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Transport ratios of reconstituted ($H^+ + K^+$)-ATPase

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Gastric ($H^+ + K^+$)-ATPase was reconstituted into artificial phosphatidylcholine/cholesterol vesicles by means of a freeze-thaw-sonication procedure. The passive and active transport mediated by these vesicles were measured (Skrabanja, A.T.P., Asty, P., Soumarmon, A., De Pont, J.J.H.H.M. and Lewin, M.J.M. (1986) Biochim. Biophys. Acta 860, 131–136). To determine real initial velocities, the proteoliposomes were separated from non-incorporated enzyme, by means of centrifugation on a sucrose gradient. The purified proteoliposomes were used to measure active H^+ and Rb^+ transport, giving at room-temperature velocities of 46.3 and 42.5 μmol per mg per h, respectively. A transport ratio of two cations per ATP hydrolyzed was also measured. These figures indicate that the enzyme catalyzes an electroneutral H^+–Rb^+ exchange.

Introduction

The enzyme ($H^+ + K^+$)-ATPase, present in the tubulovesicular system in the gastric mucosa, catalyzes the exchange of H^+ for K^+ upon hydrolysis of ATP and is involved in gastric acid secretion [1]. Most studies favour an electroneutral transport of the cations [2–4].

One of the unsolved problems in the study of ($H^+ + K^+$)-ATPase is the ratio between the ions transported and the ATP hydrolyzed. In the past ratios of 4, 2 and 1 have been postulated [5–7]. In a previous study with isolated native gastric membrane microsomes, we found that the ratio of H^+ transported per ATP hydrolyzed was dependent on the ATP concentration and approached a value of 2 at infinite ATP concentration. In addition, the kinetics indicated positive cooperativity between the transported H^+ ions [8].

Disadvantages of the latter preparation were the presence of proteins other than the ATPase in the microsomal membrane and the presence of 'broken' vesicles, both having an effect of the above measured ratio. Another limitation was the small intravesicular volume of the microsomes, so that measurement of initial transport rates was difficult, since saturaton occurred rapidly.

Recently the purified gastric ($H^+ + K^+$)-ATPase has been reconstituted in phosphatidylcholine/cholesterol liposomes, using a freeze-thaw-sonication technique [9,10]. These proteoliposomes accumulated H^+ upon addition of ATP, when K^+ was present intravesicularly. The rate of H^+ accumulation was higher when an outward-directed K^+ gradient was present than with inside and outside equilibrated K^+ concentrations. The rate increased with increasing K^+ gradient until

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saturation was reached. The enzyme also catalysed a passive H^+ transport, the magnitude of which was 5% of the active one.

In the present study, we have measured the H^+ and Rb^+ transport ratios at various ATP concentrations to establish the transport ratios. In order to do this, it was necessary to separate the incorporated enzyme from non-incorporated protein. Our measurements confirm the electroneutral behaviour of the $(H^+ + K^+)$-ATPase with the transport of 2 H^+ and Rb^+ ions per ATP hydrolyzed at high ATP concentration.

Methods

Preparation of membrane fraction. Stomachs of freshly slaughtered pigs were transported to the laboratory on ice. After flushing with tap water and cleaning with paper towels, the mucosa of the fundic region was scraped off and homogenized in a buffer comprising 150 mM sucrose/0.2 mM EDTA/5 mM Tris-HCl (pH 7.2). After three up–down strokes of the rotating pestle (1000 rev/min) of a Braun Teflon-glass homogenizer, the mixture was centrifuged for 20 min at 20000 $\times g$ (Sorvall, GSA rotor). The resulting supernatant was centrifuged for 45 min at 100000 $\times g$ (MSE, 8 × 50 rotor). The pellet was resuspended in 25 mM Tris-HCl (pH 7.4) and centrifuged on top of a gradient consisting of 7% Ficoll/250 mM sucrose in 25 mM Tris-HCl over 37% sucrose in 25 mM Tris-HCl. After 60 min centrifugation at 100000 $\times g$ (MSE, 8 × 50), the interface was diluted in 25 mM Tris-HCl and centrifuged for 60 min at 120000 $\times g$ (MSE, 10 × 10 rotor). The final pellet was resuspended in a small volume of 25 mM Tris-HCl and frozen at −30 °C. Specific ATP hydrolysis activities of these preparations were 70–90 μmol per mg per h.

