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The Structure and Biochemistry of the Vacuolar H^+ ATPase in Proximal and Distal Urinary Acidification[1]

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Vacuolar H^+ ATPases participate in renal hydrogen ion secretion in both the proximal and distal nephron. These plasma membrane forms of the vacuolar H^+ ATPase are regulated physiologically to maintain the acid–base balance of the organism. Proton transporting renal cells have requirements for constitutive acidification of intracellular compartments for normal endocytic and secretory functions. Recent experiments have begun to reveal how the kidney regulates these proton pumps independently. Vacuolar H^+ ATPases are a family of structurally similar enzyme which differ in the composition of specific subunits. Cytosolic regulatory enzymes are present in renal cells which may affect vacuolar H^+ ATPases in certain membrane compartments selectively. The vacuolar H^+ ATPase in the plasma membrane of intercalated cells resides in a specialized proton-transporting apparatus that translocates the enzyme between an intracellular membrane pool and the plasma membrane in response to physiologic stimuli.

KEY WORDS: Kidney; collecting tubule; intercalated cells; urinary acidification; acid-base balance; proton pump; V-ATPase; regulatory proteins.

INTRODUCTION

To maintain acid–base balance, the kidney must reabsorb all of the filtered bicarbonate and excrete a quantity of acid equal to that produced by metabolic proton generation. Renal hydrogen ion excretion takes place in several nephron segments (Alpern, 1990). The proximal tubule reabsorbs 90% of the filtered bicarbonate, and an additional 5% of the filtered bicarbonate is reabsorbed in a thick ascending limb. The collecting duct is responsible for the final reabsorption of bicarbonate in the regulation of net acid excretion by the kidney. In the collecting duct, hydrogen ion excretion is carried out by the intercalated cells, a specialized cell population rich in carbonic anhydrase (Brown, 1989; Madsen and Tisher, 1985). These cells employ a vacuolar H^+ ATPase as a predominant means for hydrogen ion transport (Brown *et al.*, 1988a,b). A number of years ago, our laboratory isolated the proton ATPase from bovine kidney and characterized its enzymatic and structural properties (Gluck and Caldwell, 1987, 1988; Wang and Gluck, 1990). Like other vacuolar H^+ ATPases, the kidney enzyme is a protein of $M_r \sim 580{,}000$ that is composed of a number of polypeptides. Using this purified enzyme as an antigen for immunization, we

[1] This review will focus on the structure, enzymology, and regulation of the vacuolar H^+ ATPase in the mammalian kidney. Because of space limitations, it will cover predominantly work from our laboratory. However, a number of investigators, including Brown (Brown *et al.*, 1987, 1988a,b, 1989), Burckhardt (Sabolic *et al.*, 1985; Turrini *et al.*, 1989; Simon and Burckhardt, 1990), Madsen and Tisher (Madsen and Tisher, 1985; Verlander *et al.*, 1987, 1989). Steinmetz (Steinmetz, 1986; Stetson and Steinmetz, 1986), Schwartz (Scwartz *et al.*, 1985, 1988; Satlin and Schwartz, 1989), Sabatini and Kurtzman (Sabatini *et al.*, 1990a,b), DuBose (Diaz-Diaz *et al.*, 1986; Gurich and DuBose, 1989), Al-Awqati (Van Adelsberg and Al-Awqati, 1986), and their coworkers, and many other investigators have made important contributions to this field.

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developed a monoclonal antibody to the kidney H^+ ATPase (Yurko and Gluck, 1987), used it to purify the H^+ ATPase by immunoaffinity chromatography (Gluck and Caldwell, 1987; Wang and Gluck, 1990), and characterized the structure and catalytic properties of the enzyme. The affinity-purified enzyme remained enzymatically active and retained the capacity for active proton transport when reconstituted in a phospholipid vesicle. SDS polyacrylamide gels of the purified enzyme showed polypeptides at M_r 70,000, a cluster at ~56,000, and polypeptides at 45,000, 42,000, 38,000, 33,000, 31,000, 15,000, 14,000, and 12,000 (Gluck and Caldwell, 1987; Wang and Gluck, 1990).

