight lines with different slopes which cross at about 20°. $\Delta E$ calculated from the slope of each line was $3.95 \times 10^3$ cal per mole within 0° to 20° or 0.22
Effect of pH on activity of microvillus ATPase was examined nearly at $V_{\max}$. As illustrated in Fig. 7, the dependency of ATPase activity on pH gives a bell-shaped curve with a maximum approximately at pH 7.8.

Effect of deoxycholate and ultrasonication on the structure and ATPase activity of microvilli: Sodium deoxycholate (DOC, pH 8.0) in various concentrations was added in a solution of 0.5 ml containing 0.2 to 0.25 mg microvillus protein and 1 M sodium chloride, and the mixture was incubated at 0° for 15 min. ATPase activity of the DOC-treated sample was measured in 4.5 ml of reaction medium (Fig. 8). ATPase activity remarkably decreased with the increase of DOC concentrations from 0 to 0.1% and reached 30 per cent of the original activity at concentration of 0.1%. But ATPase activity gradually decreased as DOC concentrations were increased over 0.1 per cent. The inhibitory effect of DOC was scarcely influenced by the change of incubation time from 15 min to 12 hr.

After the treatment of 0.5 mg microvilli with 1 M NaCl and various concentrations of DOC at 0° for 15 min, the sample were diluted with sufficient volume of distilled water, and then centrifuged at 40,000 $\times$ g, for 40 min. ATPase activity of the pellet suspended in 5 ml of reaction mixture was 20 to 30 per cent higher than that of the DOC-treated microvilli before
Fig. 8 Effect of DOC on microvillus ATPase. DOC (0~1.0%) was added in a solution of 0.5 ml containing 0.2 to 0.25 mg microvillus protein and 1 M sodium chloride, and incubated at 0° for 15 min, and then 4.5 ml of reaction medium was added to the mixture. ATPase activity was measured in 2 mM ATP, 5 mM MgCl₂, 0.1 M NaCl, and 0.04 M Tris-Cl (pH 7.4) at 25°.

Fig. 9 Effect of washing of microvillus membranes with distilled water on the ATPase activity Pretreated with DOC. The DOC-treatment was conducted as described in Fig. 8. The DOC-treated sample was centrifuged at 40,000 x g for 40 min and the residue was suspended in 5 ml of reaction medium. ATPase activity was measured at 25° in the same medium as described in Fig. 8. -○-, DOC treated microvilli; -●-, washed microvilli after treating with DOC.
washing with distilled water (Fig. 9). The result probably indicates that ATPase activity was partially restored by removing DOC. Fig. 10 shows the electron micrograph of microvilli treated with 0.5% DOC and negatively stained with PTA. Microvillus membranes were remarkably destroyed by DOC treatment. Fig. 11 shows an electron micrograph of microvillus sample that was washed with distilled water to remove DOC after DOC treatment. Many vesicles appear to be similar to untreated microvillus vesicles. The results suggest that the removal of DOC resulted in partial reconstitution of microvillus structure, accompanying partial restoration of ATPase activity.

Microvilli suspended in 4.5 ml reaction medium without ATP were sonicated at 20 Kc at 0° for various times and ATPase activity of the sonicated microvilli was assayed at 25° with taking time course after addition of 0.5 ml ATP. As illustrated in Fig. 12, ATPase activity is more markedly inhibited by the longer sonic treatment, and 70% cent of the total activity is lost by sonication for 4 min. The electron micrographs of the same sample showed that almost all of the microvillus membranes were destroyed (Fig. 13). Neither replacement of K⁺ by Na⁺ nor lack of Mg²⁺ in the sonication medium altered the effect of sonication on ATPase activity. The ATPase activity lost by sonication was not restored when the sonicated sample was stocked in the reaction medium at 0° for various periods (Fig. 14).

The electron micrographs of the residue obtained from the sonicated microvilli by centrifugation at 40,000×g for 40 min, showed relatively
smaller vesicles than the original microvillus vesicles (Fig. 15). Such vesicles were also observed in the supernatant. But the vesiculation of the sonicated microvilli did not accompany the restoration of ATPase activity.

