An Electrogenic Proton-translocating Adenosine Triphosphatase from Bovine Kidney Medulla

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Abstract. Urinary acidification in the mammalian collecting tubule is similar to that in the turtle bladder, an epithelium whose H^+ secretion is due to a luminal proton-translocating ATPase. We isolated a fraction from bovine renal medulla, which contains ATP-dependent proton transport. H^+ transport was found to be electrogenic in that its rate was reduced by a membrane potential. H^+ transport activity was inhibited by *N*-ethyl maleimide and dicyclohexyl carbodiimide, but not by oligomycin or vanadate; its activity did not depend on the presence of potassium, differentiating this ATPase from the mitochondrial F_0-F_1 ATPase and the gastric H^+-K^+ ATPase. H^+ transport activity had a specific substrate requirement for ATP, distinguishing this pump from the lysosomal H^+ATPase, which uses guanosine or inosine triphosphate as well. The distribution of this H^+ pump on linear sucrose density gradient was different from that of markers of lysosomes and basolateral membranes.

These results show that the kidney medulla contains an H^+-translocating ATPase different from mitochondrial, gastric, and lysosomal proton pumps, but similar to the turtle bladder ATPase.

Introduction

The mammalian kidney maintains acid-base balance by secreting as much acid into the urine as is produced by cellular metabolism. Although the bulk of hydrogen ion secretion occurs in the proximal tubule, the ultimate regulation of net acid excretion is probably achieved by the collecting duct. Physiologic studies on isolated perfused medullary collecting tubules demonstrate acidification of the tubular lumen (1) in the absence of net sodium transport (2), which is associated with a lumen-positive potential; this suggests the existence of electrogenic hydrogen ion secretion in this segment. Many of the ideas about acidification by the mammalian collecting duct have come from studies of a functional and structural analogue of this segment of the nephron, the turtle urinary bladder. Hydrogen ion transport by the bladder derives from a proton-translocating ATPase located in the luminal membrane (3). In recent studies (4), we fractionated bladder cells and identified an electrogenic proton-translocating ATPase that was distinct from that of mitochondria and lysosomes but that co-migrated with the luminal membrane; it is probably responsible for urinary acidification. We also showed that this H^+ATPase is located in endocytic vesicles that are situated under the luminal membrane (5). CO_2, a major stimulus for H^+ secretion in renal epithelia, caused these vesicles to fuse with the luminal membrane, thereby inserting the proton pumps into that membrane and increasing the rate of transepithelial H^+ secretion.

In this paper we report the identification of an electrogenic proton-translocating ATPase in membranes derived from the bovine medulla, which resembles the H^+ATPase of turtle bladder but differs from both mitochondrial and lysosomal H^+ATPases. This ATPase is probably responsible for urinary acidification in the mammalian collecting duct.

Methods

Medullary tissue fractionation. Bovine kidneys were obtained fresh on ice from a local slaughterhouse. The pelvic fat was removed and the medullary tissue was punched out with a cork borer and transected at the junction of the inner and outer medullary stripes. Medullary tissue was washed in iced homogenizing buffer (250 mM sucrose, 5 mM Tris-HCl, 1 mM $NaHCO_3$, 1 mM dithiothreitol, 1 mM ethylene-glycol-bis-(-aminoethyl ether)*N,N'*-tetra-acetic acid) and minced. The tissue was suspended 1:6 wt/vol in homogenizing buffer and homogenized in a Teflon-glass homogenizer (Wheaton Scientific, Millville, NJ) 10 strokes at 1,000 rpm. The pestle was advanced to the bottom of the tube on the first stroke by gentle up and down motions to prevent the tissue from packing, and care was taken not to pull a vacuum. The homogenate was spun at 6,000 *g* for 15 min to remove connective tissue, unbroken

