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1-Anilino-8-Naphtalene Sulfonate Probes a Gastric HK-ATPase Potassium Site Whose Access Requires Ionophores

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Abstract. 1-anilino-8-naphtalenesulfonate (ANS) is a hydrophobic dipole previously used to demonstrate that the proton for potassium exchange by the gastric HK-ATPase is electroneutral. In this paper, we demonstrate that ANS binds to gastric membranes and probes conformational changes of the HK-ATPase independently of any active H for K exchange. Conformational changes require the presence of potassium-valinomycin and are not triggered by sodium. Potassium effect is enhanced by ATP, in the presence and in the absence of magnesium and, by ADP, in the presence of magnesium. Labeling of the pig HK-ATPase K518 by fluorescein-5-isothiocyanate inhibits the enzyme activity and knocks out the ATP effect on ANS fluorescence. Scherring 28080 and the monoclonal antibody 95-111, two competitive inhibitors of K-activated ATPase dephosphorylation, do not modify K-effect on ANS fluorescence but inhibit ATP effects. This supports that ANS does not probe K-site between the H1–H2 loop. Treatment of gastric membranes with trypsin does not inhibit the ANS response to potassium but does inhibit the response to ATP. This suggests that the ATP site inducing the ANS response is cytoplasmic and the potassium site is intramembranous. Titration reveals that one mole of ANS interacts with one mole of ATPase. We suggest that ANS probes a hydrophobic potassium site of gastric ATPase and that addition of ATP and ADP-Mg embed that site.

Key words: 1-Anilino-8-naphtalene sulfonate — Fluorescent probes — Gastric HK-ATPase — Potassium-site — Trypsin digestion — Molecular modeling

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Introduction

The gastric HK-ATPase (EC 3.6.1.36) is a polytopic transmembranous protein made of two subunits, alpha and beta (Hersey & Sachs, 1995). Ten transmembrane helices were predicted for alpha and one for beta. HK-ATPase exchanges protons against potassium while hydrolyzing ATP and thus is responsible for the acidity of gastric juice. The HK-ATPase cycles between at least two conformers, E1 and E2. E1 is induced by the binding of ATP and proton to cytoplasmic sites. Change to E2 is induced by potassium on the other side of the membrane (Rabon et al., 1990).

By using chemical reagents and monoclonal antibodies, five sites important for catalysis were characterized. (i) The ATP site which, in all P-ATPases is a spatial arrangement of several lysines dispersed along the sequence of the large cytoplasmic loop between the fourth and fifth transmembrane helices (Farley & Faller, 1985; Ohta, Nagano & Yoshida, 1986; Tamura et al., 1989; Yamamoto et al., 1988). (ii) The phosphorylation site which is also highly conserved, is characterized by the consensus sequence DKTGTL (Walderhaug et al., 1985; Serrano & Portillo, 1990). (iii) The magnesium binding site which corresponds to 726D in the alpha subunit of HK-ATPase (sequence GDGXNDA), (Ovchinikov et al., 1987; Serrano & Portillo, 1990). Last, (iv–v) Two potassium sites are evoked: an activating site and a transport site. Potassium activates the ATP hydrolysis when it binds on the short luminal loop between the two first transmembrane helices, H1 and H2 (Munson et al., 1991). It activates the ATPase dephosphorylation (Wallmark & Mardh, 1979). Scherring derivatives are competitive inhibitors of that effect and were described to bind at the same site (Munson, 1991; Wallmark et al., 1987). The monoclonal antibody 95-111 is another potassium-competitive inhibitor of

ATPase activity but unlike the Scherring compounds, it binds a cytoplasmic site. Therefore, it should impair an allosteric movement of structure induced by the luminal binding of potassium to trigger the activation of dephosphorylation (Bayle et al., 1992). The second potassium-site of the ATPase should be a transport site; potassium is exchanged for proton and the exchange should, in the NaK-ATPase and HK-ATPase take place in the C-terminal domain of the alpha subunit as suggested by the fragment occluding potassium and rubidium (Rabon et al., 1993; Shainskaya & Karlish, 1994).

Fluorescent probes such as FITC (Jackson, Mendlein & Sachs, 1983) and MDPQ (Rabon et al., 1991) can detect conformational changes of the HK-ATPase because fluorescence yields are sensitive to the hydrophobicity of the probe environment. FITC binds K518 in competition with ATP and probes conformational changes induced by potassium and sodium used as substitute for proton. MDPQ is a fluorescent potassium-competitive inhibitor of the HK-ATPase.

