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THE H^+/ATP STOICHIOMETRY OF THE ($H^+ + K^+$)-ATPase OF DOG GASTRIC MICROSOMES

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Gastric microsomal vesicles isolated from dog fundic mucosa were shown to be relatively ion tight and have a low level of proton permeability. The H^+ translocase, basal ATPase and K^+-activated ATPase activities of these vesicles were measured and the H^+/ATP stoichiometry calculated using either the total K^+-ATPase or the K^+-stimulatable component (total K^+-ATPase − basal ATPase). The former estimations consistently gave stoichiometric of approximately one, whereas the use of only the K^+-stimulatable component gave widely differing values. Measurement of the dephosphorylation of the enzyme under basal conditions revealed both a labile and a stable phosphoenzyme component. The rate of decay of the labile component completely accounted for the basal ATPase activity observed. We conclude that the basal ATPase associated with our preparations is a spontaneous dephosphorylation of the phosphoenzyme occurring in the absence of K^+ and that the H^+/ATP stoichiometry of the gastric ATPase is one.

Microsomal vesicles isolated from the bullfrog gastric mucosa have been shown to contain an Mg^{2+}-dependent ATPase which was stimulated by K^+ in the presence of valinomycin [1]. Vesicles isolated from homogenates of the dog and hog fundic mucosa have been shown to contain a similar ATPase [2,3]. The ATPase is oriented in the microsomal membrane such that the site of ATP hydrolysis is external whereas the site of K^+ stimulation is internal [4]. This ATPase has been shown to exchange internal K^+ for external protons in a non electrogenic manner at the expense of ATP hydrolysis [5]. These observations and the immunocytochemical localisation of the ATPase on the parietal cell [6] have strongly suggested that this ATPase is responsible for H^+ secretion by the parietal cell. From thermodynamic considerations [7] the only H^+/ATP stoichiometry compatable with the involvement of this ATPase in the production of gastric acidity is a value of one. Previous studies on the hog gastric ATPase have reported values of 3 or 4 [5] whereas more recently values of 2 [8] and 1 or 1.5 [9] have been reported. In this paper we report determinations of the H^+/ATP stoichiometry of the dog gastric ATPase.

Gastric vesicles were prepared from dog stomachs as described previously [2]. Proton uptake by the vesicles was measured using apparatus described by Mitchell and Moyle [10] as previously reported [2]. ATPase activity was measured using (γ-^{32}P)-labelled ATP as detailed in the legends. The levels of phosphorylation of the ATPase and the rate of decay of the enzyme-phosphate complex were determined using a rapid addition apparatus. Rapid mixing was ensured by means of a rapidly rotating magnetic follower in the reaction vessel. The apparatus was characterised by mea-

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surement of the alkaline hydrolysis of 2,4-dinitrophenylacetate [11] and was found to be accurate for reaction intervals down to 0.2 s. Protein was determined by the method of Lowry et al. [12].

ATP as the magnesium salt and the ionophores valinomycin and FCCP were obtained from Sigma. Adenosine 5[γ-^{32}P]triphosphate, triethylammonium salt ([γ-^{32}]ATP) and PCS scintillant were obtained from the Amersham International, Amersham. All other chemicals were of analytical grade.

It has been reported previously [2] that the uptake of protons into gastric vesicles can be detected, under the appropriate conditions, by measurement of the removal of protons from the external medium using sensitive pH monitoring equipment. However, in using such measurements for the calculation of H^+/ATP stoichiometries the possibility of partially intact or ion leaky preparation must be considered. Thus if a vesicle preparation is partially disrupted or not completely ion tight the net measurement of proton removal from the bulk phase will underestimate the total proton translocation resulting from the hydrolysis of ATP. To investigate the intactness of the vesicle preparation the internal localisation of the site of K^+ stimulation was employed. Vesicles were equilibrated in the presence or absence of KCl and then assayed over a short (5 s) time course for ATPase activity by the addition of labelled ATP containing no KCl or KCl giving final assay concentrations of 5, 20 or 50 mM. Typical data are given in Table I and show that after 15 min equilibration in 20 mM KCl a stimulation of nearly 3-fold is observed. No stimulation of the sucrose equilibrated vesicles was found however when potassium ATP was added to a final concentration of 5 or 20 mM indicating that the preparation is essentially totally vesicular. Some stimulation over basal was observed when the external KCl concentration was 50 mM and was probably due to a slow penetration of KCl into the vesicles down the large concentration gradient during the assay period. Similar results were found for all preparation tested ($n = 5$).