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cells, and nuclei. The supernatant was then spun at 38,000 *g* for 1 h. The pellet consisted of a fluffy white upper layer and a brown mitochondrial-lysosomal button. The upper layers were removed by gentle swirling and termed the P fraction, and the buttons were called the M fraction. The P fraction was then loaded onto either a continuous gradient or a sucrose step gradient. The media used for density gradient experiments were formed by adding enough sucrose to the homogenizing medium to bring the concentration to the desired level. The continuous gradient was a 12-ml linear gradient formed from a heavy solution of 40% sucrose (wt/wt) in homogenizing buffer and a light solution of homogenizing buffer alone. 1 ml of the P fraction suspended in homogenizing buffer was loaded on top of the gradient and spun overnight at 100,000 *g*. Fractions were collected by puncturing the tube bottom. The step gradient had three steps: 40, 25, and 15% sucrose (wt/wt) in homogenizing buffer. P fraction membranes suspended in homogenizing buffer were loaded on the gradient and spun overnight at 100,000 *g*. Membrane fractions accumulating at the interfaces were collected with a Pasteur pipette. The fraction at the 15–25% interface was designated the G2 fraction, and the fraction at the 25–40% interface was called the G3 fraction. Membrane fractions used for assay were either used immediately or frozen as collected for assay the following day. Fractions used for transport were suspended in homogenizing buffer, spun down at 38,000 *g* for 1 h, and stored at −70°C in homogenizing buffer containing 3 mM ATP.

Preparation of renal cortex submitochondrial particles. Mitochondria were prepared from slices of bovine renal cortex as described by Johnson and Lardy (6), and submitochondrial particles were prepared from these as described by Beyer (7).

Preparation of soybean phospholipid vesicles. Soybean phospholipids were prepared from soybean asolectin as described by Kagawa and Racker (8). Approximately 200 mg of phospholipid was dried under N_2, then suspended in 10 ml of 50 mM potassium phosphate and sonicated with a probe tip sonicator (Branson Sonic Power Co., Danbury, CT) for 1 min in an ice bath.

Enzyme assays. For assay of the *N*-ethyl maleimide- (NEM-)[1] sensitive ATPase activity, the final composition of the assay medium was 150 mM KCl, 30 mM Tris, 30 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 6 mM $MgCl_2$, pH 7.0, 3 mM ATP±1 mM NEM in a final volume of 1 ml. The difference produced by 1 mM NEM was taken as the activity. For assay of the Na^+-K^+ ATPase activity, the final composition of the medium was 120 mM NaCl, 20 mM KCl, 30 mM Tris, 30 mM MES, 6 mM $MgCl_2$, 3 mM ATP, pH 8.0±1 mM ouabain in a final volume of 1 ml. The difference produced by 1 mM ouabain was taken as the Na^+-K^+ ATPase activity. The assays were started by the addition of 100 μl of 30 mM ATP adjusted to pH 7.0. Samples were incubated for 20 min at 37°C, and the reaction was stopped by the addition of 100 μl of 100% trichloroacetic acid. Tubes were allowed to stand on ice for 20 min, then centrifuged at 3,000 *g* for 10 min. 200 μl of supernatant was assayed for phosphate by the method of Baginski (9).

Succinate dehydrogenase was assayed by the method of King (10). *N*-acetyl glucosaminidase was assayed by the method of Ray et al. (11). Protein was assayed by the method of Bradford (12) using bovine serum albumin as a standard.

H^+ transport assays. Proton transport was assayed in a dual wavelength spectrophotometer (Johnson Foundation Machine Shop, University of Pennsylvania, Philadelphia) by measuring the ATP-induced uptake of acridine orange as described previously (4). Unless otherwise indicated, all assays were done in a medium consisting of a final concentration of 150 mM KCl, 2 mM Tris, 2 mM MES, 6 mM $MgCl_2$, pH 7.0, and containing 1 μM valinomycin to reduce the generation of a potential by the pump, which might limit the development of a pH gradient. All transport assays were performed at room temperature. Fig. 1 shows one such experiment in which the addition of ATP to renal medulla vesicles caused the uptake of acridine orange into these vesicles. Since the protonated form of acridine orange is less permeable than the weak base, the development of an acid pH in the vesicles will lead to an accumulation of the dye. At high concentrations the dye forms dimers and higher order multimers with a consequent spectral shift. The dual wavelength spectrophotometer was tuned to the signal of the monomeric form of acridine orange. A decrease in the signal, due to the uptake of acridine orange, was taken to indicate the development of a pH gradient, since the addition of the uncoupler nigericin caused the rapid discharge of the dye from the vesicles. The discharge of the dye induced by nigericin was instanteous compared with the uptake induced by ATP, showing that the permeability of the weak base acridine orange is not rate limiting to uptake.