ANS is a noncovalent fluorescent probe. It is useful to monitor the folding of proteins because it binds hydrophobic pouches: the decrease in ANS fluorescence is a good criteria to test a correct folding (Suarez Varela, Sandez Macho & Minones, 1992). ANS also binds hydrophobic pouches of actin and the probe fluorescence decreases in response to conformational changes induced by nucleotide binding (Hozumi, 1990). ANS was also used to probe transmembrane potentials. Lewin et al. (1977) demonstrated that the proton for potassium exchange catalyzed by the gastric HK-ATPase is electroneutral. In this paper, we reproduce their data and further demonstrate that, in the presence of valinomycin, ANS probes a hydrophobic potassium-site of the HK-ATPase whose conformation changes in response to ATP and ADP.

Materials and Methods

Materials

PEP, ATP (sodium salt), EDTA (disodium salt), SDS, magnesium sulfate, ANS, ADP, FITC, valinomycin, nigericin, monensin, trypsin and trypsin inhibitor were purchased from Sigma-France; BSA, HEPES, pyruvate kinase were from Boerhinger-France; ascorbic acid, sodium heptamolybdat, DMF were distributed by Merck-France; DTT, magnesium sulphate, methanol, sucrose, sodium chloride, potassium chloride, sodium dihydrogenphosphate, Tris, sodium hydroxide, sulfuric acid, acetic acid, hydrochloric acid, acrylamide/bis-acrylamide (30–0.8%) solution, TEMED were bought from PROLABO. Scherring 28080 was a gift from Dr. G. Sachs.

Methods

Proteins

Protein concentration was measured according to Bradford (1976) with Biorad kit and BSA as standard.

Preparation of hog gastric HK-ATPase membranes: The HK-ATPase membranes were prepared and enzymatic assays performed as previously described (Benkouka et al., 1989). The membrane fraction collected out of discontinuous sucrose gradient elicited a potassium-stimulated activity of 87 μmol Pi/mg proteinxh.

FITC Labeling

Freshly prepared HK-ATPase membranes were incubated at a final concentration of 750 μg/ml into a buffered solution containing 100 mM TRIS-Cl/2 mM EDTA (pH 9.2) (Jackson et al., 1983); the reaction was initiated by adding FITC in DMF (final concentration: 5 μM). Final DMF concentration did not exceed 1%. Incubation was run for 90 min at room temperature in darkness. The reaction was stopped by a 20-fold dilution (v/v) into ice-cold 250 mM sucrose-50 mM Tris-Cl solution (pH 7.4). The material was recovered by centrifugation at 100,000 × *g* in a 70 Ti rotor for 30 min (L5 65 Beckman centrifuge). Pellet was suspended in the same buffer. Only 6% of the original ATPase activity remained. The control was a fraction treated similarly but in the absence of FITC.

Monoclonal antibody 95-111: It was produced in our laboratory (Benkouka et al., 1989) and was purified with the Biorad DEAE Affligel blue (Bruck et al., 1982).

Tryptic digestion of HK-ATPase membranes: Tryptic digestion was performed as described by Rabon et al. (1993). Incubations were run for 3, 7, 13, 20, 30 min at 37°C with a protein/trypsin ratio of 20. Reactions were stopped by adding trypsin inhibitor (trypsin inhibitor/trypsin ratio = 6). Undigested material was pelleted at 300,000 × *g* for 15 min at 4°C with a TL 100 centrifuge (Beckman). All different pellets were analyzed by SDS PAGE (13.75% acrylamide) according to the Laemmli (1970).

Spectrofluorometric Measures

(i) ANS fluorescence. The fluorescence of ANS was measured in a LS 5B Perkin-Elmer spectrofluorometer at an excitation wavelength of 375 nm. Emission spectra were scanned from 420 to 520 nm or intensity was recorded at the fixed wavelength of 472 nm. Fluorescence is expressed as arbitrary units with a sensitive scale from 0.25 to 1.5. The standard conditions of assays were: to 0.93 ml of 50 mM Tris, with 0.4 mM EDTA, 2 mM DTT adjusted to pH 6.1 with acetic acid, 5 μl of a methanol solution of 2 mM ANS, 20 μl of HK-ATPase membranes (2.5 mg/ml), 5 μl of a methanol solution of 2 mM valinomycin and 40 μl of Tris buffer containing 0.80 M potassium chloride. Effects of ATP, ATP-Mg, ADP-Mg and other ligands were tested by adding less than 2.5 μl of solution. In some experiments valinomycin was replaced by nigericin and monensin.