A further possible source of error in the measurement of proton translocation by the pH method is the possible existence of a proton leak across the vesicle membrane which would lead to an underestimation of the total proton translocation. The traces in Fig. 1 show that a small leak of protons back into the bulk phase does occur subsequent to the rapid uptake of protons caused by ATP additions. Such a leak would obviously impair the accuracy of stoichiometric calculated from the total translocation of protons and ATP hydrolysed. Typically however, the rate of decay of the generated pH gradient is less than 3% of the initial rate of proton uptake and is only slightly increased when valinomycin is included to facilitate the rapid counter movement of potassium across the vesicle membrane. Thus we conclude that for freshly isolated vesicles the initial increase in external pH after the addition of ATP can be used as an accurate measurement of the rate of proton uptake into the vesicular space.

The ATPase activity of preparations was measured in the presence and absence of various levels

TABLE I

POTASSIUM PERMEABILITY OF GASTRIC VESICLES

50 μl of vesicle suspension (approx. 0.5 mg protein) was equilibrated at 25°C for 15 min in 350 μl of buffer (5 mM Mes, pH 6.1, 2 mM $MgCl_2$) containing KCl at the required concentration and sucrose to maintain osmolarity. The assay was started by the addition of 50 μl of the appropriate buffer containing [γ-^{32}P]ATP·MgATP and KCl when required to equilibrated vesicles whilst vortex mixing. (Final assay concentrations: ATP, 50 μM, KCl, as indicated). The reaction was stopped 1 to 5 s later by the addition of 2 ml 5% $HClO_4$, 1 mM P_i. The assay was standardised by allowing additional assays to proceed to completion (10 min incubation) and blanks were prepared by the addition of acid prior to the labelled ATP. The $^{32}P_i$ released was then complexed with molybdate (200 μl 4% ammonium molybdate) and extracted into 2 ml of isobutanol/toluene (50:50, v/v), 1 ml of which was taken for scintillation counting in PCS scintillant.

Equilibration buffer	Final [KCl] in assay (mM)	(nmol $P_i \cdot s^{-1}$. (mg protein)$^{-1}$)
Sucrose	0	0.822
Sucrose	5	0.813
Sucrose	20	0.826
Sucrose	50	0.934
5 mM KCl	5	1.42
20 mM KCl	20	2.34
50 mM KCl	50	2.28