The initial rate of uptake of acridine orange can be used to estimate H^+ transport. Accumulation of a weak base in an acid vesicle should follow the relationship (4):

$$\frac{[H^+]_{in}}{[H^+]_{out}} = \frac{D}{1 - D}\frac{v_{out}}{v_{in}}, \quad (1)$$

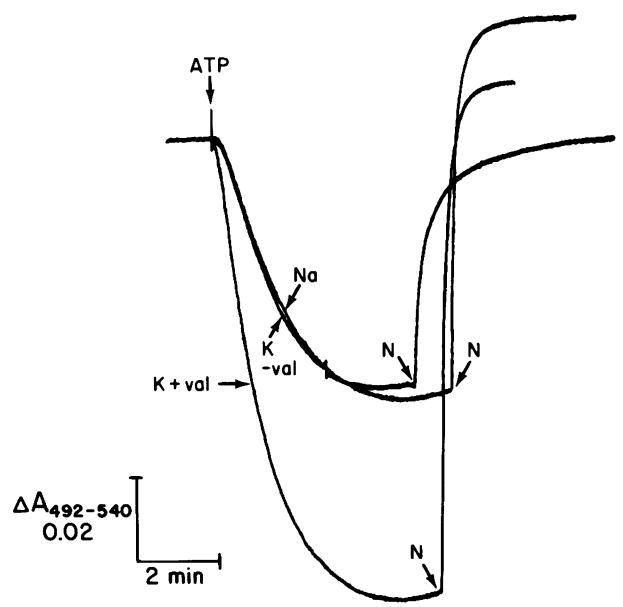

Figure 1. ATP-dependent H^+ transport in G2 vesicles measured by the uptake of acridine orange using a dual wavelength spectrophotometer at the given wavelengths. ATP was added to vesicle suspension as indicated. K−val trace was from medium containing 150 mM KCl without valinomycin. In K+val trace, 1 μM valinomycin was added to the medium. Na trace was from medium containing 150 mM NaCl instead of potassium. Nigericin was added to a final concentration of 1 μM at the point indicated (N). Each assay contained 714 μg protein and 20 μg/ml oligomycin. ΔA, change in absorbance.

1. *Abbreviations used in this paper:* DCCD, dicyclohexylcarbodiimide; MES, 2-(*N*-morpholino)ethanesulfonic acid; NEM, *N*-ethyl maleimide.

where D is the fractional change in absorption, $[H^+]_{in}$ and $[H^+]_{out}$ are the hydrogen ion concentrations inside the vesicles and in the surrounding medium, respectively, and v_{in} and v_{out} are the internal volume of the vesicles and the volume of the surrounding medium, respectively. When $D \ll 1$, the expression for the initial rate of uptake of base is given by (13):

$$\frac{dD}{dt} = \frac{d[H^+]_{in}}{dt} \frac{1}{[H^+]_{out}} \frac{v_{out}}{v_{in}}. \quad (2)$$

The validity of the assay was confirmed in two ways. First, the initial rate of uptake was found to be proportional to the vesicle protein (i.e., vesicle volume) added (Fig. 2 *A*), as predicted by Eq. 2. Second, the internal space of the vesicles was shown to be osmotically active, with no significant uptake of acridine orange due to binding. If we assume that the maximum pH attained in these vesicles is not affected by osmotic shrinking, $D/(1 - D)$ for the same pH should be proportional to the vesicle volume, as predicted by Eq. 1. Fig. 2 *B* shows that osmotic shrinking of the vesicles produced a decrease in $D/(1 - D)$ that was proportional to vesicle volume. When the line was extrapolated to zero vesicle volume (at infinite osmolality), the intercept was zero, implying that there was no significant uptake due to binding.

The accuracy of the assay is determined largely by our ability to assign a unique value to the maximum initial slope of decline of the signal. Hence, one should consider this assay only as a useful semi-quantitative measure of the rate of H^+ transport into the vesicles.

