Biochimica et Biophysica Acta, 774 (1984) 91–95
Elsevier

BBA 72170

THE H^+/ATP TRANSPORT RATIO OF THE ($K^+ + H^+$)-ATPase OF PIG GASTRIC MEMBRANE VESICLES *

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(Received January 27th, 1984)

Key words: ($K^+ + H^+$)-ATPase; Proton transport; H^+/ATP transport ratio; (Pig gastric parietal cell membrane)

Various values have been reported for the H^+/ATP transport ratio of the ($K^+ + H^+$)-ATPase of the gastric parietal cell: 4, 2 and 1. We have, therefore, reinvestigated this matter with a vesicle preparation isolated from pig gastric mucosa. The vesicles are suspended in glycylglycine buffer (pH 6.11) at 22°C, and incubated until equalization of the K^+ concentration inside and outside (75 mM). After addition of ATP, the initial rates of H^+ uptake and ATP hydrolysis are then measured. Proton uptake is inhibited in the absence of K^+ or in the presence of nigericin. The $K_{0.5}$ value for proton transport is 154 μM and the K_m value for ATP hydrolysis is 61 μM. The Lineweaver-Burk plot for ATP hydrolysis vs. ATP concentration is linear with a V_{max} of 5.5 nmol/mg protein per s, but that for H^+ uptake is not. Thus with increasing ATP concentration (6.7 to 1670 μM) the transport ratio increases from 0.3 to 1.8. Extrapolation to infinite ATP concentration gives a value of 1.89. (S.E. 0.13, $N = 5$) and a Hill coefficient of $n = 1.21$ (S.E. 0.06, $N = 5$) implying that the true transport ratio is 2 H^+/ATP with positive cooperativity between the protons.

Introduction

Resting gastric parietal cells contain a multitude of tubular vesicles, which upon isolation have been shown to possess a high activity of a K^+-stimulated ATPase. This enzyme, usually called ($K^+ + H^+$)-ATPase, has been shown to be responsible for proton uptake into the vesicles at the expenditure of ATP [1,2]. This process has been demonstrated in vesicles from pig [3,4] and dog [5,6] gastric mucosa.

Divergent values for the H^+/ATP transport ratio have been reported: 4 by Sachs et al. [7] in 1976, 2 by Rabon, McFall and Sachs [3] in 1982, and 1 by Reenstra and Forte [4] in 1981 and by Smith and Scholes [6] in 1982. Thermodynamically, a value of 4 would be unlikely in view of the $10^5:1$ gradient which can be generated in the stomach. The other two values would both be thermodynamically possible.

From a study of the condition used by these investigators, it became clear that in all but one study fixed ATP concentrations have been used. This has led us to an investigation in which a wide range of ATP concentrations is used. In addition we have determined some kinetic parameters of H^+ transport and ATP hydrolysis, and we have investigated the cooperativity of H^+ transport.

Materials and Methods

Preparation of gastric membrane vesicles. Isolation of the gastric vesicles has been carried out according to the procedure previously reported by Forte et al. [8–10], Sachs et al. [7,11,12] and Schrijen et al. [13] with some modifications.

Stomachs from freshly slaughtered pigs are ob-

* This is article no. VII in the series Studies on ($K^+ + H^+$)-ATPase.

tained from the local slaughterhouse and after removal of the contents transported to the laboratory on ice. All subsequent manipulations are carried out at 4°C. The stomachs are rinsed with tap water and the fundic region is taken and placed in 3 M NaCl. Mucus is removed by wiping the tissue with paper towels and the mucosa is scraped from the underlying muscular layer by means of a surgical blade. The scraped material (70–80 g) is placed in 200 ml homogenization buffer, containing 113 mM mannitol, 37 mM sucrose, 0.2 mM EDTA, 5 mM Tris-HCl (pH 7.8). The mixture is homogenized with a Braun teflon-glass homogenizer by three up-down strokes of the rotating pestle (250 rev./min).

The homogenate is filtered over four layers of surgical gauze and centrifuged for 20 min at 20000 $\times g$ in a Sorvall RC2-B centrifuge (rotor GSA, 4°C). The supernatant is centrifuged for 60 min at 100000 $\times g$ in an MSE Prepsin 50 centrifuge (rotor 8 × 50, 4°C). The resulting pellet is resuspended in 20 ml homogenization buffer. The suspension is layered on top of a discontinuous gradient of 20 ml 20% (w/v) sucrose and 10 ml 37% (w/v) sucrose. The gradient is centrifuged for 40 min at 80000 $\times g$ in an MSE Europe 75 centrifuge (rotor TST 28.38, 4°C).