Effect of phospholipid on the ATPase activity of the sonicated microvilli was studied by using essential phospholipid (EPL, Nathaman Co., Ltd.). EPL solution, main component of which is purified lecithin with polyenoic acid as fatty acid residues, contains 2.3% DOC for solvent of lecithin. In the following experiment, therefore, the effect of phospholipid on ATPase activity was estimated from the difference between activity in EPL-containing medium and that in the medium containing the same amount of DOC that was included in the EPL-containing medium. In Fig. 16, EPL in various concentrations was added to the sonicated suspension of microvilli (0.18~0.26 mg). After incubating at 0° for 10 min, the mixture was centrifuged to remove excess EPL, and ATPase activity of the residue was examined. Though some differences were observed in the results of each experiment, the ATPase activity lost by the sonic treatment was gradually restored as concentration of EPL was increased. The maximum restoration was obtained at 1~2 mg EPL/mg of microvillus protein, and the activity was about 30 to 40% of ATPase activity of microvillus membranes before sonication. No further increase of activity was obtained by addition of EPL in higher concentrations than 2 mg per mg of membrane protein.
Fig. 16 Effect of phospholipid (EPL) on ATPase activity of the sonicated microvilli. ATPase activity was measured in 2 mM ATP, 5 mM MgCl₂, 0.1 M NaCl, 0.01 M Tris-Cl (pH 7.4) and 0.179~0.264 mg protein/ml at 25°. ●, preparation 1; ○, preparation 2.

Effects of sucrose and D-glucose on microvillus ATPase: As described previously active transport of D-glucose, galactose, and some of other sugars across the small intestinal wall has been shown by using the everted-sac system. Crane postulated that active transport of sugars through membranes of small intestinal epithelial cells was coupled with microvillus ATPase reaction. In the present paper, for the purpose to investigate whether the ATPase of the purified microvillus membrane takes a part in the sugar transport system or not, ATPase activity of the microvillus membrane was measured in the presence of sucrose or glucose with or without phlorizin. (Na⁺ + K⁺)-dependency of the ATPase was also investigated. ATPase activity assayed under the presence of sucrose in various concentrations is illustrated in Fig. 17. Oda et al. (9, 10) demonstrated that sucrase was situated in the repeating particles observed on the surface of microvillus membrane. If glucose, which was formed from sucrase by the action of sucrase, gives some effect on ATPase activity, the possibility might be anticipated that the ATPase participates in active transport of glucose. But as illustrated in Fig. 17, sucrose, even in wide range of the concentration, gave no significant effect on ATPase activity. Effect of D-glucose on ATPase activity was also investigated (Fig. 18). Microvillus ATPase was gradually activated as concentration of D-glucose increased up to 25 mM and the activity gradually declined when the concentration was increased still more. Phloridin (0.9 mM) gave no effect on ATPase
activity under the presence of sucrose or D-glucose.

Next, effect of sodium and potassium ions on ATPase activity of microvilli was examined. Concentrations of sodium chloride and potassium chloride in assay medium were altered while the total concentration of these ions (Na\(^+\) + K\(^+\)) was maintained constantly at 100 mM. As a result (Na\(^+\) + K\(^+\))-dependency of microvillus ATPase was not detected under the present assay condition.
DISCUSSION

It is well known that many kinds of sugars, amino acids and others are absorbed actively at the small intestine. Concerning the transport mechanism CRANE advocated an idea that sugar transport through the microvillus membrane is coupled with the energy dependent sodium pumping (6). Though most of the data have been brought about by using everted sac system, it has been strongly suggested that microvillus ATPase engages in the active transport mechanism. Up to date, studies on the ATPase of the intestinal epithelial cell have almost been done in the organ, tissue, or cellular levels by using physicochemical or histochemical methods. Studies on the ATPase in subcellular or purified enzyme levels have been only a few (13-16). TAYLOR (13) has found a (Na++K+)-stimulated, ouabain sensitive ATPase in the cell-debris fraction isolated from the intestinal mucosa of guinea-pig essentially according to the method of MILLER and CRANE (8). QUIGLEY and GOTTER studied about distribution of (Na++K+)-stimulated ATPase activity in rat intestinal mucosa (15). They reported that the major portion of the (Na++K+)-stimulated ATPase in the rat intestinal mucosal cell had been isolated in the M·l fraction which was thought to be a plasma membrane fraction, free of brush border. The brush border fraction consistently was found to contain about 15% of the total cellular (Na++K+)-stimulated ATPase, but they could not determine whether this activity was an integral part of the brush border membrane or whether the activity was present on small tags of the lateral plasma membrane which remained attached to the brush border. We reported previously by biochemical and cytochemical methods that the relatively high concentration of Mg2+-dependent ATPase was localized in the microvillus membrane and that the active site of the ATPase was localized predominantly in the inner surface of the trilaminar structure of the microvillus membrane (9, 10).