Results

Several different types of proton-translocating ATPases have been described, including the mitochondrial F_0-F_1 ATPase (8), the stomach H^+-K^+ ATPase (14), the fungal plasma membrane H^+ATPase (15), the lysosomal H^+ATPase (16, 17), and the turtle bladder H^+ATPase (4). These ATPases are distinguishable by properties that include their net charge translocation (electroneutral vs. electrogenic), their inhibitor sensitivities, the presence or absence of phosphorylated intermediates, their substrate specificities, their subunit size and number, and other physical properties such as pH optima. We performed an initial characterization of the properties of the H^+ATPase in bovine medulla microsomes, which showed that it is similar to that which we described in the turtle urinary bladder (4) but that it differs from other described H^+ATPases.

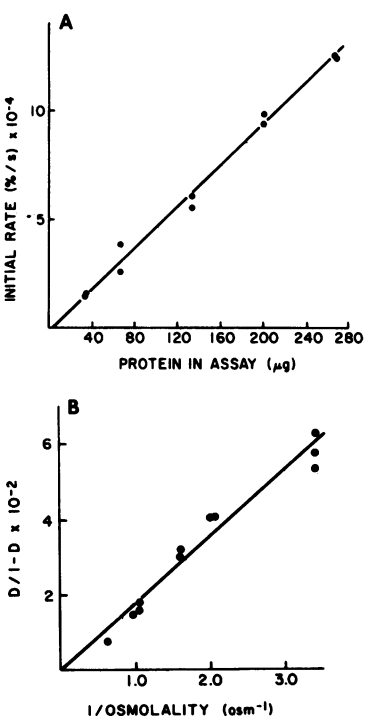

Figure 2. (*A*) Plot of the initial rate of acridine orange uptake vs. the amount of G2 vesicle protein added. Each assay contained 20 μg/ml oligomycin. (*B*) Effect of osmolality on the maximal uptake of acridine orange induced by ATP. G2 vesicles were suspended in KCl transport medium with various amounts of sucrose added to give the final osmolalities indicated in the figure. ATP was added, and acridine orange uptake was followed as in Fig. 1. The maximal uptake was recorded. D represents the fractional decrease in absorbance from the initial reading. Assays contained 138 μg protein and oligomycin at a final concentration of 20 μg/ml.

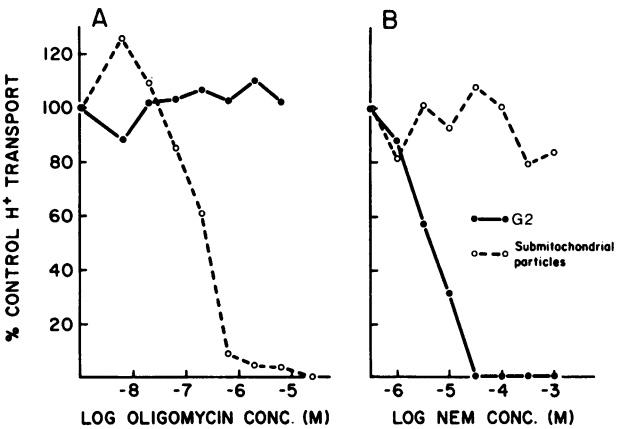

Figure 3. Effect of oligomycin and NEM on H^+ transport in G2 vesicles and submitochondrial particles. (*A*) 224 μg of G2 vesicles or renal cortex submitochondrial particles was used in each assay. The initial rate of ATP-induced acridine orange uptake in 150 mM KCl transport medium containing 1 μM valinomycin was studied at various concentrations of oligomycin as indicated and expressed as a percentage of control. (*B*) 88 μg of G2 vesicles or submitochondrial particles was used in each assay. H^+ transport was studied as above at various concentrations of NEM. Samples were allowed to equilibrate in transport medium and then incubated with either inhibitor at 22°C for 15 min before each assay.

Bovine medulla H^+ATPase is different from mitochondrial F_0-F_1 ATPase. Bovine medulla microsomes were found to have ATP-dependent proton transport (Fig. 1). This H^+ transport activity was shown in two ways not to be due to contamination by submitochondrial particles. First, in all experiments, 20 μg/ml oligomycin was added to inhibit proton transport by any mitochondrial H^+ATPase present. This concentration of oligomycin was found to inhibit proton transport completely in submitochondrial particles isolated from bovine renal cortex at similar protein concentration (Fig. 3 *A*). Second, H^+ transport in the medullary vesicles was highly sensitive to NEM, whereas proton transport in renal submitochondrial particles was not inhibited by even much higher concentrations of NEM (Fig. 3 *B*).