The vesicle fraction at the interface of supernatant and 20% sucrose layer is collected by suction. It is diluted 1 : 1 with a buffer containing 5 mM glycylglycine, 150 mM KCl and 2 mM $MgCl_2$ [5], and the pH is adjusted to 6.05–6.10. The resulting suspension is stored overnight at 4°C to equilibrate the vesicles with respect to pH and K^+ content. The next day this fraction is used for measurements of H^+ transport and ATP hydrolysis. The fraction at the interface of the 20% and 30% sucrose layers can be collected for the purification of $(K^+ + H^+)$-ATPase by centrifugation on a continuous sucrose gradient according to Schrijen et al. [13].

Proton uptake measurement. The uptake of protons from the extravesicular medium is measured at 22°C by recording the change in the medium pH after addition of ATP [3]. 4 ml of gastric membrane vesicles (4–6 mg protein), equilibrated overnight at 4°C in glycylglycine buffer, are placed in a small glass vessel. The suspension is adjusted to pH 6.11 and is magnetically stirred throughout the experiment, while the pH is continuously recorded with a Radiometer GK 2321C combined electrode connected to a Radiometer PHM 75 Research pH meter with a BD 40/60 recorder (Kipp and Sons, Delft).

Measurements are started by adding known volumes of a 5 mM MgATP solution, adjusted to pH 6.11. Any pH changes due to the addition of ATP are corrected for. Subsequently, the proton uptake is determined by titrating the suspension with 10^{-3} M HCl or 10^{-3} M KOH. The response time of the electrode is negligible (less than 2 s).

Valinomycin and nigericin are added as 1 mg/ml solutions in methanol.

ATP hydrolysis measurement. During proton uptake measurements 100-μl aliquots are taken from the vessel for phosphate determination according to a modification of the procedure of Carter and Karl [14]. The aliquots are added to 1 ml of a solution containing 0.85 M HCl, 0.3% sodium molybdate, 0.2% Bion-Ne-9 to stop the enzyme reaction. Then 320 μl malachite green solution (6.3 mg/100 ml) is added, and immediately after mixing the extinction at 625 nm is read and compared with phosphate standards similarly treated.

Protein determination. The method of Lowry et al. [15] is employed with trichloroacetic acid precipitation of the protein and bovine serum albumin as standard.

Chemicals. MgATP, nigericin and valinomycin have been purchased from Sigma (St. Louis, MO, U.S.A.), bovine serum albumin from Behringwerke (Marburg, F.R.G.), Bion-Ne-9 from Pierce (Rockford, IL, U.S.A.), malachite green from Aldrich (Milwaukee, WI, U.S.A.) and sucrose from Janssen (Beerse, Belgium), all other chemicals from Merck (Darmstadt, F.R.G.).

Results

Centrifugation on the discontinuous sucrose gradient (37%, 20% sucrose, supernatant) yields two vesicle fractions: one at the 30/37% sucrose interface, and one at the supernatant/20% sucrose interface.

The $(K^+ + H^+)$-ATPase activity in the first fraction is 40 μmol P_i/mg protein per h when measured at 37°C, pH 7.4 in the presence of 20 mM K^+ and 5 mM ATP and after correction for

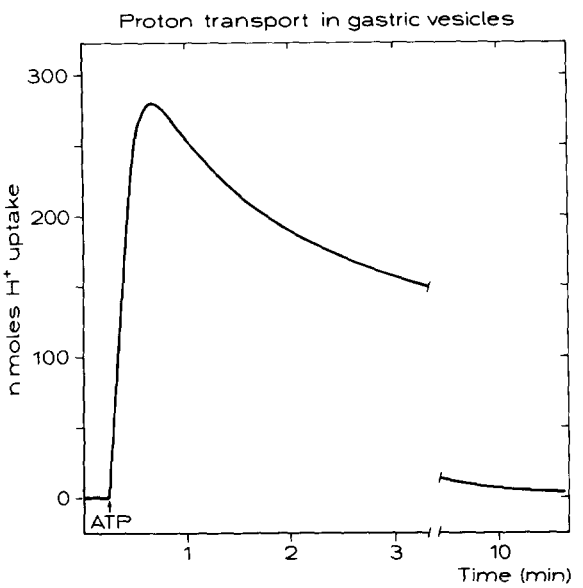

Fig. 1. Proton transport in gastric parietal cell vesicles. Typical experiment with 5.2 mg protein in 4 ml vesicle suspension. Addition of 2 mmol MgATP, final ATP concentration 455 μM. Reproduction of pH meter recording.