Preparation of liposomes. A mixture of 40% cholesterol and 60% phosphatidylcholine was evaporated under a stream of nitrogen. After washing with diethyl ether, a 1 : 1 mixture of diethyl ether and buffer (5 mM glycylglycine/2 mM $MgSO_4$/50 mM K_2SO_4 (pH 6.1)) was added and the solution was thoroughly mixed on a Vortex mixer, while diethyl ether was again slowly evaporated by a stream of nitrogen. Afterwards liposomes were sonicated for 30 min in a Branson sonicator bath (at output 10). They were immediately used or were stored up to 1 week at 4 °C.

Solubilization and reconstitution. Gastric microsomes (20–24 mg/ml) in 25 mM Tris-HCl were treated with 1.8% (w/v) recrystallized cholate (final concentration). This preparation was then added to a 6-fold larger volume of liposomes, giving a lipid/protein ratio of 15 (on weight basis).

After thorough mixing, the preparation was frozen in liquid nitrogen, thawed at room temperature and sonicated for 2 min at output 10 in a Branson sonicator bath.

Gradient centrifugation. The reconstituted mixture was layered on top of a discontinuous gradient of 0, 10, 20 and 40% sucrose (w/v) in glycylglycine buffer (pH 6.1) and centrifuged for 90 min at 80000 $\times g$ in a TST 28–38 swing out rotor (MSE centrifuge). Afterwards fractions of 1–1.5 ml were collected from the top of the tube and the pattern at 280 nm was measured with an Uvicord ultraviolet cell.

Proton uptake measurement. The uptake of protons from the extravesicular medium was measured at 22 °C by recording the change in the medium pH after addition of ATP [5]. 150 μl of gradient purified proteoliposomes was added to 850 μl uptake buffer, comprising 5 mM glycylglycine/2 mM $MgSO_4$/50 mM K_2SO_4 (pH 6.1). The interior volume of the proteoliposomes was of the same composition.

MgATP was added to the suspension, while the pH was continuously recorded with a Radiometer GK 2231 C combined electrode, connected with a Radiometer PHM 75 Research pH meter with a BD 40 recorder (Kipp & Sons, Delft, The Netherlands).

Calibration of protein uptake measurements was performed by titrating the suspension with known volumes of 1 mM HCl.

ATP hydrolysis measurements. 50 μl aliquots of purified proteoliposomes were added to 150 μl of a medium containing 95 mM Tris-HCl, 16 mM $MgSO_4$, 0.3 mM ouabain, 0.3 mM EDTA and 32 mM choline chloride or 16 mM K_2SO_4 (pH 7.0). 200 μl of a stock solution containing 0.87 mM ATP and tracer [γ-^{32}P]ATP (for maximal ATP hydrolysis, otherwise less ATP) was then added

and the mixture was incubated at room temperature.

The reaction was terminated by adding 800 μl 5% trichloroacetic acid 10% Norit (w/v) (Norit binds the unhydrolyzed ATP). After 10 min the mixture was vortexed again, and centrifuged for 10 min at 5000 × *g* (Hereaus Christ). 500 μl of the supernatant was taken and counted in a Philips Liquid Scintillation Analyser.

Rb^+ transport measurement. Transport of Rb^+ was measured by adding 400 μl of purified proteoliposomes to 100 μl of a buffer containing 120 mM Tris-HCl, 500 mM Rb_2SO_4 and tracer ^{86}Rb. After 2 h loading at room temperature, the mixture was incubated with 25 μl of a buffer comprising 83.3 mM Tris-HCl/41.6 mM $MgSO_4$/41.6 mM ATP (pH 7.0) (for maximal initial transport, otherwise less ATP; control was without ATP), for 30 s at room temperature.

Reaction was stopped by adding 100-μl samples to Dowex columns [11]. Proteoliposomes were then eluted with 1 ml of a solution of 250 mM sucrose and 25 mg/ml bovine serum albumin. The eluate was counted in a Philips Liquid Scintillation Analyser by measuring Cerenkov radiation.

Protein measurement. Protein was determined with the method of Lowry et al. [12] or with the use of a fluorimeter with an excitation wavelength of 278 nm and an emission wavelength of 340 nm. Bovine serum albumin was used as standard.

Lipid phosphorus measurement. Phosphate content of the gradient was determined with the method of Fiske and SubbaRow [13]. Samples of 100 μl were digested with 0.2 ml concentrated $H_2SO_4/HClO_4$ for 1 h at 180 °C. The tubes were then cooled below 50 °C. In case the destruction was incomplete, 0.1 ml 30% H_2O_2 was added to the tubes, and the destruction was continued for at least 15 min, until the samples were colourless. After cooling, 4.75 ml of a freshly prepared mixture of 50 ml of a solution containing 2.60 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 2.2 ml of a solution containing 30.1 mg $Na_2S_2O_5$, 11 mg $Na_2S_2O_3$ and 55 mg aminonaphthalenesulphonic acid [14] was added.

