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INTERACTION OF FLUORESCEIN ISOTHIOCYANATE WITH THE ($H^+ + K^+$)-ATPase

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Fluorescein isothiocyanate was used to covalently label the gastric ($H^+ + K^+$)-ATPase. FITC treatment of the enzyme inhibited the ATPase activity while largely sparing partial reactions such as the associated *p*-nitrophenylphosphatase activity. ATP protected against inhibition suggesting the ligand binds at or near an ATP binding site. At 100% inhibition the stoichiometry of binding was 1.5 nmol FITC per mg Lowry protein a value corresponding to maximal phosphoenzyme formation. Binding occurred largely to a peptide of 6.2 isoelectric point, although minor labelling of a peptide of p*I* 5.6 was also noted. Fluorescence was quenched by K^+, Rb^+ and Tl^+ in a dose-dependent manner, and the $K_{0.5}$ values of 0.28, 0.83 and 0.025 mM correspond rather well to the values required for dephosphorylation at a luminal site. Vanadate, a known inhibitor of the gastric ATPase produced a slow Mg^{2+}-dependent fluorescent quench. Ca^{2+} reversed the K^+-dependent loss of fluorescence and inhibited it when added prior to K^+. This may relate to the slow phosphorylation in the presence of ATP found when Ca^{2+} was substituted for Mg^{2+} and the absence of K^+-dependent dephosphorylation. The results with FITC-modified gastric ATPase provide evidence for a conformational change with K^+ binding to the enzyme.

Introduction

Ion transport by plasma membrane ATPases involves changes in protein conformation associated with binding of ligands such as nucleotides, Mg^{2+} and the ion(s) to be transported. Thus, in the case of the ($Na^+ + K^+$)-ATPase there is a Na^+ conformation, a K^+ conformation and an occluded K^+ form of the enzyme, defined kinetically [1,2]. Various methods have been used to monitor these changes, such as intrinsic protein fluorescence [3], effects of trypsin [4] and fluorescent analogs of ATP such as formycin [5] and trinitrophenyl-ATP [6]. In particular, fluorescein isothiocyanate has been a useful probe of Na^+- and K^+-induced conformations of the enzyme. The compound is thought to bind to a lysine group at or near the active site and in so doing inhibits the ATPase but not the associated *p*-nitrophenylphosphatase activity of the enzyme. ATP protects against FITC inhibition and Na^+ reverses the fluorescence quench induced by K^+ [7]. FITC has also been used as a probe of conformational changes in the Ca^{2+}-ATPase [8,9].

In the case of the gastric ($H^+ + K^+$)-ATPase, conformational changes have not been described, although there are many similarities between this enzyme and the ($Na^+ + K^+$)-ATPase. To obtain direct evidence for a K^+ conformation of the enzyme, FITC was used as a labelling reagent and the effects of a variety of cations and other ligands on the FITC fluorescence provided direct evidence for a K^+-dependent change in conformation.

Abbreviations: FITC, fluorescein isothiocyanate; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetate.

Methods

Gastric membrane vesicles were prepared by previously described methods and involved scraping of hog gastric mucosa, homogenization, differential and density gradient centrifugation [10]. ATPase assays were performed at 37°C as previously described [11] with either ATP or Mg^{2+} added to initiate the reaction. The inorganic phosphate liberated was quantitated by butyl acetate extraction of the molybdate complex [12]. *p*-Nitrophenylphosphatase activity was quantitated by color development in alkaline solutions [13]. Protein was determined by a modified phenol method with included 1% SDS in reagent A [14]. Bovine serum albumin was employed as the standard. Disodium ATP, *p*-nitrophenyl phosphate, and FITC isomer I were purchased from Sigma. Isoelectric focusing gels were run employing LKB ampholines in the pH range 3.5–10 [15]. Following FITC labelling, the samples were diluted into 50 mM Tris-HCl (pH 7.4), centrifuged at $100000 \times g$ for 45 min and the pellets dissolved in equal volumes of 2% SDS and 20% NP-40. Gels for fluorescent analysis were placed in ethanol/acetic acid/H_2O (25 : 9 : 65, v/v) overnight with stirring to remove ampholines. The gels were sliced into 4 mm slices, extracted with 1% Triton, homogenized and 700 μl of H_2O added. Following centrifugation the fluorescence of each extract was determined in the spectrofluorimeter.

