Biochimica et Biophysica Acta 860 (1986) 131–136
Elsevier

BBA 73233

H^+ transport by reconstituted gastric ($H^+ + K^+$)-ATPase

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(Received February 24th, 1986)

Key words: ($H^+ + K^+$)-ATPase; Membrane reconstitution; H^+ transport; (Pig stomach)

Gastric ($H^+ + K^+$)-ATPase was reconstituted into artificial phosphatidylcholine/cholesterol liposomes by means of a freeze-thaw-sonication technique. Upon addition of MgATP, active H^+ transport was observed, with a maximal rate of 2.1 μmol · mg^{-1} · min^{-1}, requiring the presence of 100 mM K^+ at the intravesicular site. However, in the absence of ATP an H^+-K^+ exchange with a maximal rate of 0.12 μmol · mg^{-1} · min^{-1} was measured, which could be inhibited by the well-known ATPase inhibitors vanadate and omeprazole, giving the first evidence of a passive K^+-H^+ exchange function of gastric ($H^+ + K^+$)-ATPase. An Na^+-H^+ exchange activity was also measured, which was fully inhibited by 1 mM amiloride. Simultaneous reconstitution of Na^+/H^+ antiport and ($H^+ + K^+$)-ATPase could explain why reconstituted ATPase appeared less cation-specific than the native enzyme (Rabon, E.C., Gunther, R.B., Soumaron, A., Bassilian, B., Lewin, M.J.M. and Sachs, G. (1985) J. Biol. Chem. 260, 10200–10212).

Introduction

Early work by Lee et al. [1] demonstrated that gastric vesicles are able to actively transport H^+ as the intact mucosa does. This property was correlated with the presence of an ATPase later called ($H^+ + K^+$)-ATPase which actively exchanged H^+ for K^+ [2,3]. The low unspecific permeability of native vesicles allowed a good characterization of ATPase transport properties and it was shown that, in the absence of ATP, ($H^+ + K^+$)-ATPase can act as a passive K^+/K^+ exchanger [4].

Reconstition of gastric ($H^+ + K^+$)-ATPase has recently been achieved by means of free-thaw-sonication of a mixture of phosphatidylcholine-cholesterol liposomes and solubilized membranes [4,5]. As found with native membranes, in the presence of K^+, proteoliposomes accumulated acridine orange dye after the addition of MgATP, suggesting vesicular H^+ accumulation. Moreover, as seen with native membranes, reconstituted ($H^+ + K^+$)-ATPase also catalyzed passive exchange of K^+ in the absence of ATP [4,5]. However, the rate of active H^+ transport could not be measured because of the dye technique used.

In the present paper, we used a pH electrode to measure the rate of active H^+ transport [6] and took advantage of the larger intravesicular volume to test the existence of an ATPase-mediated passive H^+-K^+ exchange thought to be present in native membranes [4].

Methods

Preparation of the membrane fractions. Stomachs from freshly slaughtered pigs were transported to the laboratory on ice; after flushing with tap water and cleaning with paper towels, the mucosal layer was removed from the underlying tissue and homogenized in a buffer containing 250 mM sucrose and 50 mM Hepes-Tris (pH 7.2). This homogenate was centrifuged for 10 min at 2000 rpm; the pellet

was homogenized once more and centrifuged again. The supernatants were pooled and centrifuged for 7 min at 25000 rpm (70 Ti rotor, Beckman L5-65 centrifuge); the supernatant from this step was afterwards centrifuged for 30 min at 39000 rpm.

The resulting pellet was resuspended in the buffer and centrifuged on top of 30% sucrose (w/w) for 2 h at 40000 rpm. The opalescent 8.5%–30% sucrose interface was diluted twice in 50 mm Hepes-Tris (pH 7.2) and centrifuged for 30 min at 40000 rpm. The resulting pellet, which contained the $(H^+ + K^+)$ATPase-enriched membrnes, was resuspended in the buffer and stored at −30°C [4].

Preparation of liposomes. A mixture of 60% egg phosphatidylcholine and 40% cholesterol in chloroform was evaporated with a stream of nitrogen. After washing with diethyl ether, a 1:1 (v/v) mixture of diethyl ether and buffer was added, and the solution was thoroughly mixed on a vortex while ether was again slowly evaporated by a stream of nitrogen.