The contents of each tube were mixed and incubated for 20 min in a boiling water bath. After cooling with tap water and standing for 30 min, the 820 nm absorbance was measured against water. In each determination, a series of standard P_i samples was incubated and similarly treated.

Dowex columns. Commercially available Dowex was converted from the hydrogen form to the Tris form. Columns for the assay were packed in Pasteur pipettes up to a height of 2 cm [15]. Before use, they were treated with 1.5 ml of a solution of 250 mM sucrose and 25 mg/ml bovine serum albumin.

Chemicals. MgATP, cholesterol and Dowex 50 W-X4 (200–400 mesh) were purchased from Sigma (St. Louis, MO, USA). Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham, AL, USA).

[γ-^{32}P]ATP and ^{86}Rb were from Amersham (U.K.) and all other chemicals were from Merck (Darmstadt, F.R.G.), except Ficoll from Pharmacia Fine Chemicals (Uppsala, Sweden) and sucrose from Janssen (Beerse, Belgium).

Results

Gradient purification of proteoliposomes

As shown in Fig. 1, a separation of the reconstitution mixture in three bands was obtained after 90 min of centrifugation. The three bands centrifuged at densities of 1.014, 1.039 and 1.094, respectively. Although after 90 min no equilibration was reached, this centrifugation time was applied because after longer centrifugation times, diffusion of the separate bands occurred.

All fractions of the gradient were collected and tested for their ability to hydrolyse ATP and to transport H^+ and Rb^+. A typical pattern of a gradient is shown in Fig. 2. The first (lightest) band showed nearly no ATPase activity and no transport of H^+ or Rb^+. Both other bands possessed ATPase activity, whereas the middle band had a transport capacity, both for Rb^+ (Table I) and H^+. Measurement of the protein concentrations by means of a sensitive fluorimetric method, yielded only small amounts in the lightest band, indicating that this fraction contained liposomes without protein incorporated and that the lipids were responsible for the absorption peak at 280 nm (Fig. 1). Inorganic phosphate, determined after complete destruction of the fractions, was only found in the two bands with the lowest density (Fig. 2) suggesting that liposomes were present

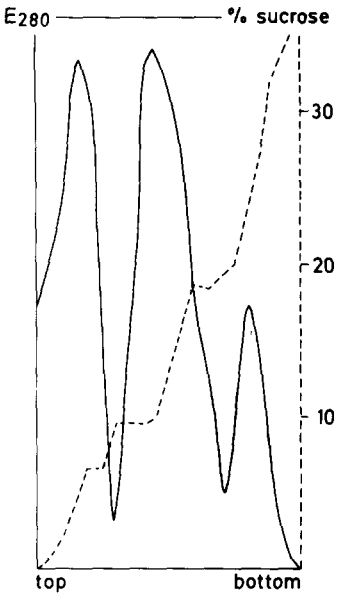

Fig. 1. Absorbance spectrum of proteoliposomes centrifuged on a discontinuous sucrose gradient. The absorbance of the gradient was continuously recorded by the passage of the fractions through a Uvicord ultraviolet cell with an wavelength of absorbance of 280 nm. Details of gradient are described in the Methods section.

only in these bands. The peak with the highest density probably contained non-incorporated enzyme. In the further part of this study, only the middle band was used, since it contained the proteoliposomes.

The fact that in the third (heaviest) band no phosphate is detected indicates only that the amount of enzyme is too small to measure the lipid phosphorus originating from the enzyme.

TABLE I

EXTRUSION OF RUBIDIUM FROM PROTEOLIPOSOMES

Rubidium extrusion from proteoliposomes initiated by MgATP during first 30 s of incubation at 22° C. See Methods.

Fraction number [a]	Rb^+ transport (μmol/mg per h)
8	26.2 (S.E. = 4.2, $n = 3$)
9	30.7 (S.E. = 3.4, $n = 3$)
10	42.5 (S.E. = 1.6, $n = 3$)
11	15.6 (S.E. = 3.0, $n = 3$)
12	3.2 (S.E. = 2.9, $n = 3$)

[a] The other fractions showed no Rb^+ transport capacity.