Bovine medulla H^+ATPase is electrogenic. To demonstrate that the medulla H^+ pump is electrogenic, we showed that collapsing the potential in the vesicles accelerates the rate of proton

transport (Fig. 1). Vesicles were preincubated for several hours in transport buffer that contained 150 mM KCl, and the rates of proton transport were compared before and after the potassium ionophore valinomycin was added. As shown in Fig. 1, the addition of valinomycin stimulated proton transport, suggesting an electrogenic pump. To show that this effect of valinomycin was due to the collapse of the potential in the vesicles and not to an increased availability of potassium to the inside of the vesicles, proton transport was studied in a potassium-free transport medium, which contained 150 mM NaCl instead of KCl. As shown, proton transport in the sodium buffer was identical to that in the potassium buffer without valinomycin. The addition of valinomycin in the sodium transport medium had no effect (not shown).

Inhibitor sensitivities of H^+ transport. To further examine the properties of this H^+ pump, we next tested the sensitivity of H^+ transport to several inhibitors (Table I). As shown, the pump was resistant to the mitochondrial inhibitors efrapeptin and rutamycin and was also resistant to vanadate. However, H^+ transport was inhibited by dicyclohexylcarbodiimide (DCCD), and by the sulfhydryl reagents NEM, *p*-chloromercuriphenylsulfonic acid, and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole. The resistance to efrapeptin and rutamycin provides additional evidence that this H^+ATPase is different from that of the mitochondria. The lack of inhibition by vanadate is similar to that of the turtle bladder H^+ATPase and distinguishes this pump from those with a phosphorylated intermediate in their catalytic cycle, such as the stomach H^+-K^+ ATPase and the yeast plasma membrane H^+ATPase.

Table I. Inhibitor Sensitivities of H^+ Transport. Effect of Inhibitors on the Initial Rate of Uptake of Acridine Orange in G2 Vesicles

Inhibitor	Control	Experimental	Percent of control
	change in OD/s per mg protein		
Efrapeptin (5 μg/ml)	6.07×10^{-3}	6.10×10^{-3}	105
Rutamycin (20 μg/ml)	6.07	4.25	70.0
Na_2HVO_4 (500 μM)	4.57	3.98	87.1
DCCD (200 μM)	6.07	0.0	0.0
NBD-Cl (10 μM)	5.95	0.62	10.4
PCMBS (10 μM)	2.49	0.0	0.0
NEM (1 mM)	2.49	0.0	0.0

Protein in assays ranged from 113 to 234 μg. The assay media contained 10 μg/ml oligomycin and 1 μM valinomycin. NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; PCMBS, *p*-chloromercuriphenylsulfonic acid.

The effect of the inhibitors on H^+ transport in these vesicles was measured as a reduction in the rate and extent of development of a pH gradient across the membrane, an effect that could result from dissipation of a gradient as well as from inhibition of the ATPase. To test for the ability of these inhibitors to act as proton conductors, we prepared asolectin vesicles and loaded them with buffer at pH 5, added them to buffer at pH 7, and measured the decay of the pH gradient by the uptake and release of 9-aminoacridine as described by Nicholas et al. (18), using 10 μM 9-aminoacridine. As expected, nigericin collapsed this gradient instanteously. Except for DCCD, none of the inhibitors used above increased the rate of collapse of the pH difference. DCCD at concentration of 200 μM rapidly dissipated the gradient. Whether this effect was due to a contaminant in the prepartion, to lysis of the vesicles, or to a true ionophore effect remains to be established.

Fig. 4 shows typical H^+ transport in the presence and absence of vanadate. As shown, in control H^+ transport experiments it was characteristic for acridine orange to be released slowly after its initial maximum uptake, implying that the pH gradient in the vesicles was slowly collapsing. The tracing in Fig. 4 demonstrates that the addition of vanadate had little effect on either the initial rate or maximal uptake of acridine orange. However, the pH collapse was markedly reduced. In several experiments, the rate of collapse of the pH gradient in control vesicles was $10.25 \pm 1.95 \times 10^{-3}$ change in OD/s per mg protein, whereas in vanadate treated vesicles it was only $0.37 \pm 0.18 \times 10^{-3}$. We have no explanation at present either for the rate of collapse of the pH gradient in control vesicles or for the effect of vanadate on it.