other ATPase activity by replacing K^+ with 20 mM choline chloride. The activity is not stimulated by valinomycin addition and the vesicles do not exhibit proton transport. So these vesicles are presumably leaky, and they have not been used in any of the experiments reported below. The supernatant/20% sucrose interface fraction has a ($K^+ + H^+$)-ATPase activity of 12 μmol/mg per h when determined under the same conditions. This activity can be stimulated 80–100% by the addition of 10 μl valinomycin (1 mg/ml in ethanol), and the vesicles in this fraction exhibit H^+ uptake capacity. This fraction is assumed to consist of closed vesicles, and it has therefore been used in all further experiments.

A typical curve for proton uptake is shown in Fig. 1. Proton uptake reaches a maximum within 30 s after addition of ATP. Afterwards passive efflux of protons dominates and the medium pH returns to the initial value after 5–10 min. The proton efflux can be enhanced by addition of the K^+ ionophores valinomycin and nigericin, in which case the pH returns to the initial value within 90 s. Addition of nigericin before ATP removes the ability of the vesicles to accumulate protons, so that no pH change occurs upon addition of ATP. Addition of valinomycin before ATP does not have this effect, because there is no K^+ gradient between the equilibrated vesicles and the buffer medium. However, addition of valinomycin after ATP addition, when the uptake of H^+ has caused an equivalent extrusion of K^+, causes K^+ influx in exchange for H^+, thus speeding up the return of the medium pH.

From the plot of phosphate release against time (Fig. 2) we can calculate that the maximal medium pH is reached when 80–90% of the added 100 nmol ATP has been consumed. When the vesicles are equilibrated in choline chloride the ATPase activity is only about 5% of the activity after equilibration with KCl, and the proton transport is negligible. When the vesicles are washed to remove the last traces of K^+, and then equilibrated with choline chloride, the ATPase activity is only 1% and proton transport is zero. These observations agree with the findings reported by Rabon et al. [3].

The rates of H^+ transport and ATP hydrolysis during the first 10 s are constant, and heve therefore been used in determining the H^+/ATP transport ratio. This ratio has been determined at different ATP concentrations in the following way. Lineweaver-Burk plots have been constructed for

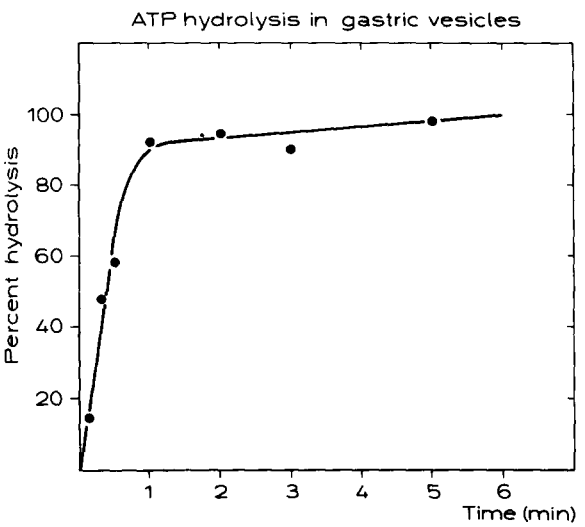

Fig. 2. ATP hydrolysis by gastric parietal cell vesicles. Typical experiment with 3 mg protein in 4 ml vesicle suspension. ATP hydrolysis is plotted against time following addition of 100 nmol MgATP.