FITC labelling of enzyme. For fluorescent studies, freshly prepared enzyme was incubated at a final concentration of 750 μg/ml in a reaction mixture containing 100 mM Tris-HCl/2 mM EDTA (pH 9.2). The reaction was initiated by the addition of FITC in dimethylformamide to a final concentration of 5 μM. The reaction was allowed to proceed for 30 min at room temperature and was terminated by dilution into ice-cold 50 mM Tris-HCl (pH 7.4), +0.25 M sucrose followed by centrifugation at 33000 rpm in a Ti 70 rotor for 45 min. On occasion, the enzyme preparation was applied to a Sephadex G-25 column (1×7 cm) equilibrated in 50 mM Tris-HCl + 0.25 M sucrose (pH 7.4) to separate bound from free ligand.

The 30-min incubation period was chosen to maximize fluorescent quenching upon the addition of KCl [7] and resulted in a loss of approx. 50% of total ATPase activity.

Fluorescent measurements. Measurements were performed with a Perkin-Elmer MPF-44 fluorescence spectrophotometer. Excitation wavelength was set at 495 nm and emission wavelength at 517 nm with slit widths of 6 nm and a response time of 0.3 sec. The signal was amplified 2.5 times for most measurements.

Stoichiometry of FITC binding. The stoichiometry of FITC binding to fully inhibited and ATP protected enzyme was estimated at 495 nm with 2% SDS solubilized enzyme in 20 mM Tris-HCl buffer using an extinction coefficient of 75 $mM^{-1} \cdot cm^{-1}$ as determined by Carilli et al. [16] for FITC reacted with lysine. This value was independently confirmed in this laboratory. ATPase reactions were run in parallel with each of the stoichiometric determinations to insure 95% inactivation or protection of enzyme in the presence of ATP. FITC bound per mg inactive enzyme was obtained by subtraction of the absorbance reading of the ATP protected enzyme.

Phosphoenzyme formation. Experiments were conducted with 20–40 μg of protein in a total volume of 1 ml on ice. Final concentrations in the standard reaction were 2 mM divalent cation, 40 mM Tris-HCl (pH 7.4), 0.3 mM CDTA, and 0.5 μM [γ-^{32}P]ATP (specific activity 100–250 cpm/pmol). Phosphorylation was initiated with the addition of 100 μl of [γ-^{32}P]ATP to a 900 μl (800 μl for dephosphorylation experiments) volume of protein preincubated 30 min. Ligands (100 μl) were added in the dephosphorylation studies to a 900 μl volume of protein preincubated with [γ-^{32}P]ATP. Reactions were stopped with the addition of 500 μl ice-cold 40% trichloroacetic acid containing 5 mM H_3PO_4 and 1 mM unlabelled ATP. Trichloroacetic acid precipitated proteins were filtered using a 3 μm HAWP millipore filter and were washed three times with 5-ml portions of 3% trichloroacetic acid containing 1 mM H_3PO_4. Filters were then counted in an LKB scintillation counter.

Results

Inhibition of ATPase and related activities

Fig. 1 depicts the time-dependent inactivation of K^+-dependent ATPase activity in the presence of 5 μM FITC and retention of activity in the