Substrate specificity of the H^+ATPase. In addition to net charge translocation and inhibitor sensitivities, a third way to distinguish among different H^+ATPases is by the substrate requirements for H^+ transport. Fig. 5 shows that of the nucleotides tested, only ATP induced H^+ transport by the medulla H^+ATPase. This distinguishes this ATPase from that of lysosomes, which were found to use guanosine and inosine triphosphate as well as ATP for H^+ transport (16).

Cellular localization of the H^+ATPase. H^+ATPases are known to be present in both the mitochondria and lysosomes. To identify which membrane fractions contained the medulla H^+ATPase, medullary microsomes were separated on a linear sucrose density gradient and assayed for markers of several cellular fractions (Fig. 6). Proton transport was assayed along the gradient by measuring the initial rate of uptake of acridine orange. As shown, proton transport had a major peak at a density of 1.11 g/ml and separated well from the Na^+-K^+ ATPase activity (basolateral membranes), which had a density of 1.15 g/ml, and from mitochondrial and lysosomal markers, which had a density of 1.17 g/ml. We tried to measure in these fractions an ATPase activity that might correspond to the enzymatic equivalent of the proton pump. Inhibition by NEM in the presence of ouabain

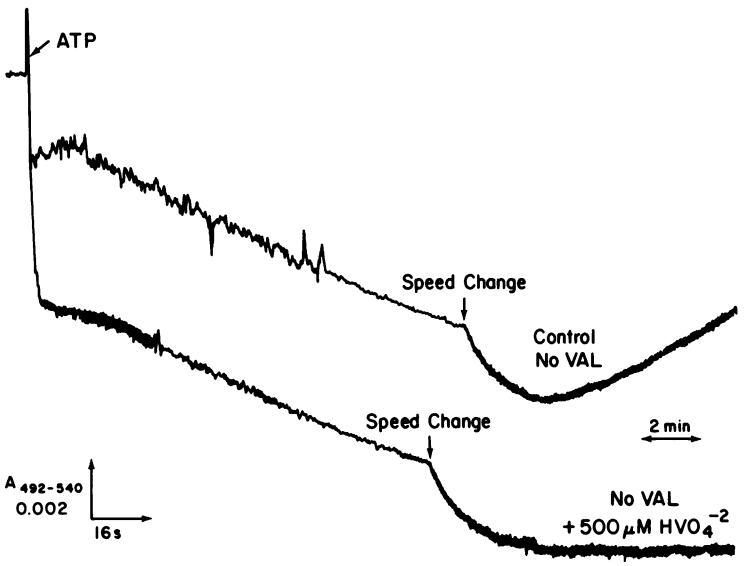

Figure 4. Effect of vanadate on H^+ transport in G2 vesicles. 163 μg of G2 vesicles was allowed to equilibrate in 150 mM KCl transport medium without valinomycin (VAL). Vanadate or buffer was added, and each sample was allowed to stand 15 min at 22°C before assay. Speed of trace was changed as indicated. A, absorbance.

and oligomycin correlated well with proton transport, as seen in Fig. 6. Unfortunately, this activity constituted a small fraction of the total ATPase activity. More work will be needed to confirm that this ATPase activity represents the H^+ pump. The medulla H^+ pump lies in a membrane fraction separate from basolateral membranes, mitochondria, and lysosomes. The smaller peak of NEM-sensitive ATPase and H^+ transport at a density of 1.17 g/ml probably represents some lysosomal contamination of the microsomes. Based on these continuous gradients, step gradients were designed to yield a membrane fraction enriched in the medulla H^+ATPase. (The separation chosen for the sucrose step is indicated by the arrow in Fig. 6.) The marker enrichment of the sucrose step fractions is indicated in Table II. The G2 fraction was used for all the transport assays reported here.