(ii) Titration of binding sites. The number of ANS molecules bound to the HK-ATPase membranes was calculated using the Klotz's equation (1947), modified by Augustin and Hasselbach (1973), (Eq. 1)

$$P(xD)^{-1} = (n)^{-1}\,[1 + K_s/(1 - x)(D)] \qquad (1)$$

where n is the number of ANS sites; P the ANS concentration; D the concentration of protein in the assay; x, the F/F_0 ratio of ANS fluorescence (F) being the fluorescence in the assay and (F_0) the maximal fluorescence determined from the Lineweaver and Burk's plot of 1/ANS fluorescence *vs.* 1/ANS concentration. In our conditions, D is 50 μg proteins/ml and corresponds to 0.25 μM of HK-ATPases. K_s, is the apparent affinity of ANS (21 μM in our assay).

(iii) Efficiency of energy transfer. Tryptophan fluorescence of

HK-ATPase was excited at 295 nm and measured at 336 nm (Matsumoto & Hamnes, 1975). A volume of 5 μl of ANS (66 to 200 μM) was added to 995 μl of the buffer containing valinomycin (10 μM), potassium chloride (50 mM) and HK-ATPase membranes 50 μg/ml and emission spectra were registered from 310 to 510 nm. When the ANS probe is added to the HK-ATPase membranes, the tryptophan fluorescence is quenched and emission is shifted at 472 nm. The plot of quenching (in %) as a function of 1/ANS concentration allowed to calculate the efficiency of energy transfer E at infinite ANS concentration (Augustin & Hasselbach, 1973). This efficiency is function of the donor-acceptor distance (r) as calculated by the Stryer's equation (1967) (Eq. 2)

$$E = (R_O/r)^6/(R_O/r)^6 + 1 \quad (2)$$

where R_O is the donor-acceptor distance corresponding to 50% transfer of energy. For tryptophan-ANS interaction $R_O = 21.6$ A (Matsumoto & Hamnes, 1975).

Molecular Modeling

The structure of ANS was constructed using Hyperchem and was energy-minimized using the Osiris energy equation (Brasseur, 1995; Lins & Brasseur, 1995). The isosurfaces of molecular hydrophobicity potential (MHP) (Brasseur, 1991) were calculated and drawn using WinMGM (Ab Initio Technology).

Abbreviations

The abbreviations used are: ADP, adenosine diphosphate; ANS, 1-anilino-8-naphtalenesulfonate; ATP, adenosine triphosphate; BSA, bovine serum albumin; DMF, N,N-dimethylformamide; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; FITC, fluorescein-5-isothiocyanate; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; mAb, monoclonal antibody; MDPQ, 1-(2-methylphenyl)-4-methylamino-6-methyl-2,3-dihydropyrrolo(3,2-c) quinoline; MHP, molecular hydrophobicity potential; P-ATPase, phospho-adenosine triphosphatase family; PEP, phosphoenolpyruvate; SDS PAGE, sodium dodecylsulfate polyacrylamide electrophoresis gel; Tris, Tris-(hydroxymethyl) aminomethane; TEMED, N,N,N′,N′,tetramethylenediamine.

Results

ANS is Hydrophobic

The structure and the MHP of ANS are shown in Fig. 1. After energy minimization the probe is dipolar; mostly hydrophobic with a small hydrophilic patch due to the negatively charged sulfonate and to the amine moieties. ANS binds hydrophobic pouches of proteins (Suarez Varela et al., 1993).

ANS Probes a Potassium Site

In the absence of gastric HK-ATPase membranes, excitation of ANS at 375 nm results in a weak emission of fluorescence from 420 to 520 nm (Fig. 2). Yield of fluorescence is increased by valinomycin and nigericin. Potassium increases the ionophore effect (Fig. 2). This suggests that the valinomycin-potassium and the nigericin-potassium complexes interact with ANS.

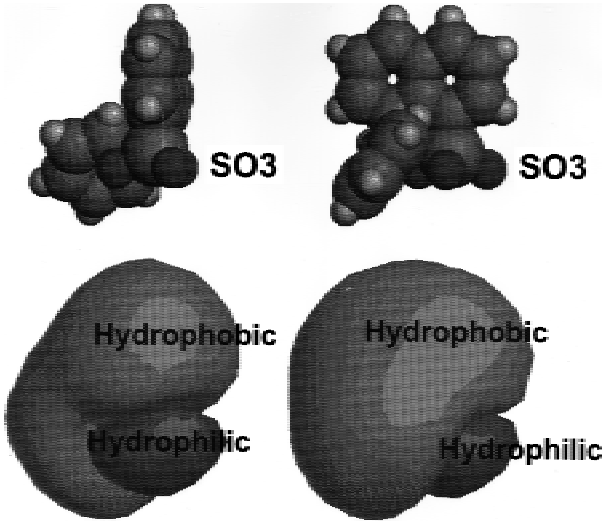

Fig. 1. Perpendicular views of ANS structure: The molecule was prepared using hyperchem software and minimized using the energy equation developed by Brasseur et al. (1995) since this force field was demonstrated to give a good approach of the folding of small hydrophobic molecule in solvents (Lins et al., 1996). MHP isopotential profiles (+ and − 25 Kcal) demonstrates that minimized ANS is largely hydrophobic (dark grey surface) with a small hydrophilic patch (light grey surface).