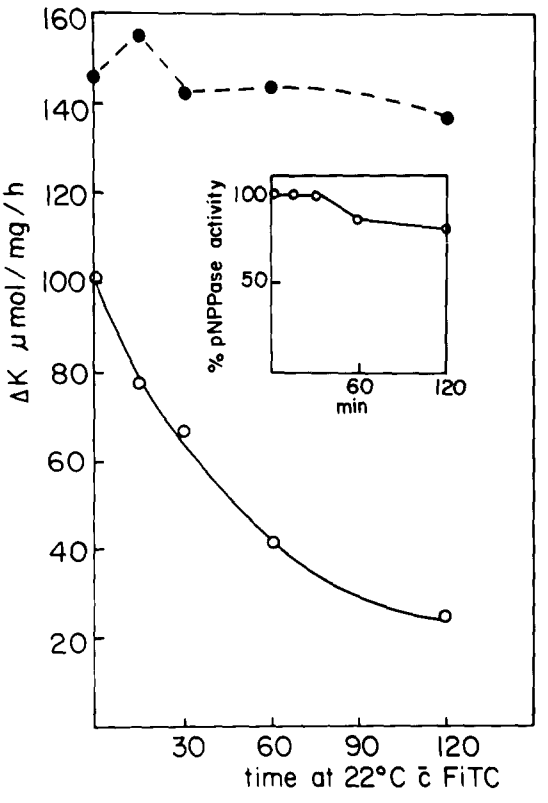

Fig. 1. Inhibition of ATPase activity by FITC. Freshly prepared enzyme (225 μg) was incubated at room temperature in 100 mM Tris-HCl buffer containing 2 mM EDTA (pH 9.2) in a total volume of 300 μl. FITC in dimethylformamide was added to a final concentration of 5 μM (open circles) or an identical sample containing 1 mM ATP (closed circles). At the time points indicated, aliquots were diluted into ice-cold ATPase reaction mixtures. The inset depicts the K^+-stimulated *p*-nitrophenylphosphatase (pNPPase) activity in the presence of 5 μM FITC. The initial *p*-nitrophenylphosphatase activity was 76 μmol/mg per h.

presence of ATP. An initial rapid inactivation (30%) of the FITC-treated enzyme was noted during the first 1–2 min of treatment, the time required for sampling and dilution into the ATPase reaction mixture. Inactivation proceeded in a non-linear fashion, and following 2 h incubation, approx. 15% of the original activity remained. The ATP protected sample retained 95% of the original activity. In contrast to the inactivation of K^+-ATPase activity by FITC, the inset of Fig. 1 demonstrates the relative insensitivity of the K^+-stimulated *p*-nitrophenylphosphatase activity to

TABLE I

STOICHIOMETRY OF FITC BINDING

1.5 mg of lyophilized enzyme was incubated in the presence of 10 μM FITC at pH 9.2 for 10–15 min depending upon the experiment. The time course of inhibition was followed in parallel ATPase assays. The reactions were terminated by dilution into 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM ATP and either 1 mM glycine or lysine. Following centrifugation the pellets were dissolved in 20 mM Tris-HCl/2% SDS, pH 7.4. Protein determinations were performed and samples read at 495 nm.

Experiment	nmol/mg	%ATPase inhibition
1	1.60	100
2	1.55	100
3	1.42	97
4	1.39	96
Average	1.49 ± 0.17	

inhibition. Approx. 80% of this activity was retained after the 120 min incubation.

Stoichiometry of FITC binding

Table I presents data obtained from four sep-

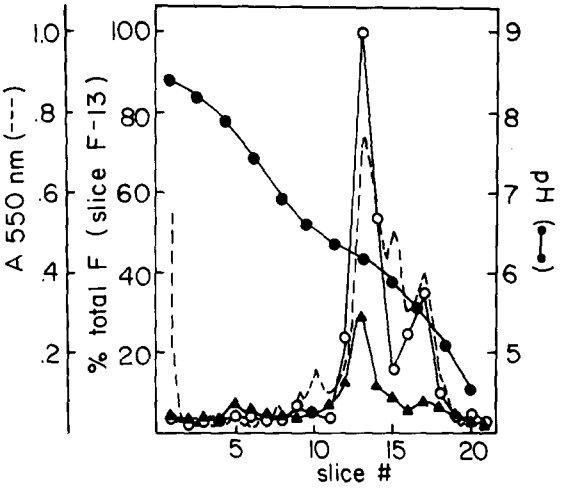

Fig. 2. Isoelectric focusing profile of FITC enzyme. A reaction mixture containing 450 μg of lyophilized enzyme in a total volume of 600 μl was reacted with 5 μM FITC for 60 min with or without 1 mM ATP. The fluorescence of the gel slice extracts from unprotected (○———○) or ATP protected (▲———▲) enzyme is plotted. The peptide pattern of Coomassie blue-stained gel (— — —) is also shown. Approx. 100 μg protein was applied to each gel.

arate experiments using three different enzyme preparations. Control enzyme activity ranged from 140 to 160 μmol/mg per h and protection by ATP was at least 95% of control activity. Typically, ATP protected enzyme gave absorbance values equivalent to 0.2–0.3 nmol FITC bound per mg enzyme. This value is probably within the limits of experimental error since enzyme prepared in the absence of FITC gave absorbance readings equivalent to these values when 2% SDS/20 mM Tris-HCl was employed as a blank. Complete inactivation of the enzyme was achieved with the binding of approx. 1.5 nmol FITC per mg enzyme.