After all ether had disappeared, liposomes were filtrated through a series of Millipore filters (WP type) from 1.2 down to 0.45 μm. Then the mixture was sonicated for 20 min in a Branson B 12 sonicator bath. The final solution contained 50 mg phospholipids per ml. The vesicles could be stored at 4°C up to 1 week.

Solubilization and reconstitution of the enzyme. Gastric microsomes (15–19 mg/ml), suspended in a buffer containing 40 mM Hepes (pH 7.2), were treated with 1.3% (w/v) recrystallized cholate (final concentration). This preparation was added to a 4–5-fold larger volume of liposomes at a lipid-protein ratio of 15 (on weight basis).

After thorough mixing, the reconstitution mixture was frozen in liquid nitrogen, thawed at room temperature and sonicated for 2 min in a Branson B 12 bath sonicator. Detergent was removed from proteoliposomes by centrifuging 150-μl aliquots over a Sephadex G-25 coarse column in a 1.5 ml syringe for 2 min at 700 rpm (Diamon/IEC PC-6000 centrifuge). This step was repeated once and the final eluate was used for transport studies. Control experiments with [^{3}H]cholate demonstrated that the final eluate contained less than 0.02% cholate (w/v).

Proton-uptake measurements. The uptake of protons from the extravesicular medium was measured at 22°C by recording the change in the medium pH, with and without addition of MgATP.

For each experiment 150 μl of Sephadex-eluted proteoliposomes were added to 850 μl glycylglycine buffer. All experiments started at pH 6.05–6.10 and the pH was continuously recorded with a Tacussel TCBC 11/HS combined electrode connected to a Tacussel ISIS 20.000 ph meter with a SEFRAM recorder. Calibration of proton uptake was done by titrating the suspension afterwards with 10^{-3} M KOH or 10^{-3} M HCl. Response time of the electrode was less than 1 s.

Final concentrations of vanadate and omeprazole were 0.5 mM and 0.2 mM, respectively. Omeprazole was prepared as a 20 mM solution in 1 mM HCl immediately before use and kept in darkness.

The ionophores CCCP and valinomycin were dissolved in ethanol and diluted to a final concentration of 25 μM. The same volume of ethanol alone had no effect.

Protein determination. Protein was measured either by the Coomassie blue staining according to Bradford [7] or according to Lowry et al. [8] using bovine serum albumin as standard.

Chemicals. Dithiothreitol, valinomycin, MgATP, CCCP (carbonylcyanide *m*-chlorophenylhydrazone) and cholesterol were purchased from Sigma (St. Louis, MO, U.S.A.).

Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Sephadex G-25 coarse from Pharmacia Fine Chemicals (Uppsala, Sweden) and omeprazole was a gift from Dr. B. Wallmark (Hassle AB, Sweden). Filter type WP were purchased from Millipore SA (Molesheim, France).

All other chemicals were from Prolabo (Paris, France).

Results

Active transport of H^+

Proteolipisomes, reconstituted by freeze-thaw sonication of a mixture of gastric membranes, cholate and liposomes, took up H^+ in the presence of K^+ and in response to the addition of MgATP (Fig. 1). MgATP induced-H^+ uptake was reversed

by the addition of CCCP plus valinomycin, when both intra- and extra-proteoliposomal medium contained K^+ (Fig. 1). Addition of CCCP alone triggered only part of the depletion which required valinomycin to be 95–100% completed. This was in agreement with previous results on native vesicles preparations and suggested that measured uptake mostly resulted from active exchange of H^+ for K^+ which required both H^+ (CCCP) and K^+ (valinomycin) conductances to be collapsed. Using phosphatidylcholine/cholesterol mixtures, 10-fold higher concentrations of ionophores than used with native membranes were required. Similar concentrations had no effect on pure liposomes.

Rates of active H^+ uptake were measured in three different ionic conditions: (i) no potassium present, (ii) equilibrated potassium concentrations, (iii) potassium concentration gradient with K^+ inside proteoliposomes and no K^+ outside. Those experiments demonstrated that the highest rates of transport were mesured in the presence of sulfate as anion and of an outwardly directed K^+ gradient (with sucrose in the extravesicular medium). With Na^+ present in the extravesicular medium, a lower H^+ uptake was observed, due to the existence of an Na^+-H^+ exchange, counteracting the H^+-K^+ exchange, as will be shown later.