In the presence of gastric HK-ATPase membranes, the fluorescence of ANS is larger with a spectral optimum of emission at 472 nm (Fig. 3). This suggests that ANS binds to hydrophobic domains of gastric HK-ATPase membranes. Addition of valinomycin (10 μM) or nigericin (10 μM) reproduces the slight increase of fluorescence previously seen in the membrane-free medium. By contrast, addition of potassium has a larger effect than in the membrane-free medium. The presence of ionophore is required and the gain in ANS fluorescence is twice with valinomycin than with nigericin (Fig. 3). ANS response to potassium is saturable with a K_D of 9 and 5.5 mM in the presence of 10 μM nigericin and valinomycin respectively (Fig. 4). Sodium with and without ionophores (monensin, valinomycin and nigericin) has no effect. The requirement for ionophore and the specificity for potassium suggests that this cation must reach an hydrophobic site to trigger the ANS response.

Actin is contaminating our membrane preparations. It accounts for less than 5% of the proteins as estimated by scanning the Coomassie blue-stained SDS-PAGE of our preparations. However, since actin binds ANS, we tested ANS on 50 μg/ml of commercial actin in a separate experiment. This is the concentration of gas-

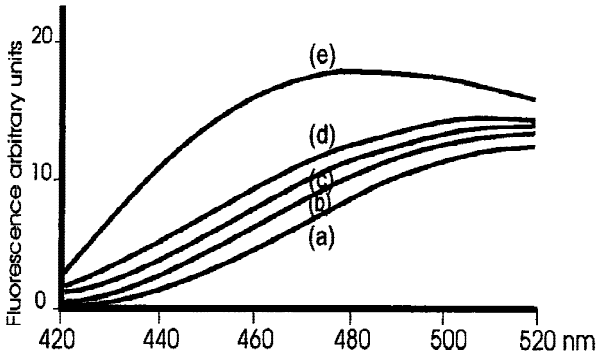

Fig. 2. Emission spectra of ANS (10 μM) expressed as fluorescence units in Tris-EDTA (40 mμ, 0.4 mμ) adjusted to pH 6.1 with acetic acid. Excitation wavelength 375 nm, emission spectra from 420 to 520 nm. (*a*) ANS alone (*b*) + nigericin 10 μM; (*c*) + nigericin 10 μM + KCl 50 mM. (*d*) + valinomycin 10 μM; (*e*) + valinomycin 10 μM + KCl 50 mM.

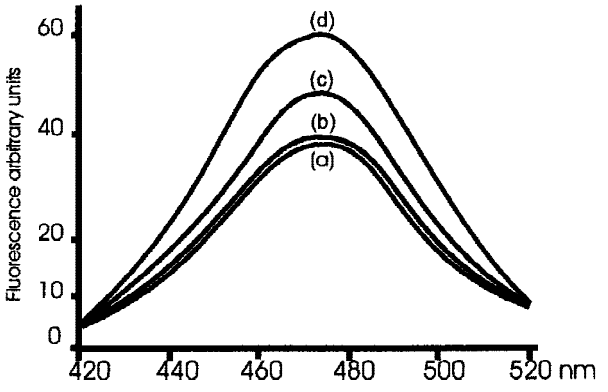

Fig. 3. Fluorescence spectra of ANS (10 μM) in the same buffer as Fig. 2 but in the presence of HK-ATPase enriched membranes (50 μg/ml). (*a*) ANS alone; (*b*) + nigericin (10 μM) or valinomycin (10 μM); (*c*) ANS (10 μM) + nigericin (10 μM) + KCl (50 mM); (*d*) + valinomycin (10 μM) + KCl (50 mM).

tric membranes in our assays and thus, should be more than 20-fold over the concentration of actin. With this high concentration, ANS response was much smaller than that obtained with gastric membranes ruling out a significant role of actin in the data.