Sites of covalent FITC binding

The gastric $(H^+ + K^+)$-ATPase when purified by free-flow electrophoresis exhibits a major peptide region in SDS gels of M_r 100000 which constitutes approx. 80% of the total protein. Electrofocusing of this preparation reveals heterogeneity of the M_r 100000 region with prominent peptides at approximate p*I* values of 8.4, 6.2, 6.0 and 5.7 [15].

Fig. 2 presents isoelectric focusing gels of FITC enzyme labelled in the presence and absence of ATP. As shown, the majority of fluorescence was associated with a peptide exhibiting a p*I* of approx. 6.2. A smaller amount of fluorescence was also associated with a peptide with a p*I* of 5.5–5.6. The ATP protected enzyme also revealed a small amount of fluorescein incorporation into the p*I* 6.2 peptide, but this incorporation could be virtually eliminated by the inclusion of 1 mM ATP and lysine in the dilution buffer prior to centrifugation and solubilization of the enzyme.

Ligand-induced conformational changes

Addition of low concentrations of K^+ to fluorescein-labelled enzyme in 50 mM Tris-HCl (pH 7.4) induced a rapid quenching of 17–20% of the total fluorescence (Fig. 3A). As shown in Fig. 3B, the addition of 2 mM Mg^{2+} prior to K^+ resulted in approx. 30% inhibition of quenching as compared to potassium alone. In analogy with the $(Na^+ + K^+)$-ATPase, these data suggest that binding of potassium ions in the highly fluorescent E_1 state of the enzyme induced a rapid conformational change to the E_2K^+ state as reflected by the quench in fluorescence. Titration of the change in fluorescence with respect to added K^+ is depicted in Fig. 4. The inset is a double reciprocal plot from which a $K_{0.5}$ of 0.25 mM was derived. Three separate titrations of different FITC-labelled enzyme preparations gave an average $K_{0.5}$ value of

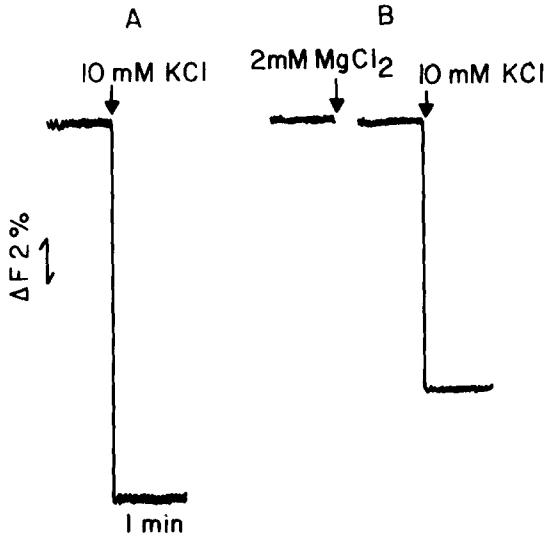

Fig. 3. Fluorescent quenching induced by KCl. FITC-labelled enzyme (20 μg) was suspended in 50 mM Tris-HCl buffer (pH 7.4) in a total volume of 750 μl. In (A), a small aliquot of KCl was added to give a final concentration of 10 mM. In (B), 2 mM $MgCl_2$ was added prior to the addition of KCl.

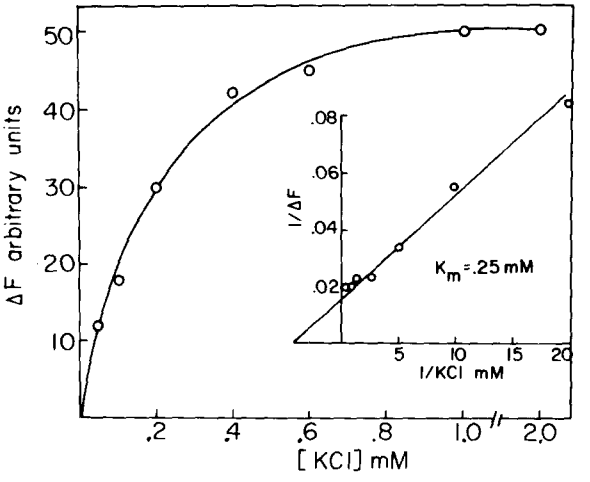

Fig. 4. Titration of fluorescent quench with KCl. The change in fluorescence is plotted as a function of KCl concentration. The inset is a double reciprocal plot of the data yielding a $K_{0.5}$ of approx. 0.25 mM in the absence of Mg^{2+}.