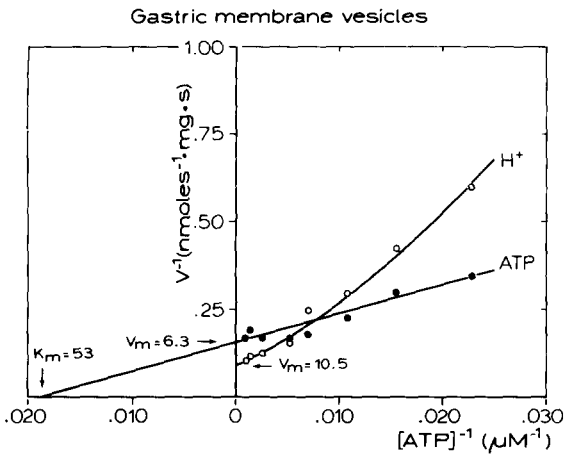

Fig. 3. Lineweaver-Burk plots of transport rate and ATP hydrolysis rate versus ATP concentration. The straight line through the experimental points for ATP hydrolysis has been determined by linear regression analysis. K_m and V_{max} values are determined as the intercepts on the abscissa and ordinate. The best fitting curve through the experimental points of H^+ transport is drawn visually, and V_{max} is extrapolated as intercept on the abscissa. All points have been determined with 5.2 mg protein in 4 ml vesicle suspension.

the rates of ATP hydrolysis and H^+ transport in the initial 10 s period against the average ATP concentrations during this period (ATP concentration at zero time plus that at 10 s, divided by 2). Fig. 3 gives a typical example of such Lineweaver-Burk plots. The two plots are not parallel, which has been consistently found in all experiments. We conclude that the transport ratio increases with the ATP concentration to a maximal value of nearly 2 at infinite ATP concentration.

Table I summarizes the results of five experiments. The average transport parameter for proton transport are $V_{max} = 10.2$ nmol H^+/mg per s (S.E. 0.3) and $K_{0.5} = 154$ μM (S.E. 5), and for ATPase activity $V_m = 5.5$ nmol P_i/mg per s (S.E. 0.3) and $K_m = 61$ μM (S.E. 7). The average H^+/ATP transport ratio at infinite ATP concentration is 1.89 (S.E. 0.13), which value is significantly higher than 1 and not significantly different from 2. The curved Lineweaver-Burk plot suggests the presence of an allosteric effect in proton transport. Hill plots constructed from these data are linear and yield a Hill coefficient of 1.21 (S.E. 0.06), which is significantly higher than 1 and thus confirms positive cooperativity in proton transport.

TABLE I

RESULTS FOR PROTON TRANSPORT BY GASTRIC MEMBRANE VESICLES

	V_m for H^+ transport (nmol·$mg^{-1}\cdot s^{-1}$)	V_m for ATPase activity (nmol·$mg^{-1}\cdot s^{-1}$)	H^+/ ATP ratio	Hill coefficient
	10.0	4.6	2.16	1.40
	10.0	5.9	1.69	1.19
	11.1	4.8	2.31	1.06
	10.5	6.3	1.67	1.30
	9.5	5.7	1.68	1.08
Mean	10.2	5.5	1.89	1.21
S.E.	0.3	0.3	0.13 [a]	0.06 [b]

$K_{0.5} = 154 \pm 5$ μM $K_m = 61 \pm 7$ μM

[a] Not significantly different from 2.
[b] Significantly higher than 1 ($P = 0.03$).

Discussion

We find a transport ratio increasing from 0.4 at 8 μM ATP to a maximum value of 1.89 at infinite ATP concentration. This may explain the different ratios reported by Reenstra and Forte [4] and Smith and Scholes [6], who found a ratio of 1, and by Rabon et al. [7], who observed a ratio of 2. Smith and Scholes used a low concentration of 50 μM ATP, while Rabon and co-workers used four different concentrations giving a wide spread of ratios, up to a ratio of 2 for the highest concentration of 255 μM ATP. In view of our results, we may conclude that the different transport ratios of Smith and Scholes and Rabon et al. are due to the different ATP concentrations used. It does not explain the low transport ratio of 1 reported by Reenstra and Forte [4] for they used a high concentration of ATP (1000 μM), which in our experiments gives a ratio significantly greater than 1. Their low ratio might be due to their way of preparing and storing the vesicles, particularly the storage of the material as a frozen suspension. The freezing of the material could very well damage the integrity of the vesicles leading to a high outward leakage of protons during proton uptake.