Using the purified ATPase as an immunogen, Brown *et al.* (1988a,b) prepared and used high-affinity polyclonal antibodies to the enzyme to localize the distribution of the enzyme in the mammalian kidney. In the proximal tubule, vacuolar H^+ ATPase staining was abundant on the brush border microvilli in the initial portion of the proximal tubule, and in the invaginations of the base of the apical microvilli throughout the proximal tubule. Moderate plasma membrane staining for H^+ ATPase was also observed in the thick ascending limb and the distal convoluted tubule. In the connecting tubule and collecting duct, heavy staining was observed in the proton-transporting intercalated cells. Rapid freeze deep etch micrographs confirmed that the vacuolar H^+ ATPase resides on the luminal membrane of the acid secreting cells (Brown *et al.*, 1987). In these cells, proton pumps were present at a density of over 16,000 per square micron. Vacuolar H^+ ATPases are also present in many of the intracellular vacuolar compartments of eukaryotic cells (Yurko and Gluck, 1987), where they serve in the processing of membranes and intraluminal molecules in endocytosis and secretion. However, immunocytochemical studies suggest that the proton pump in these intracellular compartments is present at far lower densities than in the plasma membrane of proton transporting cells (Rodman *et al.*, 1991).

The H^+ ATPase in the plasma membrane of proton-transporting cells therefore displays several distinct features not evident in most other mammalian cells: cell-specific amplification, polarization to one or more plasmalemmal domains, and physiologic regulation to preserve acid–base homeostasis (discussed in more detail below). How is the enzyme able to accomplish this highly specialized function of transcellular proton transport while functioning to acidify a variety of intracellular compartments?

PROPERTIES OF THE VACUOLAR H^+ ATPase IN RENAL EPITHELIAL CELLS

Several lines of evidence suggest that vacuolar ATPases exist as a family of enzymes with differences in structure and function. An initial observation that suggested this concept was the finding that proton ATPase isolated from bovine kidney microsomes by HPLC ion exchange contained two partially resolved peaks of ATPase activity which differed in their polypeptide composition (Gluck and Caldwell, 1987). Subsequently, we found that H^+ ATPases isolated from separate membrane functions from bovine kidney had different enzymatic and structural properties (see references in Wang and Gluck, 1990; Wang and Gluck, manuscript in preparation; and Table I). H^+ ATPase was isolated from a purified brush border membrane fraction, a kidney lysosomal fraction, and from kidney microsomes (a mixture of membranes containing plasma membranes from intercalated cells) by immunoaffinity chromatography on identical monoclonal antibody columns. The H^+ ATPase isolated from these fractions differed in pH optimum, in effects of added lipids on activity, and in sensitivity to divalent and trivalent cations (Wang and Gluck, 1990), and had subtle differences in structure that could be detected by immunoblotting on two-dimensional gels. The brush border H^+ ATPase had a pH optimum of ~7.3, the approximate intracellular pH of the proximal tubule cell. The proximal tubule functions primarily in bulk bicarbonate reabsorption and is not the site for fine regulation of net hydrogen ion excretion. The pH optimum of the brush border H^+ ATPase is, therefore, poised for the enzyme to function maximally at a normal intracellular pH. In contrast, the pH optimum of the microsomal enzyme was ~6.3, a one pH unit difference from the intracellular pH of intercalated cells. H^+ ATPase in these cells may therefore respond with an increase in activity under conditions where intracellular pH decreases, such as a decrease in the extracellular fluid bicarbonate concentration. However, the magnitude of the change is only about 30% of the total activity between pH 6.3 and 7.3, and it is therefore likely that other regulatory mechanisms control the rate of transport. The pH optimum of the kidney lysosomal H^+ ATPase was ~6.7 (Table I).

Cu^{2+} ion inhibited the microsomal enzyme activity by 100%. A concentration of copper up to 5 mm inhibited the brush border H^+ ATPase by approximately 50%. The lysosomal H^+ ATPase was relatively

Table I. Properties of H^+ ATPase in Different Membrane Compartments

	Compartment		
	Microsomes	Brush border	Lysosomes
pH optimum	6.3	7.4	6.9
Substrate specificity	ATP:GTP selectivity 4:1	ATP:GTP selectivity 2:1	ATP:GTP Selectivity 3:1
Multivalent cation effects	Inhibited 100% by Cu^{2+}, 100% by Zn^{2+} No inhibition by Al^{3+}	Inhibited 50% by Cu^{2+} 100% by Zn^{2+} No inhibition by Al^{3+}	Inhibited 23% by Cu^{2+}, 62% by Zn^{2+} Inhibited 80% by Al^{3+}
Anion effects	No Cl reqt. Inhibited by NO_3^-, SO_3^{2-}, SO_4^{2-}, and CO_3^{2-}	No Cl reqt. Inhibited by NO_3^-, SO_3^{2-}, SO_4^{2-}, and CO_3^{2-}	No Cl reqt. Inhibited by NO_3^-, SO_3^{2-}, SO_4^{2-}, and CO_3^{2-}
Structure: 31 kD	1 principal 31-kD polypeptide	Additional lower-mobility immunoreactive 31-kD polypeptides	1 principal 31-kD polypeptide
Structure: 56 kD	Both 56 kD and 58 kD polypeptides. Several different pIs for 56 kD	Both 56 kD and 58 kD polypeptides. Fewer pIs for 56 kD than in microsomes	Little or no 58-kD polypeptide. Fewer pIs for 56 kD than in microsomes