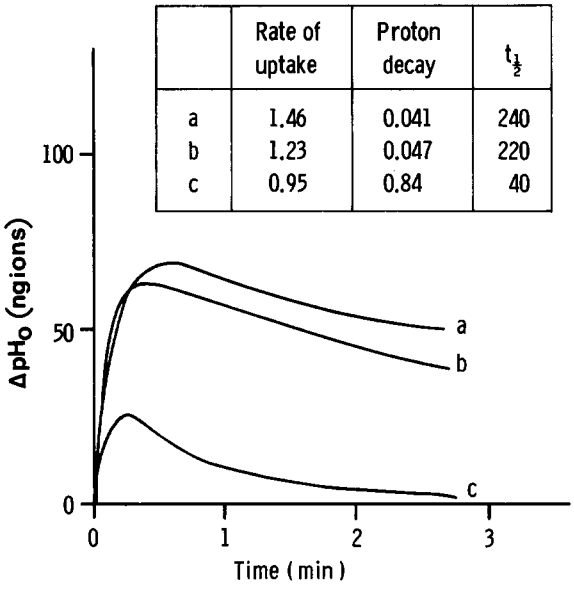

Fig. 1. The proton leak of the gastric vesicles. 0.5 ml of vesicle suspension (approx. 5 mg protein) was equilibrated at 25°C for 15 min in 3.5 ml of (a) KCl buffer (150 mM KCl, 5 mM glycylglycine, 2 mM $MgCl_2$, pH 6.1); (b) KCl buffer + valinomycin (2.5 μg/ml) and (c) KCl buffer + valinomycin + FCCP (5 μg/ml). Proton uptake was initiated by the addition of 10 μl of 10 mM MgATP, pH 6.1 and the change in the external pH (ΔpH_o) recorded. The apparatus was calibrated after each addition by back titration with standardised HCl (10 mM). The rates (in ngions·s^{-1}·(mg protein)$^{-1}$) of proton uptake and decay (at maximum ΔpH_o) are given as well as the $t_{1/2}$ of the decay in seconds.

of KCl over short (5 s) time courses. Extrapolation of these values to zero-time gave values at $t = 0$ in excess of the blank values estimated by addition of the acid prior to the labelled ATP. The blank determinations differ from the timed determinations in that the enzyme is denatured prior to the addition of [γ-^{32}P]ATP and so no phosphorylation of the ATPase will occur. Indeed the difference between the extrapolated zero and the blank was consistently of the same order as the level of enzyme phosphorylation under the same assay conditions and it is likely that the treatment with molybdate and subsequent extraction with organic solvent could, to some degree, cause the dissociation of P_i from the phosphorylated enzyme. Consequently the rates of ATP hydrolysis were calculated from the rates of P_i release determined at 1-s intervals over the first 5 s of the reaction.

The rates of proton translocation and ATP hydrolysis were measured in the presence and absence of various levels of KCl after 15 min equilibration at 25°C. The stoichiometry of proton translocation to the total and K^+-stimulated ATPase activity are presented in Table II.

The use of the total K^+-ATPase rate to calculate the stoichiometry gave values of approximately one. Using the K^+-stimulated component of ATPase, however, gave stoichiometries of a non integer value which approach unity as the basal ATPase activity becomes a less significant portion of the total K^+-ATPase rate. These data suggest that the basal ATPase of these preparations is in fact an activity associated with the gastric ($H^+ + K^+$)-ATPase evident under zero K^+ conditions and it may in fact be a spontaneous dephosphorylation of the phosphoenzyme formed in the initial partial reaction of the ATPase which could be represented by the reaction scheme.

$$\text{ATP} + \text{Enz} \rightarrow \text{EnzP} \xrightarrow{K^+} \text{Enz} + P_i$$

$$\text{basal}$$
$$\text{Enz} + P_i$$
$$0\ K^+$$

If this were the case then the turnover of the enzyme phosphate complex in the absence of added K^+ should account for the observed basal ATPase activity.

Using the rapid addition apparatus we were able to detect the breakdown of the enzyme phosphate complex under basal conditions and typical data is presented in Fig. 2. The levels of labelled enzyme-phosphate complex measured over the first 5 s after the addition of excess unlabelled ATP were found to consist of a relatively labile rapidly dephosphorylating component and a much more stable component. The rapidly dephosphorylating component demonstrated a first order exponential decay with a $t_{1/2}$ of 0.278 ± 0.028 s (S.E., $n = 6$) corresponding to a decay constant (K_d) for this component of 2.48 ± 0.25 s^{-1} (S.E., $n = 6$).

Determination of the basal ATPase activity and the amount of labile enzyme-phosphate complex under the same conditions allowed the calculation

TABLE II

THE STOICHIOMETRY OF THE GASTRIC ($H^+ + K^+$)-ATPase

Vesicles were equilibrated for 15 min at 25°C in the appropriate buffer containing KCl as indicated and sucrose to maintain osmolarity. Subsequently the rate of H^+ uptake or the ATPase activity was determined as described in Fig. 1 and Table I, respectively. In addition for each preparation tested the 'basal ATPase' activity was assayed after equilibration in sucrose buffer. The stoichiometries presented were then calculated from the rate of H^+ uptake and either the total ATPase activity or the K^+-stimulated ATPase activity (total ATPase − basal ATPase). Results are presented as mean ± S.E. (number of preparations).