The apparent affinity of ANS is 21 μM. Using Klotz's equation (1947) modified by Augustin and Hasselbach (1973) (Eq. 1), the mean number of ANS binding sites is 1.0 ± 0.1 site per molecule of ATPase (or 5 ± 0.5 nmol/mg of protein). The ATPase concentration was titrated using the mAb 95-111 as previously described (Robert et al., 1990).

These data support that ANS binds to gastric membranes and that potassium in the presence of an ionophore, but not sodium modifies the ANS environment. We suggest that ANS probes a hydrophobic potassium-site.

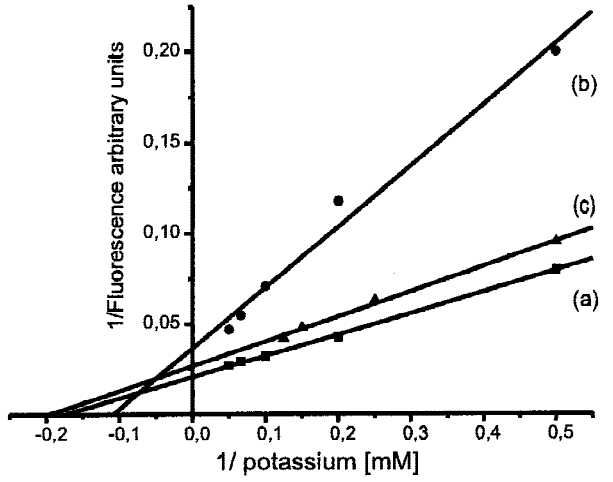

Fig. 4. Lineweaver and Burk's plot of the fluorescence of ANS (1/ fluorescent arbitrary units) as a function of potassium concentrations (1/potassium) in the same conditions as in Fig. 3 (ANS 10 μM and HK-ATPase membranes 50 μg/ml) in the presence of (*a*) valinomycin (10 μM) and (*b*) nigericin (10 μM) (*c*) is FITC-treated membranes in the presence of valinomycin (10 μM).

ANS Probes a Structural Change Dependent on HK-ATPase Activity

As previously reported by Lewin et al. (1977), in the presence of potassium and valinomycin, ANS fluorescence is increased by the addition of Mg-ATP to gastric membranes (Fig. 5*a*). The apparent affinity for ATP is 8 μM in the presence of a saturating concentration of potassium (50 mM). The apparent affinity for potassium is 3 mM in the presence of a saturating concentration of ATP (100 μM). This is in agreement with previous data from Lewin et al. (1977) who reported 11 μM and 15 mM for ATP and K affinities respectively: they concluded that ANS probed a structural change dependent on a potential and H^+ gradient.

In our experimental conditions, 10 μM ANS does not modify the HK-ATPase activity.

ANS Fluorescence is Enhanced in the Absence of Active Proton:Potassium Exchange

However, hydrolysis of ATP is not strictly required to obtain an ANS response. In the presence of EDTA the HK-ATPase activity is null but ATP does increase the ANS fluorescence. Occurrence of that effect requires the presence of potassium-valinomycin. The fluorescence increase is not as large as in the presence of magnesium but it plateaus, in agreement with the absence of ATP hydrolysis (Fig. 5*b*). If magnesium is added at equilibrium, a burst of fluorescence is obtained prior to a decrease to the baseline. The apparent affinity for ATP-

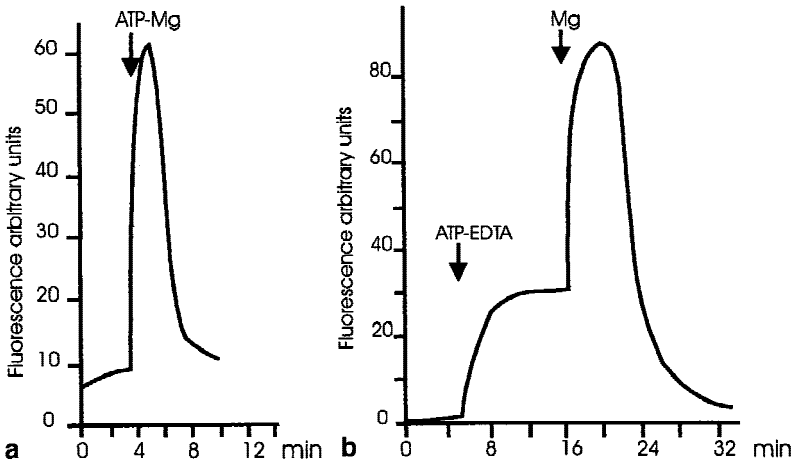

Fig. 5. Effect of ATP; the fluorescence of ANS was kinetically recorded at 472 nm after addition of ATP (50 μM) with (Fig. 5*a*) and without (Fig. 5*b*) 1 mM magnesium. The medium contained valinomycin (10 μM), KCl (50 mM) and HK-ATPase membranes (50 μg/ml). Additions of ligands are specified on the figure. The second addition in Fig. 5*b* is Mg 5 mM.