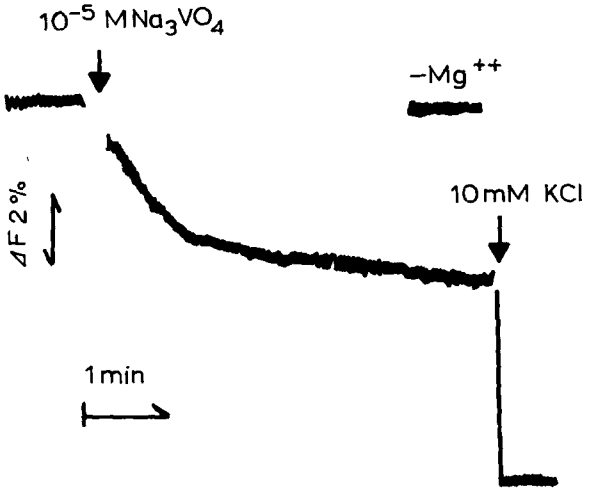

Fig. 5. Vanadate-induced fluorescent changes. FITC-labelled enzyme was incubated in the presence or absence of 2 mM $MgCl_2$ and 10^{-5} M Na_3VO_4 added as indicated. After 5 min incubation a final concentration of 10 mM KCl was added to the sample containing $MgCl_2$ and vanadate.

0.28 mM. Similar studies with the K^+ congeners, Tl^+ and Rb^+ resulted in $K_{0.5}$ values of 0.025 mM and 0.83 mM, respectively. Li^+ (20 mM) or Na^+ up to 150 mM did not induce detectable conformational changes under these conditions.

The effect of vanadate ions in the presence and absence of 2 mM Mg^{2+} is presented in Fig. 5. In the absence of Mg^{2+}, no detectable quench was observed after addition of 10^{-5} M vanadate. In the presence of Mg^{2+}, vanadate induced a slow loss of fluorescence, requiring about 5 min. The addition of K^+ induced an additional quenching.

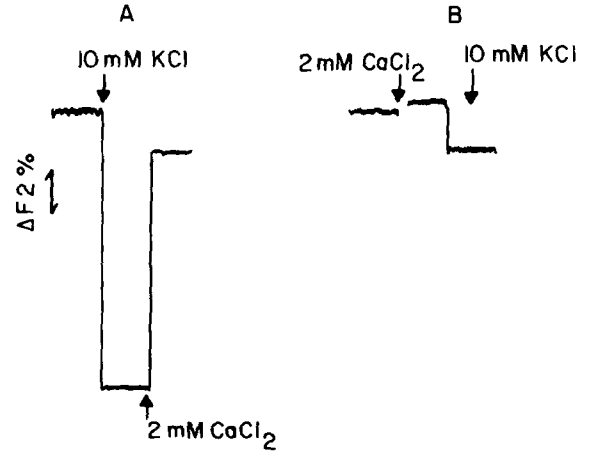

Fig. 6. Effect of Ca^{2+} on the K^+-induced change in fluorescence. Conditions were as described in Fig. 3A. In (A), 2 mM $CaCl_2$ was added following the KCl quench. (B) The inhibition of quench when the order of cation addition was reversed.

The effect of Ca^{2+} on the fluorescent change induced by K^+ is shown in Fig. 6. Addition of 2 mM Ca^{2+} resulted in a rapid increase in fluorescence to a level 85–90% of control. Addition of Ca^{2+} prior to K^+ (Fig. 6B) resulted in almost 90% inhibition of the K^+-induced conformational change.