Dilution of K^+-loaded proteoliposomes (25 mM K^+) in K^+-free medium induced a slow uptake of H^+ which was strongly accelerated by MgATP (Fig. 2). When maximal uptake was reached, valinomycin could still stimulate H^+ uptake.

This ionophore-dependent uptake was reversed by CCCP, suggesting that maximal H^+ uptake in the absence of ionophores was limited by the availability of intravesicular K^+.

It is to be noticed that addition of valinomycin induced a rapid drop of H^+ uptake, suggesting that a transient H^+ conductance was created. This result is not clearly understood. Addition of CCCP enhanced this drop, confirming that valinomycin had created a vesicular potential outside negative

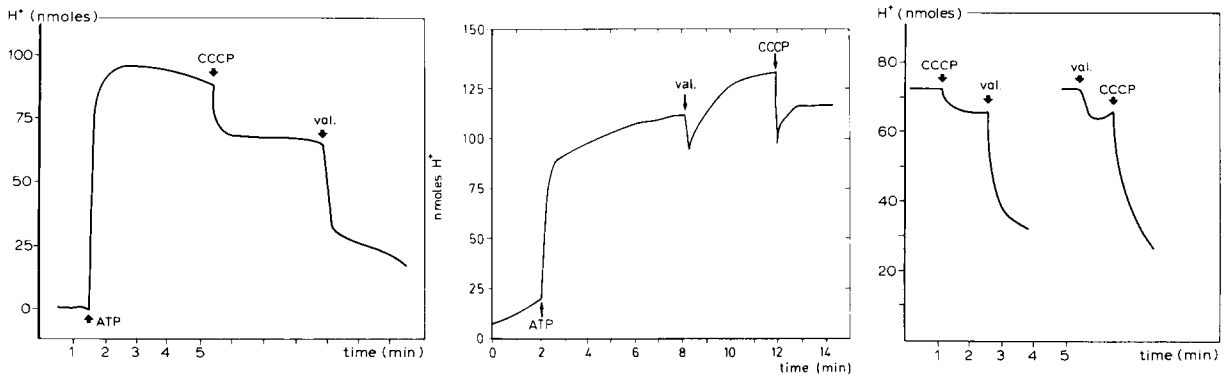

Fig. 1. (left-hand figure) Active H^+ uptake by reconstituted ($H^+ + K^+$)-ATPase. 200 μl 5 mM MgATP are added to 1 ml of a suspension of reconstituted ($H^+ + K^+$)-ATPase (0.375 mg protein) in a buffer containing 5 mM glycylglycine, 2 mM $MgSO_4$ and 12.5 mM K_2SO_4 (pH 6.1). Final concentrations of CCCP and valinomycin are 25 μM and 0.1 mM respectively, added as methanol solutions.

Fig. 2. Active H^+ transport in the presence of a K^+ gradient. ($H^+ + K^+$)-ATPase is reconstituted into liposomes prepared in 5 mM glycylglycine, 2 mM $MgSO_2$ and 12.5 K_2SO_4 (pH 6.1) and diluted 6.7-times into a buffer containing 5 mM glycylglycine, 2 mM $MgSO_4$ and 1% sucrose (pH 6.1). Final protein concentration is 0.375 mg/ml. Initial ATP concentration is 0.25 mM and final concentrations of valinomycin and CCCP are 25 μM.

Fig. 3. (right-hand figure) H^+ and K^+ conductances of PC/cholesterol proteoliposomes. ($H^+ + K^+$)-ATPase is reconstituted into liposomes prepared in 5 mM glycylglycine, 2 mM $MgSO_4$ and 1% sucrose (pH 6.1) and diluted 6.7-times into a buffer containing 5 mM glycylglycine, 2 mM $MgSO_4$ and 12.5 mM K_2SO_4 (pH 6.1). Final concentrations of CCCP and valinomycin are 25 μM and 0.1 mM, respectively, added as methanol solutions. Final protein concentration is 0.375 mg/ml.

by recycling K^+ into the vesicle.

Rate of active H^+ uptake depended upon the concentration of ATP used. Maximal rate was 2.1 μmol per min per mg protein, in the presence of 160 mM K^+ inside and 1 mM ATP.