EDTA is 25 μM in the presence of a saturating concentration of potassium plus valinomycin.

In the absence of ATP hydrolysis and thus in the absence of active transport of proton and potassium, the fluorescence of ANS cannot be explained by a potential generated by the recycling of potassium. Therefore, we suggest that ANS probes conformational changes of ANS environment induced by potassium and ATP.

Effects of HK-ATPase Ligands

Effect of ADP

In the presence of magnesium, ADP effects the fluorescence of ANS. No signal is obtained in the absence of this bication. Apparent affinity for ADP is lower than that for ATP (Km 250 μM).

Effect of Phosphate

High concentrations of phosphate (up to 50 mM) are necessary to obtain any change on the ANS fluorescence (+15%) in the presence of potassium and valinomycin. In the presence of ATP-Mg, phosphate inhibits by 85% the ANS response: we suggest that high concentrations of phosphate should displace magnesium from ATP. To check the effect of the phosphate hydrolyzed from ATP, we tested the effect of 2 mM phosphate over ADP-Mg and no modification of the fluorescence of ANS initially obtained with ADP was obtained.

Effect of HK-ATPase Inhibitors

FITC

When HK-ATPase enriched-membranes are treated with FITC, hydrolysis of ATP is inhibited. On the ANS fluorescence of FITC-treated membranes, potassium has the same effect with the same apparent affinity (Fig. 4: 6 mM as compared to 5.5 mM in absence of FITC). However, the response to ATP is inhibited (Fig. 6). Inhibition concerns Mg-dependent as well as the Mg-independent responses. Since FITC binds one lysine of the ATP site, these results support that ATP effects on ANS are due to its binding to the HK-ATPase.

Scherring 28080

Scherring 28080 inhibits the HK-ATPase activity by competing with potassium at its luminal site. When tested on ANS fluorescence, Scherring 28080 does not modify the apparent affinity for potassium. This suggests that ANS does not probe the same potassium-site as Scherring 28080 does. On the other hand, Scherring 28080 inhibits the ATP effect on ANS fluorescence: in the presence of ATP and no magnesium, inhibition by 55 nm Scherring 28080 is noncompetitive: there is no change of the affinity for ATP but a decrease of the maximal ANS response (Fig. 7). In the presence of magnesium (1 mM), the burst of fluorescence induced by 250 μM ATP is decreased by 50% with 250 nM of Scherring 28080 (*data not shown*).

Monoclonal Antibody 95-111

MAb 95-111 inhibits the HK-ATPase activity and binds to the alpha subunit. Its epitope is located between S529 and D562, on the cytoplasmic loop between the fourth and fifth transmembrane helices H4–H5 (Benkouka et al., 1989; Bayle et al., 1992). Inhibition of activity is not competitive with respect to ATP but competitive with respect to the potassium activation of dephosphorylation. Since mAb 95-111 binds on the cytoplasmic side of HK-ATPase and potassium on the opposite side of the membrane, mAb should compete with potassium by prevent-

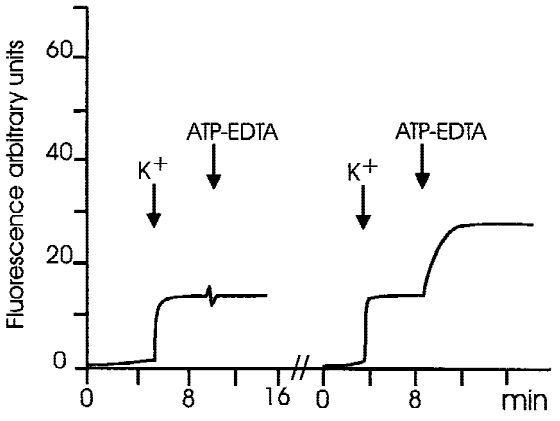

Fig. 6. Comparative assays of control and FITC-treated membranes. Time course of ANS response to potassium and ATP-EDTA, left: on FITC-treated membranes (50 μg/ml) and right on control membranes (50 μg/ml).