[KCl] (mM)	Rate of H^+ uptake	Rate of H^+-uptake
	Total ATPase	K^+-stimulated ATPase
50	1.03 ± 0.04 (5)	1.54 ± 0.27 (5)
20	1.03 ± 0.12 (4)	1.64 ± 0.31 (6)
5	1.1 ± 0.09 (5)	2.04 ± 0.27 (5)

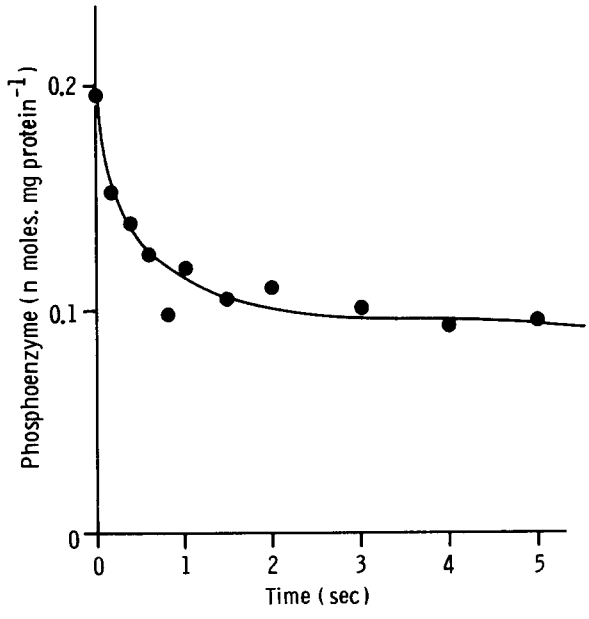

Fig. 2. Decay of the enzyme-phosphate complex. Vesicles were diluted 1 : 4 with buffer (5 mM Mes, pH 6.1, 2 mM $MgCl_2$, 230 mM sucrose) and equilibrated at 25°C for 15 min. The phosphorylation reaction was started by the addition of 100 μl labelled ATP (100 μM in sucrose/$MgCl_2$ Mes buffer) to 100 μl of the diluted preparation (approx. 2.5 mg/ml). Dephosphorylation of the enzyme-phosphate complex was measured by the addition of a large excess of unlabelled ATP (100 μl of 100 mM in the appropriate buffer) after 2 s of phosphorylation and stopped after various times by the addition of 500 μl of ice-cold 5% $HClO_4$ containing P_i and ATP at 1 mM. The levels of phosphoenzyme were determined by filtration of the protein precipitate on Whatman GF/C filters followed by 4×4 ml washes with stopping solution. (protein retention after such treatment was determined to be 98% efficient). The filters were then counted in PCS scintillant. The data shown represent the decay of the enzyme-phosphate complex after the addition of excess unlabelled ATP. Combined data from six preparations gave a $t_{1/2}$ of decay = 0.278 ± 0.028 s and a K_d = 2.48 ± 0.25 s^{-1} (S.E., $n = 6$).

of the apparent rate constant for the basal ATPase assuming the reaction proceeds solely by way of the dephosphorylation of the labile E − P component, i.e.

$$V = K[E - P_{labile}], \text{ thus } K = V/[E - P_{labile}]$$

The calculated apparent rate constant was 2.8 ± 0.15 s^{-1} (S.E., $n = 6$), a value which is not significant different from the observed decay constant.

We therefore conclude that the basal ATPase associated with our preparations is due to a spontaneous dephosphorylation of the labile component of the enzyme phosphate complex in the absence of internal potassium, whereas in the proesence of internal potassium dephosphorylation would occur by the potassium dependent pathway. Furthermore we conclude that the H^+/ATP stoichiometry of the dog gastric ATPase is unity. This is in agreement with the value reported by Reenstra and Forte [9] for the hog gastric ATPase and is compatible thermodynamically with the involvement of this ATPase in gastric acid production.

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