Maximal quantity of H^+ taken up by this material (in the presence of 160 mM K^+ inside and outside the vesicles) was 270 nmol per mg protein, which could be compared to the 54 nmol previously found with native membrane vesicles in the presence of the same ATP concentrations [6]. This was in agreement with a larger size of proteoliposomes as seen by electron microscopic examination (200 $\pm$ 90 nm in diameter), which could also account for the slower time course of vesicle loading. Maximum of uptake was reached after 70 s in proteoliposomes and 20 s in native vesicles.

Passive permeability

H^+ and K^+ conductances. The passive H^+ and K^+ conductivities in proteoliposomes were determined using ionophores in the absence of ATP. In the presence of a K^+ gradient (inside 25 mM K^+/outside no K^+), addition of valinomycin created a slow H^+ leak, which was increased after CCCP addition (Fig. 3). This suggests that the proteoliposomes have a low H^+ conductance. Addition of CCCP prior to valinomycin created a very small H^+ conductance. Addition of CCCP prior to valinomycin created a very small H^+ leak, which was suggestive of a low K^+ conductance (Fig. 3).

K^+-H^+ exchange. Passive transport of H^+ in the absence of ionophores was found; it required the availability of a K^+ or Na^+ counter-gradient across the proteoliposomal membrane. When K^+ or Na^+ was present inside the proteoliposomes, H^+ influx occurred, which was an H^+ efflux under opposite conditions (Na^+ or K^+ outside). The results suggested the existence of both H^+-K^+ and H^+-Na^+ exchanges. Upon increasing the K^+ counter concentration the exchange rate was saturated. The maximal rate represented approx. 5% of them aximal active H^+ transport rate in the same preparation (Fig. 4).

Effect of inhibitors

Active transport of H^+ was 80–90% inhibited by 0.5 mM vanadate and by 0.2 mM omeprazole

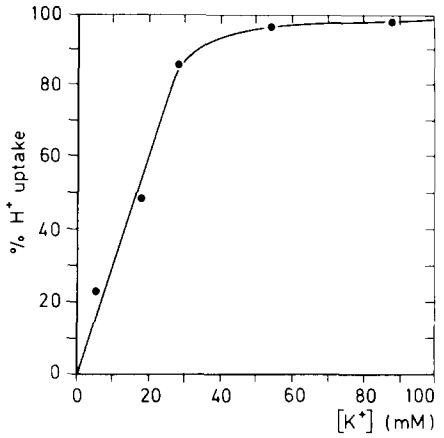

Fig. 4. Saturation of passive transort by reconstituted (H^+ + K^+)-ATPase. ($H^+ + K^+$)-ATPase is reconstituted into liposomes prepared in 5 mM glycylglycine, 2 mM $MgSO_4$ and varying K_2SO_4 concentrations (pH 6.1) and diluted 6.7-times into a buffer containing 5 mM glycylglycine, 2 mM $MgSO_4$ and varying concentrations of sucrose to maintain osmolarity (pH 6.1). Rates of passive transport are measured at a protein concentration of 0.375 mg/ml.

($ED_{50} = 25$ μM). Omeprazole inhibition required membranes which were free of dithiothreitol or other SH-reducing agents (Fig. 5). These findings suggested that active H^+ transport was catalyzed by the ($H^+ + K^+$)-ATPase.

Passive H^+-K^+ exchange was also inhibited by

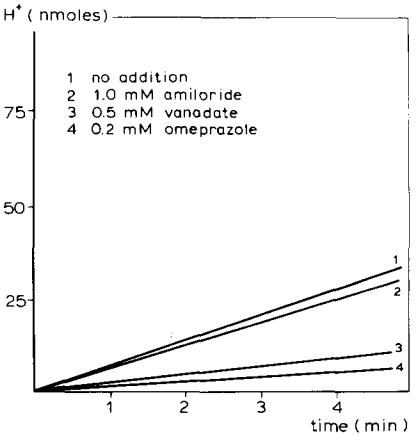

Fig. 5. Passive transport by reconstituted ($H^+ + K^+$)-ATPase. ($H^+ + K^+$)-ATPase is reconstituted into liposomes prepared in 5 mM glycylglycine, 2 mM $MgSO_4$ and 50 mM K_2SO_4 (pH 6.1). Final protein concentration is 0.375 mg/ml.