These results may be viewed in light of experiments with Ca^{2+} substitution for Mg^{2+}. Fig. 7 compares the formation of phosphoenzyme by the $(H^+ + K^+)$-ATPase in the presence of Mg^{2+} or Ca^{2+}. While the formation of the Ca^{2+} dependent phosphoenzyme form was considerably slower than

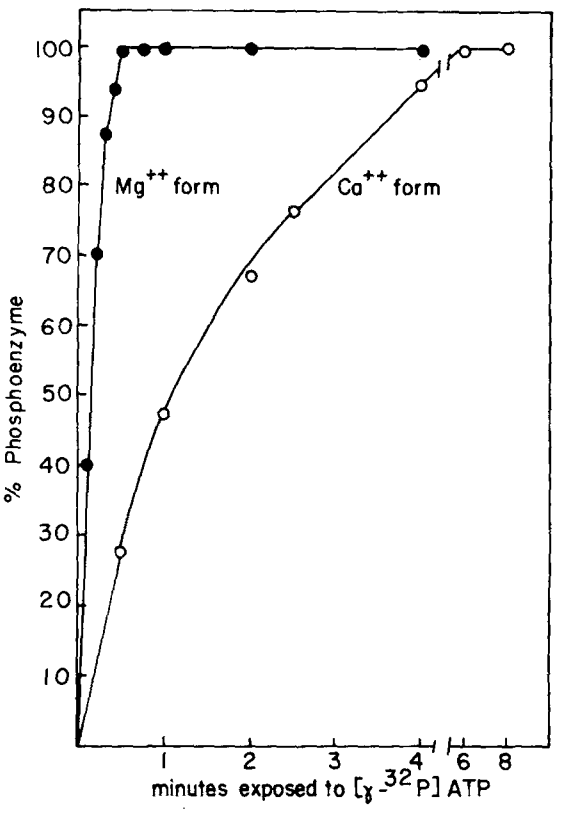

Fig. 7. Phosphoenzyme formation in the presence of 2 mM Mg^{2+} or Ca^{2+}. Phosphoenzyme formation was followed as a function of time. Maximal phosphoenzyme formation was 641 or 545 pmol/mg in the presence of Mg^{2+} or Ca^{2+}, respectively.

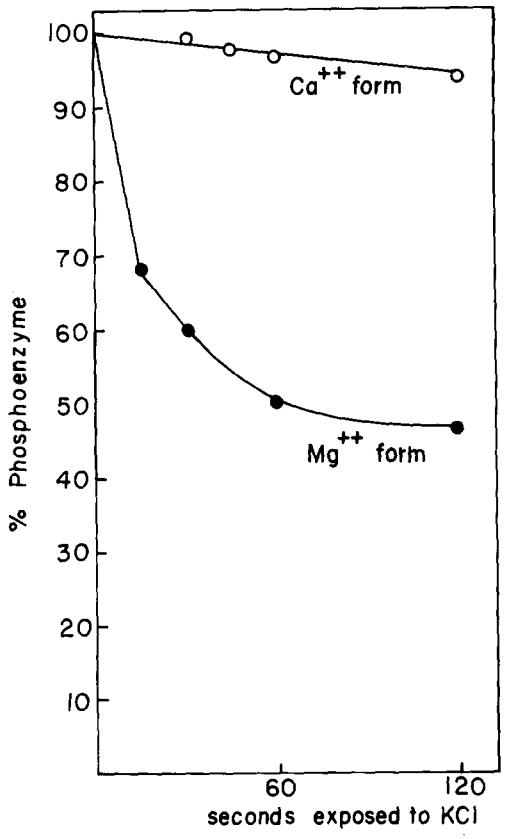

Fig. 8. Dephosphorylation of the Mg^{2+} and Ca^{2+} generated phosphoenzymes by KCl. Maximal amounts of phosphoenzyme were formed and at zero time, 0.1 mM KCl was added. Dephosphorylation was followed during the time course shown.

that generated in the presence of Mg^{2+}, nearly equivalent levels of phosphoenzyme were reached. Fig. 8 illustrates that while the Mg^{2+} generated form of phosphoenzyme was discharged in the presence of 0.1 mM KCl, the Ca^{2+} generated phosphoenzyme was essentially insensitive to potassium addition.

Discussion

Previous studies of vesicles containing the gastric ATPase have established the ability of these preparations to generate proton gradients upon the addition of external ATP and Mg^{2+} provided the vesicles contain internal K^+. These studies have indicated that the enzyme acts as an electroneutral pump exchanging H^+ for K^+ [10]. Kinetic studies [17] have indicated that the enzyme undergoes ATP-dependent phosphorylation at a high-affinity ATP site to a maximum of 1.5 nmol/mg Lowry protein and that the phosphoenzyme is an intermediate in the overall ATPase reaction.