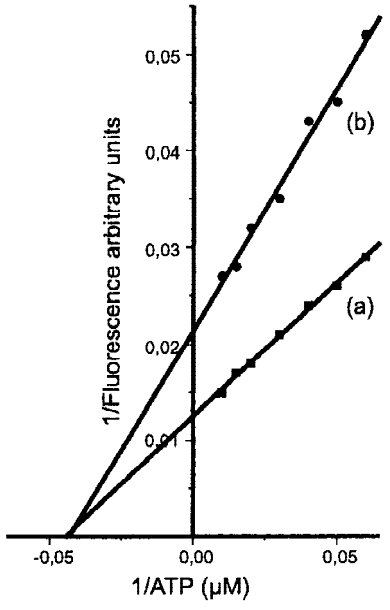

Fig. 7. Lineweaver and Burk's plot of the effect of Scherring 28080 on ANS fluorescence at 472 nm (1/fluorescent arbitrary units) as a function of ATP-EDTA concentrations (1/ATP). The incubation medium contained valinomycin (10 μM), potassium chloride (50 mM), EDTA 0.4 mM and HK-ATPase membranes (50 μg/ml). (*a*) in the absence of Scherring 28080; (*b*) in the presence of Scherring 28080 (0.55×10^{-7} *M*).

ing an allosteric transmembrane movement of the ATPase normally triggering the activation of dephosphorylation. However, mAb 95-111 does not inhibit the effects of potassium on the fluorescence of ANS but inhibits those of ATP (Fig. 8). As concluded from Scherring 28080 data, this supports that ANS does not probe the potassium-luminal H1–H2 site of the HK-ATPase but that the ATP effects are induced by its binding to the HK-ATPase.

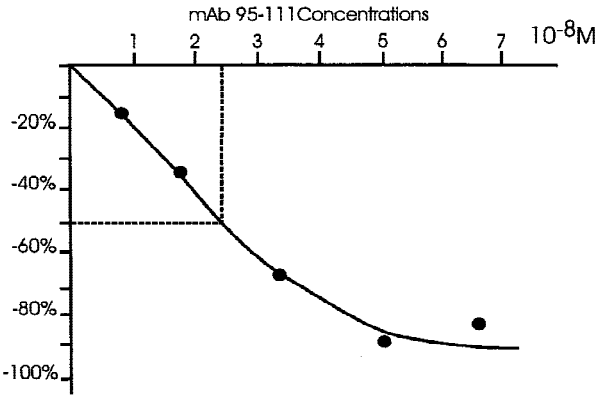

Fig. 8. Effect of mAb 95-111 on ANS fluorescence. The fluorescence of ANS was recorded at 472 nm in the presence of varying concentrations of mAb 95-111, fluorescence of ANS. Incubation medium contained valinomycin (10 μM), potassium chloride (50 mM) and HK-ATPase membranes (50 μg/ml) and ATP (50 μM) without magnesium; the 50% inhibition is obtained with 2.6×10^{-8} M of mAb 95-111.

Effect of Membrane Trypsinization

Using conditions of proteolysis as described by Rabon et al. (1993) in the presence of 200 μM vanadate, ANS binds to trypsinized membranes. In the presence of 10 μM valinomycin, 10 or 20 mM potassium increases the ANS fluorescence. The potassium response is not modified by the proteolysis but the ATP effect is lost. This is in agreement with the implication of the cytoplasmic ATP site destroyed by proteolysis. It also supports that the potassium-site probed by ANS is intramembranous since it is not destroyed after trypsinization.

Quenching of Tryptophan Fluorescence by ANS

In the absence of ANS, excitation of gastric membranes at 295 nm results in a maximal emission at 336 nm specific of tryptophan emission (Fig. 9*a*). Addition of ANS (3.3 to 10 μM) progressively quenches the tryptophan emission and shifts the optimum of emission towards 472 nm specific of ANS. This supports energy transfer from tryptophan to ANS as described by Augustin and Hasselbach (1973). The existence of an isoemissive point at 427 nm provides further evidence for energy transfer since it demonstrates that there is no change in quantum yield (Fig. 9*a*). A plot of the decrease of fluorescence (%) *vs.* 1/ANS concentration indicates a quenching limit of tryptophan fluorescence corresponding to an effective transfer of 36% of the energy (Fig. 9*b*). This suggests that the mean distance between ANS and tryptophans is 24 Ångstroms.

Discussion

Potassium activates HK-ATPase by stimulating its rate of dephosphorylation (Wallmark & Mardh, 1979). Ac-

tivation of dephosphorylation involves a luminal potassium site, between the two first transmembrane helices H1 and H2 of the alpha subunit (Munson et al., 1991) and the binding of potassium induces conformational changes transmitted down to the cytoplasmic domain. The cytoplasmic sites of trypsin attack are changed (Helmich-de-Jong, van Emst-de-Vries & de Pont, 1987), ATP site is modified (Jackson et al., 1983) and the rate of ATPase dephosphorylation is increased.