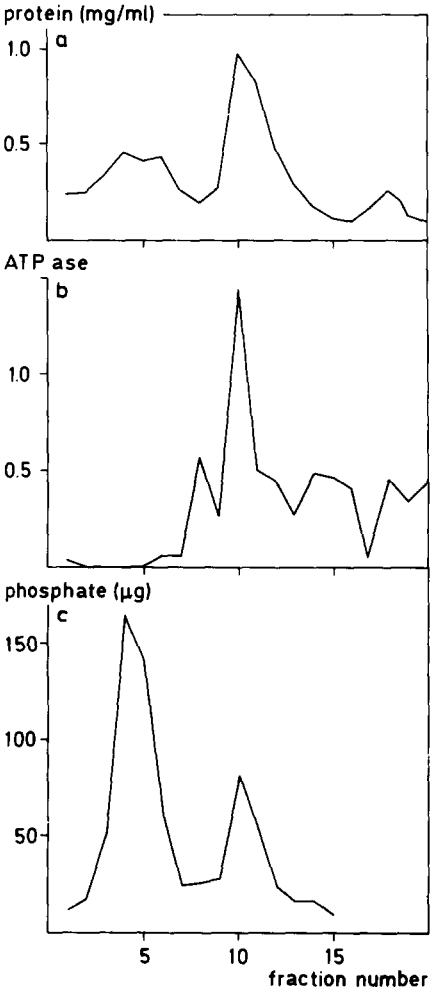

Fig. 2. Sucrose gradient as shown in Fig. 1. Curve a shows the protein content (mg/ml) of the fractions measured fluorimetrically. ATPase activity (curve b) was determined as described in the Methods section. Phosphate content was determined after heat destruction of the fractions (curve c), according to Fiske and SubbaRow [13]. Fractions of 1.5 ml were collected from the gradient, and for the above determinations 100-μl samples were used.

ATP hydrolysis

Initial velocities were measured in the first minute of incubation with ATP. In the experimental conditions used, hydrolysis was linear for this period of time. The highest value obtained in the presence of 5 mM ATP was 22.0 μmol per mg per h (S.D. = 2.6, $n = 6$). In the presence of 0.1% cholate (w/v), this figure was nearly doubled, reaching 37.1 μmol per mg per h (S.D. = 5.0, $n = 4$).

This indicates that in the middle peak 40% of the enzyme molecules are right-side-in and 60% right-side-out orientated. Upon addition of ATP only the latter molecules transport H^+ and Rb^+. Bearing in mind that the activities were measured at 22° C, these values suggest higher activities than found for native purified enzyme, with known activities of 70–90 μmol per mg per h [16], since at 37° C, ATPase activity is about 3-times higher than at 22° C (for purified membrane fraction).

H^+ and Rb^+ transport

Proton transport in gastric vesicles is mostly measured at pH 6.1, since at this pH hydrolysis of ATP itself does not result in proton gain or loss. Since ATP hydrolysis and Rb^+ transport was measured at pH 7.0, we compared the rate of proton transport at pH 7.0 and 6.1. At pH 7.0 the rate corrected for proton formation was due to ATP hydrolysis was 95% of the rate at pH 6.1. These results are in agreement with those of Reenstra and Forte [17]. We therefore used for convenience pH 6.1 for the study of proton transport.

At room temperature a maximal value for proton transport of 46.3 μmol H^+ per mg per h (S.D. = 5.6, $n = 5$) and for rubidium transport of 42.5 μmol per mg per h (S.D. = 1.6, $n = 3$) was found, both in the presence of 5 mM ATP. These velocities are not significantly different, indicating the same transport velocities for these processes.

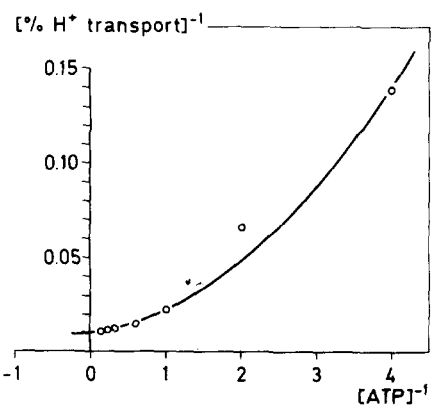

Fig. 3. Lineweaver-Burk plot of proton transport versus MgATP concentration in the extravesicular medium. ATPase activity was measured as described in the Methods section and H^+-transport was measured with the use of a pH electrode. Initial transport velocities during first 10 s were calculated and plotted against the average amount of ATP in this period. This figure is typical of five experiments.