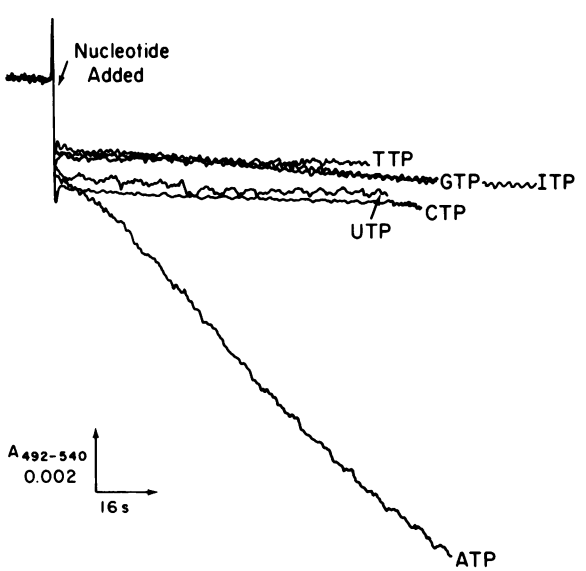

Figure 5. Substrate specificity for H^+ transport in G2 vesicles. 67 μg of G2 vesicles was used in each assay. Vesicles were allowed to equilibrate in 150 mM KCl transport medium containing 1 μM valinomycin. Nucleotides were added at point indicated to a final concentration of 0.4 mM. CTP, GTP, ITP, TTP, and UTP; cytosine, guanine, inosine, thymidine, and uridine triphosphate, respectively. A, absorbance.

Discussion

The results show that the bovine medulla contains an electrogenic proton-translocating ATPase whose properties and membrane localization are different from the proton pumps of mitochondria and lysosomes, but which closely resembles the H^+ pump from the turtle urinary bladder.

Ion-translocating ATPases fall into two general categories. The E_1-E_2 type ATPases are characterized by (*a*) a phosphorylated intermediate in their catalytic cycle; (*b*) a simple subunit structure, composed of at most two subunits; (*c*) a catalytic subunit of ~100 kD; and (*d*) the presence of the ATPase portion as deeply imbedded integral membrane proteins. This class includes the Na^+-K^+ ATPase, the Ca^{++}ATPase, the stomach H^+-K^+ ATPase, and the yeast plasma membrane H^+ATPase. All of these ATPases are inhibited by vanadate at concentrations of <100 μM. In the case of the Na^+-K^+ ATPase, vanadate has been found to bind to the same site, which is phosphorylated during its catalytic cycle (19). This has led to a general hypothesis that vanadate, whose structure resembles that of phosphate, inhibits all phosphorylated ion-translocating ATPases by binding to the phosphorylation site (20).

The other principal type of ion-translocating ATPase is the

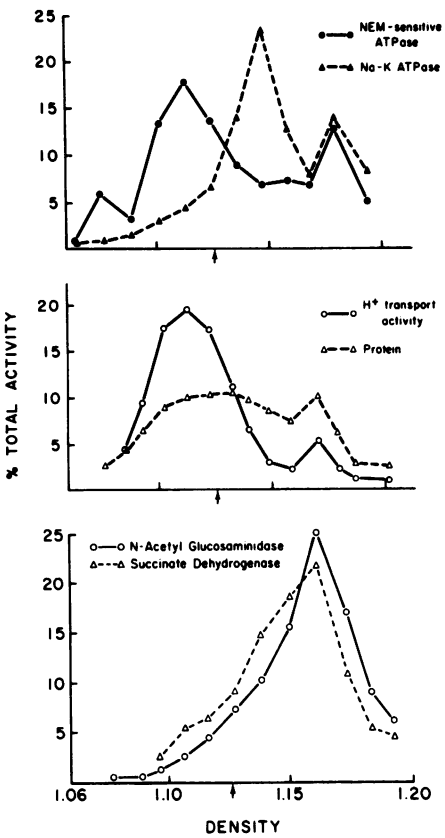

Figure 6. Distribution of markers of P fraction membranes on a 0–40% sucrose (in homogenizing buffer) continuous density gradient. The total activities loaded on the gradient in μmol/min were: NEM-sensitive ATPase, 0.0734; Na^+-K^+ ATPase, 0.775; *N*-acetyl glucosaminidase, 0.231; succinate dehydrogenase, 0.0356. Total H^+ transport activity loaded on was 9.94×10^{-3} change OD/s, and the total protein was 1.56 mg. The arrows represent the separations chosen for the sucrose step.