0.5 mM vanadate and by 0.2 mM omeprazole ($ED_{50} = 25\ \mu M$). However, these inhibitions occurred only when K^+ was used as counter ion, not when Na^+ was used. By contrast, passive H^+-Na^+ exchange was inhibited by amiloride, which did not affect H^+-K^+ exchange. This suggested the existence of two distinct passive transporters: the H^+/K^+ exchanger inhibited by ATPase inhibitors and the H^+/Na^+ exchanger inhibited by amiloride.

Discussion

It has been previously demonstrated using native gastric vesicles that the use of a pH electrode is a reliable way to quantitate H^+ transport parameters [6,9]. In this paper, we used the technique to monitor H^+ transport parameters of reconstituted ($H^+ + K^+$)-ATPase. Sensitivity was high: due to the large intravesicular volume of the proteoliposomes, maximal uptake per mg protein was 5-times that of the microsomes we have previously used [6]. The relatively slow response of the electrodes [9] was less problematic because filling of vesicles lasted longer as compared to native vesicles. This is due to the dilution of the ATPase in the artificial membrane. Initial rates of passive exchanges were maintained for at least 10 min.

Rate of active H^+-K^+ exchange was dependent upon K^+ concentration and maximal rate of 2.1 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ was obtained under K^+ saturating conditions. It was higher than we had previously observed with gastric microsomes (0.6 $\mu mol \cdot mg^{-1} \cdot min^{-1}$) [6] and improvement could be partly due to the higher purity of the membrane preparation used for reconstitution, as seen in specific K^+-stimulated ATPase activities of the preparation. In fact, the transport rate was closer to that estimated for gradient-purified native membrane (1.4 $\mu mol \cdot mg^{-1} \cdot min^{-1}$) [2]. Gain due to reconstitution was also found in the easy availability of efficient reconstituted preparations as compared to the lability of H^+ transport function in the native fractions.

Reconstituted ATPase actively transported H^+. Higher rates of transport weree measured in the presence of sulfate as compared to malonate. This should not be explained by a difference in anionic permeability, but we suggest that reconstitution in the presence of malonate was less efficient.

From a previous study, reconstituted ($H^+ + K^+$)-ATPase was known to elicit active H^+-K^+ exchange in the presence of MgATP and a passive K^+:K^+ one in the absence of ATP [4]. Evidence that it also catalyzed passive K^+-H^+ exchange specifically inhibited by omeprazole and vanadate is new. This indicates that passive activity of ($H^+ + K^+$-ATPase reflects all aspects of its active properties. Such a proposal has already been made for ($Na^+ + K^+$)-ATPase, which passively exchanges K^+ for Na^+ or K^+ [10–13]. Passive ($Na^+ + K^+$)-ATPase rates were 10–15% of the maximal active ones.

It is at this point interesting to note that passive K^+-K^+ exchange rates were very close to the maximal ATPase transport rate [5], whereas we found that passive K^+-H^+ exchange was only 5% of it. This suggests that it is not the K^+ transport which is rate limiting for the H^+-K^+ passive exchange capacity, but the H^+ transport.

Reconstituted ($H^+ + K^+$)-ATPase was suggested to be less specific for K^+ than the native enzyme, because the rate of H^+ transport was high when intravesicular Na^+-loaded vesicles were diluted in $RbSO_4$ [5]. We demonstrate here that reconstitution also led to the incorporation of an amiloride-sensitive Na^+/H^+ exchanger into the liposomes. It could account for the apparent low specificity of the ATPase because, with Na^+-loaded vesicles, an Na^+-H^+ exchange coupled to an H^+-K^+ one would rapidly drive K^+ (or Rb^+ in the reported study) into the proteoliposomes; H^+-K^+ passive exchange being driven by both isnide-in and inside-out reconstituted ATPase. An amiloride-sensitive Na^+ transport has been previously described in gastric isolated stomachs which accounted for Na^+ apical absorption [14]. Whether this transport is driven by the transporter here defined is still unclear.

Acknowledgements

The authors would like to thanks Dr. S.L. Bonting for his useful comments on the manuscript and Gabriel Péranzi for electron microscopy examinations of proteoliposomes. This work was supported by a FEBS fellowship.

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