Our maximal transport ratio of 1.89 could be somewhat low for two reasons. First the total proton uptake might be underestimated because of the contribution of protons leaking out during the

first 10 s of measurements, which would result in a somewhat lower peak in the plot of pH versus time. Secondly a small part of the amount of hydrolyzed ATP could be due to the presence of leaky vesicles, which are able to hydrolyze ATP but not to transport protons. It is unlikely that the presence of other ATPases would lower the ratio, since in the absence of Na^+ and Ca^{2+} neither $(Na^+ + K^+)$-ATPase, nor $(Ca^{2+} + Mg^{2+})$-ATPase could be active.

Thus we assume a true transport ratio of 2 protons per ATP. Thermodynamically this ratio is the highest possible, as shown by the following calculations. A ΔpH of 4.5 units is found to be maximally generated by gastric membrane vesicles [17]. The equation for the needed transport energy is as follows:

$$\Delta G = RT \ln \frac{c_2}{c_2} = RT \ln 10^{4.5} = 6.0 \text{ kcal/mol}$$

If two protons are transported, this would thus require cost 12 kcal. The hydrolysis of ATP has a free energy of -13.4 kcal/mol, which is sufficient for the transport of 2 H^+/ATP, and in addition transport of K^+ against a gradient which is necessary when an electroneutral pump is assumed [17].

The curved shape of the Lineweaver-Burk plot for H^+ uptake and the Hill coefficient of 1.21 (derived from plotting $\log(v/(V_{max} - v))$ versus log[ATP]) indicate positive cooperativity of two proton sites involved in proton transport. The curved shape of the proton uptake plot explains why the H^+/ATP transport ratio increases at increasing ATP concentration. The Hill coefficient larger than 1 indicates that the transport of one proton facilitates the transport of a second proton. The low ratios at low ATP concentration are probably due to the increase of proton leakage from the vesicles relative to the active proton uptake.

Acknowledgements

We are grateful to Mr. Herman Swarts for his valuable advise and assistance with the pH measurements. This study has been supported in part by the Netherlands Organization for Basic Scientific Research through the Netherlands Biophysics Foundation.

References

1 Bonting, S.L., De Pont, J.J.H.H.M., Van Amelsvoort, J.M.M. and Schrijen, J.J. (1980) Ann. N.Y. Acad. Sci. 341, 335–356
2 De Pont, J.J.H.H.M. and Bonting, S.L. (1981) in Membrane Transport (Bonting, S.L. and De Pont, eds.), pp. 209–234, Elsevier/North-Holland Biomedical Press
3 Rabon, E.C., McFall, T.L. and Sachs, G. (1982) J. Biol. Chem. 257, 6296–6299
4 Reenstra, W.W. and Forte, J.G. (1981) J. Membrane Biol. 61, 55–60
5 Lee, J., Simpson, G. and Scholes, P.B. (1974) Biochem. Biophys. Res. Commun. 60, 825–832
6 Smith, G.S. and Scholes, P.B. (1982) Biochim. Biophys. Acta 688, 803–807
7 Sachs, G., Chang, H.H., Rabon, E.C., Shackman, R., Lewin, M.J.M. and Saccomani, G. (1976) J. Biol. Chem. 25, 7690–7698
8 Forte, J.G., Ganser, A.L. and Tanisawa, A.S. (1974) Ann. N.Y. Acad. Sci. 242, 255–267
9 Lee, H.C. and Forte, J.G. (1978) Biochim. Biophys. Acta 508, 339–356
10 Ray, T.K. and Forte, J.G. (1976) Biochim. Biophys. Acta 443, 451–467
11 Saccomani, G., Stewart, H.B., Shaw, D., Lewin, M.J.M. and Sachs, G. (1977) Biochim. Biophys. Acta 465, 311–330
12 Schackman, R., Schwartz, A., Saccomani, G. and Sachs, G. (1977) J. Membrane Biol. 32, 361–381
13 Schrijen, J.J., Luyben, W.A.H.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1980) Biochim. Biophys. Acta 597, 331–344
14 Carter, S.G. and Karl, D.W. (1982) J. Biochem. Biophys. Methods 7, 7–13
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
16 Forte, J.G., Adams, P.H. and Davies, R.E. (1963) Nature 197, 874–876
17 Rabon, E.C., Chang, H. and Sachs, G. (1978) Biochemistry 17, 3345–3353