insensitive to copper, but highly sensitive to aluminum ion, whereas aluminum had no effect on the brush border and microsomal H^+ ATPases. These studies serve to demonstrate that differences in enzymatic properties can be detected in H^+ ATPases from different kidney membrane fractions. These observations also pose somewhat of a structural enigma. Other lines of evidence suggest that the 70,000 molecular weight subunit vacuolar H^+ ATPases is the locus of the ATP hydrolytic site involved in catalysis (Forgac, 1989). Two-dimensional gels of the H^+ ATPase immunoaffinity-purified from different kidney membrane fractions have revealed only one M_r 70,000 polypeptide (Wang and Gluck, 1990; Wang and Gluck, manuscript in preparation). To date, only one isoform encoding the subunit has been reported by *c*DNA cloning from a single species. If there is only a single type of M_r 70,000 subunit, it would suggest that the $M_r \sim 56{,}000$

Table II. Properties of Inhibitor and Activator of H^+ ATPase

	Inhibitor	Activator
Actions	Inhibits solubilized immunoaffinity-purified H^+ ATPase Inhibits ATP-dependent proton transport	Inhibits solubilized immunoaffinity-purified H^+ ATPase
Mode of action	Saturable effect on H^+ ATPase. Hill coefficient of 1.46 Nonenzymatic effect. Probably works by binding, although inhibitor cannot be washed off readily at any pH	Saturable effect on H^+ ATPase. Hill coefficient of 1.0 Nonenzymatic effect. Probably works by binding. Activator binds more tightly at pH < 7.5
pH effects	% Inhibition increases for pH > 7.2	Binding optimum at pH 6.5. % activation increases for pH < 6.5
Composition	Heat labile protein. M_r 6300 polypeptide on SDS gels. Active MW of $\sim 12{,}000$ by gel filtration; probably active as a dimer	Heat-stable protein. Active size of $\sim 40{,}000$ by gel filtration
Specificity	Highly specific for vacuolar H^+ ATPases. Slight effect on F_0F_1 H^+ ATPase Not selective for subclass of vacuolar H^+ ATPase	Highly specific for vacuolar H^+ ATPases. Slight effect on F_0F_1 H^+ ATPase. Selective for brush border > microsomal $\gg$ lysosomal H^+ ATPase

subunit, which has sequence homology with the regulatory α subunit of the $F_0F_1H^+$ ATPases, may be a regulatory subunit which affects the overall enzymatic properties of the vacuolar H^+ ATPases.

Although studies from other laboratories had suggested that the vacuolar H^+ ATPases were stimulated directly by chloride, we found no evidence for this in any of the H^+ ATPases examined. Increasing concentrations of chloride added to the ATPase assay buffer decreased ATPase activity slightly. Other anions were found to have more profound effects on ATPase activity as described in the fungal chromaffin granule, coated vesicle, and other H^+ ATPases (Bowman *et al.*, 1989; Moriyama and Nelson, 1989; Arai *et al.*, 1989). Nitrate is an inhibitor of H^+ ATPase activity at concentrations above 5 mM. Our results suggest that this effect is due to an uncompetitive type inhibition on ATPase activity and not due to dissociation of subunits from the cyto-plasmic domain of the enzyme (Wang and Gluck, 1990). Sulfite, and to a lesser extent sulfate, had similar inhibitory effects on the H^+ ATPase. These observations suggest that the kidney H^+ ATPase may have an oxyanion binding site which regulates activity. We tested the effect of different concentrations of bicarbonate on the enzyme under conditions of constant pH and found little effect. However, under conditions where greater than 0.1 mm carbonate ion was present, as estimated from the bicarbonate–carbonate proton K_d, a 20–30% inhibition of ATPase activity was observed (Z-Q. Wang and S. Gluck, unpublished). Hence one function of the anion binding site of the kidney H^+ ATPase may be as a receptor for carbonate ion. Carbonate ion would be present at significant concentrations only in an alkaline cytosol, or in the setting of a significant unstirred layer of bicarbonate ion, and it might serve to inhibit further proton extrusion from the cell.