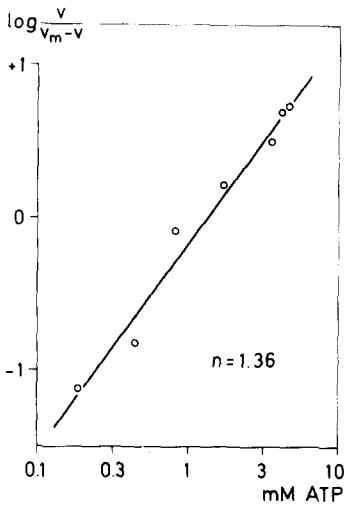

Fig. 4. Hill plot of the maximal H^+ transport velocities versus the MgATP concentrations, Hill coefficient is calculated from the slope of the curve. The values obtained for the initial velocities during first 10 s were used for this curve and plotted against the average ATP concentration during this period. This figure is typical of five experiments.

Both transport mechanisms could be inhibited by 0.2 mM vanadate and in the case of Rb^+, both the passive loading of the proteoliposomes during preincubation and the active extrusion after ATP addition were inhibited. This inhibition was concentration-dependent, at concentrations of 0.2 mM maximal inhibition was obtained.

When detergent, i.e., cholate, was added, even low concentrations (0.1% (w/v)) were sufficient to block the transport completely, suggesting an opening of closed vesicular systems. ATP hydrolysis was not affected by these concentrations: it was even enhanced.

Transport velocities increased at higher MgATP concentration (Fig. 3), resulting in the above-mentioned maximal values. When these results were plotted in a Hill plot, an average Hill coefficient was found of 1.25 (SD = 0.13, $n = 5$). In all experiments this value was greater than 1, indicating a positive cooperativity between the two protons, as was earlier found in native gastric membrane vesicles [8]. A typical example of such a Hill plot is shown in Fig. 4).

From the above values, a ratio between H^+

transported and ATP hydrolyzed of 2.10 (S.D. = 0.17) could be calculated. For Rb^+ transport, the ratio was 1.93 (S.D. = 0.24). These values are not significantly different from 2.

Discussion

In earlier studies, the proton transport capacity of native gastric membrane vesicles [8,18] and reconstituted $(H^+ + K^+)$-ATPase [9,10] has been demonstrated upon addition of MgATP. Several methods have been described to quantitate the transport velocities, such as accumulation of fluorescent dyes and of radioactive base. In our studies, we have used the pH meter method to measure H^+ transport.

Several different H^+/ATP ratios have been reported: a ratio of 1 was found by Reenstra and Forte [6], and by Smith and Scholes [7]; a ratio of 2 was described by Rabon et al. [5] and by ourselves [8], and even a ratio of 4 was reported by the group of Sachs [2]. Reconstitution of $(H^+ + K^+)$-ATPase in artificial liposomes and the purification of these proteoliposomes gives a preparation in which 'real' transport velocities can be measured, since few other proteins are present to decrease specific activities. Therefore, reconstitution can be used to settle the question of ratios by determination of maximal transport and hydrolysis rates. To determine these rates, different incubation conditions have been used. The results can be compared, however, since for each process maximal rates were measured. Proton transport has been measured at pH 6.1 for practical reasons, since this is the isoprotonic pH for ATP hydrolysis. At higher pH values proton transport after ATP addition has to be corrected for ATP hydrolysis, but the proton uptake rates are not significantly different from those at pH 6.1 (Ref. 17 and this study). After reconstitution, purification of the proteoliposomes is necessary, since non-incorporated ATPase, capable of ATP hydrolysis, but not of transport would lead to an underestimate of the H^+/ATP and Rb^+/ATP ratios.

The proton transport velocity of 46.3 μmol per mg of protein per h is higher than reported previously for native membrane vesicles [8]. This is presumably due to the effect of the $(H^+ + K^+)$-ATPase purification. The transport ratio of 2 protons transported per ATP hydrolyzed was, however, not affected. In parallel studies on Rb^+ transport (used as a marker for K^+ transport) we found that the maximal velocity of this transport was not significantly different from that of the H^+-transport.

These findings imply the electroneutral functioning of $(H^+ + K^+)$-ATPase; two H^+ ions would be exchanged for two K^+ ions in each transport cycle. Earlier, electroneutrality was suggested by the absence of vesicular accumulation of lipophilic ions during ATPase activity [2,3] and by the absence of a membrane potential [4]. In the present study, direct evidence for the electroneutrality of the ATPase reaction is given, whereas part of the reaction cycle has recently been shown to be electrogenic (Van der Hijden, H.T.W.M., personal communication).

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