In the absence of ATP, potassium is transported by the HK-ATPase via a passive potassium:potassium or a potassium:proton exchange (Soumarmon, Rangachari & Lewin, 1984; Skrabanja et al., 1986). Whereas ATP blocks this exchange, addition of magnesium transforms it into an active transport, driving proton outside and potassium inside the cell. In NaK-ATPase, a very similar ion pump, potassium is occluded in the C-terminal domain of the alpha subunit (Shainskaya & Karlish, 1994) supporting that potassium channel is there.

ANS fluorescence is increased in hydrophobic environments of some globular proteins (Sheperd & Hamnes, 1976; Stryer, 1965; Matsumoto & Hamnes, 1975; Suarez-Varela et al., 1992) and of some membranous proteins as P-ATPases (Lewin et al., 1977; Teruel & Gomez-Fernandez, 1987). In our assays using gastric membranes containing HK-ATPase, potassium selectively enhances the ANS fluorescence; this effect is not mimicked by sodium. The specificity for potassium as well as for other HK-ATPase ligands as ATP, ADP support that the ionic pump is involved. ANS was also shown to bind to the Ca-ATPase (Augustin & Hasselbach, 1973). The apparent affinity and binding capacity of ANS to gastric HK-ATPase are similar to those found for preparations of sarcoplasmic reticulum Ca^{++} ATPase: the Kd for ANS is 21 μM as compared to 19.4 for the calcium pump and the binding capacity is 5 nmol/mg protein as compared 12 nmol/mg protein. The mean distance calculated between tryptophans and ANS is also comparable (24 and 20 Ångstroms, respectively). Teruel et al. (1987) calculated that distance between ANS and FITC in the Ca^{++}-ATPase is 51 Ångstroms and the authors suggested that ANS should be located in the intramembranous core. The absence of ANS effect on the HK-ATPase activity also supports that the probe is not near the ATP binding site.

Through its binding on the loop between the two first helices of alpha, H1 and H2, potassium induces a conformational change of the ATPase and activates the rate of dephosphorylation. This loop has carboxylic residues. Binding of potassium should however not explain the present ANS results, because Scherring 28080 a competitive antagonist of the H1–H2 potassium site fails to inhibit K-induced ANS fluorescence. Therefore, we probe another potassium binding event.

Potassium is transported across the membrane, passively in the absence of ATP and actively in its presence. The cation is not occluded by the gastric HK-ATPase, unless vanadate is present (Rabon et al., 1993). This supports the existence of a hydrophobic potassium site that is not freely accessible. We suggest that valinomycin gives potassium-access to that site and that ANS probes the conformational changes induced when potassium binds. Binding of ATP to the ATPase catalytic loop induces an ANS response and thus, another conformational change. Assuming the hypothesis that ANS probes a K-occlusion site, ATP effect in the absence of magnesium could be related to the inhibition of passive K:K exchange by ATP (Soumarmon et al., 1984).

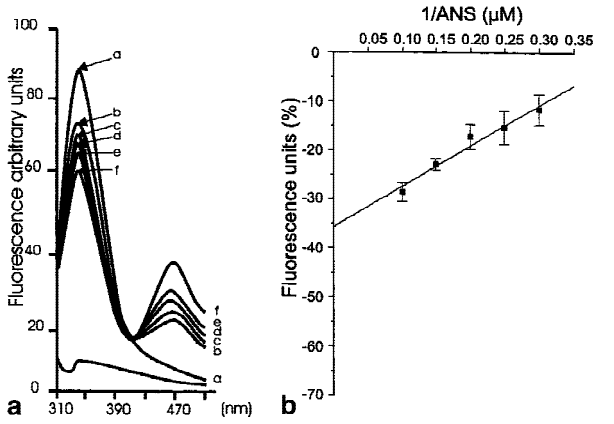

Fig. 9. Fig. 9*a;* emission spectra of HK-ATPase membranes (50 μg/ml) expressed as fluorescence arbitrary units in Tris-acetic acid buffer, + valinomycin (10 μM) and potassium chloride (50 mM). Excitation wavelength 295 nm, emission spectra from 310 to 510 nm with a maximum at 336 nm. (*a*) HK-ATPase membranes only (*b, c, d, e, f*) HK-ATPase membranes + varying concentrations of ANS from 3.3 to 10 μM. An isoemissive point is seen at 427 nm. Emission of fluorescence of ANS increases at 472 nm. Figure 9*b* shows the percentage of quenching of fluorescence emission at 336 nm as a function of ANS concentration (1/ANS). The maximal quenching at infinite ANS concentration is 36%.

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