In this communication we reported general properties of the ATPase in the purified microvillus membranes and its correlation with the active transport of sugars was also studied. The microvillus ATPase was markedly inhibited with glycerol in high concentration, K_m and V_max values for ATPase were 0.9×10^{-3}M and 0.3~0.5 μmole per mg protein, respectively. The ATPase activity depended on either Mg2+ or Ca2+. Though it was not clear whether the Mg2+- and Ca2+-dependent ATPases were the same or not, the affinity of ATPase to Ca+ was very low comparing with that to Mg2+. Temperature dependency of the microvillus ATPase was somewhat conspicuous. Namely, the Arrhenius plot gave two straight lines.
The structure of microvillus membranes was damaged by the treatment with deoxycholate or ultrasonication, resulting in the decrease of ATPase activity. The partial restoration of both structure and activity was obtained by removal of DOC or addition of phospholipid after the treatments. These results suggest that activity of microvillus ATPase depends on the membrane-structure somewhat similar to mitochondrial ATPase (17, 18).

Binding of glucose to intestinal brush border has been reported by Faust et al. (19, 20) and Eichholz et al. (21). Eichholz et al. studied about glucose binding to isolated intestinal brush borders by using a sensitive method based on the difference in binding of L-(14C) glucose and D-(3H) glucose. And they found most of the D-glucose specific binding to be localized to the D fraction of the disrupted brush borders, which contains core plus other heavier, contaminating materials. Only 10% of the binding activity at most was associated with the membrane fraction. In our experiments, contrary to expectation, D-glucose, sucrose or sodium and potassium concentrations did not give any significant effect on the activity of microvillus ATPase. Ouabain and phloridin gave no effect on the ATPase activity either. The possibilities which are thought of from the above results are as follows: 1) The microvillus ATPase was modified during the purification process of the microvillus membrane. 2) Microvillus membranes do not participate in active transport of sugars. 3) Active transport of sugars in the microvillus membrane does not occur by the action of (Na\(^{+} + K\(^{+}\))-dependent ATPase but by another unknown mechanism. Although there is no doubt that certain sugars are actively transported from the intestine, and many investigators suppose that the microvillus border takes part in the active transport of sugars, the direct evidence of the latter obtained by using the isolated microvillus membrane is not quite so conclusive. For this reason, studies on the physiological functions of the microvillus ATPase would be one of the important problems remaining in the field of the intestinal absorption.

**SUMMARY**

For the purpose to investigate the physiological functions of microvillus ATPase, general properties of the enzyme were studied on the microvillus membranes isolated from rabbit intestinal epithelial cells.

1) ATPase of the microvillus membranes was activated with Mg\(^{2+}\). Mg·ATP complex was thought to be a substrate of the enzyme. The
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Michaelis constant for ATP of the ATPase was a value of 0.8 to 1.0 mM.

2) The microvillus ATPase was also activated with Ca$^{2+}$, but the affinity was lower than a half of that of Mg$^{2+}$.

3) The optimum pH of the ATPase was about 7.8.

4) Activity of the microvillus ATPase was markedly inhibited by treating with deoxycholate (DOC), and the activity inhibited was partially restored by washing the microvillus membrane with distilled water. The structure of the membranes destroyed by treating with DOC was also partially restored by the same procedure.

5) Ultrasonic treatment also markedly destroyed the microvillus membrane and inhibited ATPase activity. Damaged ultrastructure and ATPase activity both were partially restored by treating with phospholipid, EPL.

6) Simultaneous presence of Na$^+$ and K$^+$ stimulated scarcely the ATPase of purified microvillus membranes.

7) The microvillus ATPase was slightly activated in the presence of D-glucose. Phloridin gave little effect on the activity of the microvillus ATPase.

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REFERENCES


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