F_0-F_1 type ATPase. These enzymes are characterized by (*a*) the lack of a phosphorylated intermediate; (*b*) a complex subunit structure, often with 10 or more subunits; (*c*) a catalytic subunit much smaller than 100 kD, usually in the 50–60 range; and (*d*) the presence of the ATPase portion as an extrinsic membrane protein that is bound to an intrinsic membrane protein channel spanning the membrane. The catalytic portion is large (~360 kD) and visible as a sphere extrinsic to the lamina of the plasma membrane. These ATPases are known only to transport H^+, and none is inhibited by vanadate. This class includes the mitochondrial, bacterial, and chloroplast pumps. The H^+ pump described here is more similar to the F_0-F_1 type than to the gastric H^+ pump. However, without more molecular information it will be impossible to ascertain to which class of ATPases the renal H^+ pump belongs.

The location of this H^+ pump in the tubular cell is not completely resolved by the present investigation. However, it seems reasonable to conclude that since the H^+ pump-containing vesicles do not migrate with the Na^+-K^+ ATPase, they are not located in the basolateral membrane. Recently, an ATP-driven proton pump has been found in endocytic vesicles (21–23), Golgi complex (24), and rough and smooth microsomes (25) derived from various epithelial and nonepithelial tissues. The proton pump(s) of intracellular organelles bears many similarities to the one described here; it is electrogenic, inhibited by NEM

Table II. Activity of Enzymatic Markers in Differential Centrifugation and Sucrose Step Gradient Fractions

Fraction	Specific activity	Fold increase	Percent of total
	nmol/min per mg protein		
NEM-Sensitive ATPase			
H	9.1±0.64		100
S	6.9±0.68	0.75	51.1±6.7
P	18.9±0.53	2.07	8.3±2.1
M	18.7±0.45	2.05	6.8±1.0
G2	52.4±0.70	5.75	3.0±0.2
G3	17.8±0.80	1.95	2.6±0.4
Na^+-K^+ATPase			
H	91.9±0.7		100
S	42.0±2.0	0.46	28.3±0.9
P	301±44	3.28	15.9±2.2
M	144±19	1.57	5.1±0.9
G2	228±45	2.48	1.6±0.2
G3	675±44	7.34	11.5±1.8
N-Acetyl glucosaminidase			
H	121±7.7		100
S	64.8±3.2	0.54	35.2±1.6
P	70.6±3.1	0.59	2.4±0.2
M	255±29	2.13	7.0±1.2
G2	28±5.6	0.23	0.14±.03
G3	90.1±7.3	0.75	0.95±.04
Succinate dehydrogenase			
H	13.6±0.7		100
S	5.1±0.5	0.37	21.9±1.5
P	12.2±5.9	0.90	3.2±0.2
M	66.2±2.5	4.87	13.7±0.6
G2	8.9±0.8	0.65	0.29±.06
G3	27.1±0.5	1.99	2.4±0.1

H, crude homogenate; S, 6,000 *g* supernatant; P, fluffy upper portion of the 38,000 *g* pellet; M, lower dark part of that pellet; G2, fraction at the 15–25% interface of the discontinuous sucrose gradient; G3, membranes sedimenting at the 25–40% interface of the gradient.

but not by oligomycin or vanadate, and does not seem to require any alkali metal cation to function. We have shown previously that the proton pump of the turtle urinary bladder is packaged in endocytic vesicles (5). Recent studies (Schwartz, G. J., and Q. Al-Awqati, manuscript in preparation) have also shown that the cortical and medullary collecting tubules of rabbit kidney endocytose luminal material into vesicles whose contents are acidified by the action of proton pumps, and that CO_2 caused exocytotic secretion of the endocytosed content, much as it does in the turtle bladder. Hence, it seems likely that the proton pump responsible for urinary acidification in the collecting tubule is also packaged in endocytic vesicles. The vesicles we have isolated in the present study greatly resemble endocytic vesicles, since they are of low density and contain a proton pump. However, in the absence of an unequivocal label for the luminal membrane of acid-secreting cells, we will have to await immunocytochemical localization of this proton-translocating ATPase before any conclusions about its role in urinary acidification can be made.

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