The covalent binding of the FITC molecule to the gastric ATPase resulted in a time-dependent loss of ATPase activity while largely sparing the associated *p*-nitrophenylphosphatase activity. The protection of activity by inclusion of ATP suggests that as in the case of the $(Na^+ + K^+)$-ATPase [7], the ligand is binding at or near the active nucleotide site region.

Being a covalent probe, it was possible to determine the FITC binding stoichiometry. At 100% inactivation, the binding of 1.5 nmol FITC per mg Lowry protein corresponded to the maximal 1.5 nmol phosphoenzyme formed from [γ-^{32}P]ATP in the presence of Mg^{2+} [17]. It should also be noted that the Lowry method using bovine serum albumin as a standard overestimates the protein by about 35% when compared with quantitative amino acid analysis of this preparation hence, the actual value of binding or phosphorylation in this preparation is 2 nmol per mg protein (Catersan, B., unpublished observations; Ref. 18). Carilli et al. [16] reported a stoichiometry of FITC bound per mg of $(Na^+ + K^+)$-ATPase for complete inactivation which also corresponded to one site per high-affinity ATP or ouabain-binding site.

Although highly enriched preparations of the gastric ATPase show that the major peptide constituent (80%) is found at a M_r of 100000, isoelectric focusing demonstrates heterogeneity of this peptide region [15]. Under the particular conditions employed, of the three major peptides, those at p*I* 5.6 and 6.2 were FITC labelled and labelling was prevented by the presence of ATP in both cases. Thus, one or both of these peptides may play a role in enzyme activity. It may be noted that the peptide of p*I* 6.2 labels with [^{32}P]N_3-ATP [15].

Transport of H^+ into gastric vesicles and rapid dephosphorylation of phosphoenzyme requires K^+ at a high affinity ($K_{0.5}$ 0.2–0.8 mM) internal site. An additional lower affinity K^+ site ($K_{0.5}$ 8 mM) is located on the external surface of the vesicle which inhibits phosphoenzyme formation [19].

The interaction of ionic ligands with the FITC-

treated enzyme in the case of K^+, Rb^+ and Tl^+, resulted in a large fluorescent quench. The $K_{0.5}$ values suggested that the effects were due to cation binding to the luminal or internal face of the enzyme, in contrast to what has been suggested for the $(Na^+ + K^+)$-ATPase. The argument could be made that in the absence of ATP cation affinities on both sides of the enzyme are identical, but this would not be consistent with the K^+-dependent inhibition of phosphorylation found by rapid kinetic methods [17]. Thus, the possibility arises that K^+ binding on the luminal side of the transport barrier affects the fluorescent probe at the ATP binding site on the cytosolic face, suggesting proximity of the two sites or large transmitted conformational changes.

While tryptic digestion of the $(Na^+ + K^+)$-ATPase in the presence of Na^+ or K^+ led to the definition of a Na^+ or E_1 and a K^+ or E_2 ligand induced conformational states [20,21] a similar approach with the gastric ATPase failed to detect specific monovalent cation forms [22].

Based on the data presented here, it would appear that the FITC-labelled $(H^+ + K^+)$-ATPase exists in the E_1 conformation and that the quenching of fluorescence reflects the formation of E_2K^+. It is known that vanadate in the presence of Mg^{2+} inhibits the gastric ATPase by a slow reaction. Since vanadate substitutes for phosphate, the inhibitory conformation in the $(Na^+ + K^+)$-ATPase is thought to be $E_2 \cdot VO_4$. In the FITC-labelled gastric ATPase, vanadate induced a slow decrease of the fluorescence, as expected for a shift to the E_2 conformation.

Finally, ATPase and phosphorylation data in the presence of Ca^{2+}, rather than Mg^{2+}, suggest that the phosphoenzyme slowly formed in the presence of Ca^{2+} is resistant to dephosphorylation by K^+. This is reflected in the FITC results in that Ca^{2+} either reverses or prevents the effect of K^+. One interpretation is that Ca^{2+} effectively competes for the K^+ site, but in the dephosphorylation experiments, even very high concentrations of KCl failed to accelerate dephosphorylation. Hence, as for the $(Na^+ + K^+)$-ATPase, there is a Ca^{2+} form [23] of the gastric enzyme, which, based on the FITC effects, is maintained in the E_1 form even in the presence of K^+.

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