The structure of the microsomal brush border lysosomal ATPases was compared on two-dimensional gels, and significant differences were noted in both the M_r 31,000 subunit and the $M_r \sim 56{,}000$ subunit among the different ATPase preparations (Wang and Gluck, 1990). The M_r 31,000 subunit has an isoelectric point of approximately 8.1 with a large pH spread. In the microsomal ATPase, the subunit appeared as a single polypeptide band at M_r 31,000. The M_r 31,000 subunit in the brush border microvillar H^+ ATPase appeared as a predominant polypeptide at 31,000 with several polypeptides at an identical pI, but of lower mobility (higher relative molecular mass; references in Wang and Gluck, 1990). Our laboratory previously isolated a *c*DNA clone encoding the M_r 31,000 subunit and determined its complete predicted protein sequence (Hirsch *et al.*, 1988). By analyzing the sequence, we predicted that the carboxy terminus of the subunit would be located on an external accessible site of the molecule (Hemken *et al.*, 1992). Mice were immunized with a 10-residue peptide from the carboxy terminus, and a series of monoclonal antibodies reacting with subunit were isolated and characterized. Several of these reacted with the native M_r 31,000 subunit by immunoblot analysis. However, when these antibodies were examined by immunoblotting on two-dimensional gels and immunocytochemistry, significant differences in reactivity were discovered (Hemken *et al.*, 1992). Antibody E11 yielded a pattern of immunocytochemistry in rat kidney identical to the pattern that we had described previously using rabbit polyclonal antibodies. E11 stained the brush border microvilli in rat proximal tubules, the invaginations at the base of the microvilli, the apical and basolateral poles of cortical intercalated cells, and the apical poles of medullary intercalated cells. On two-dimensional immunoblots, E11 reacted with a single M_r 31,000 polypeptide on two-dimensional immunoblots of the affinity-purified microsomal H^+ ATPase. However, E11 reacted with a series of polypeptides on two-dimensional immunoblots of the affinity-purified brush border H^+ ATPase. The polypeptides had the same isoelectric point as the M_r 31,000 subunit, but the additional polypeptides had a lower mobility, a pattern identical to that observed on the two-dimensional protein gels of the affinity-purified brush border H^+ ATPase. A second antibody, H8, also reacted on immunoblots with the native M_r 31,000 subunit from kidney microsomes. On immunocytochemical staining of rat kidney, antibody H8 stained the invaginations at the base of the mcirovilli and the intercalated cells with a pattern identical to that observed with antibody E11. However, H8 showed no staining at all of the brush border microvilli. On immunoblots, this antibody stained the single M_r 31,000 polypeptide in microsomal ATPase but had nearly absent immunoreactivity against the brush border enzyme. These results suggest that the structure of the M_r 31,000 subunit differs in brush border, appearing as several polypeptide forms with a mobility lower than the most prevalent form of the subunit found in the kidney. The physiologic role of the M_r 31,000 subunit remains to be determined, but studies on the biosynthesis of the vacuolar H^+ ATPase in LLC-PK1 cells suggest that it controls the assembly of

the enzyme on the membrane (J.-Y. Fu and S. Gluck, unpublished).

The basis for the lower-mobility forms of the M_r 31,000 subunit is unclear. Bovine kidney *c*DNA libraries were screened at low stringency with the M_r 31,000 *c*DNA probe in an attempt to isolate isoforms of the subunit, but none were obtained by this method. More recently, we looked for isoforms of the subunit using genomic cloning (Hemken *et al.*, 1992). A Southern blot was performed on human leukocyte DNA, cleaved with different restriction enzymes and probed with a PCR fragment spanning the carboxy terminal portion of the coding region of the M_r 31,000 *c*DNA and a small portion of the 3′ untranslated region. This probe hybridized to several fragments on Southern blots of leukocyte genomic DNA. A full-length human M_r 31,000 *c*DNA was isolated from a human kidney library and used to screen a human genomic library. Several genomic clones were obtained. Two of these were purified and the lambda DNA was cleaved with the same restriction enzymes as used for the genomic Southern blots and analyzed for hybridization to the same PCR fragment. The two clones accounted for all but one of the fragments observed on the Southern blots. Sequence analysis of the two clones showed that one was a true gene with a stretch of nucleotides identical to the human *c*DNA. The second genomic clone appeared to be a pseudogene, as it contained a stretch with a predicted protein sequence highly similar to human *c*DNA, but with several frame shift changes (Hemken *et al.*, 1992). The clone was also missing the stop codon found on the human M_r 31,000 *c*DNA, and the carboxy terminal five amino acids were absent in all three reading frames. Nevertheless, this clone was used to perform an RNA hybridization on RNA from different tissues, and a single transcript of about 1.2 kb was detected uniform in human kidney, heart, lung, liver, placenta, brain, and other tissues. Hence, this clone and the unidentified DNA fragments hybridizing to the PCR-derived M_r 31,000 *c*DNA fragment on the genomic Southern may represent isoforms of the subunit, and might explain the lower-mobility immunoreactive polypeptides detected. Work is in progress to examine this possibility.

An alternative basis for the lower-mobility immunoreactive forms of the M_r 31,000 subunit may be that they are the result of post-translational modifications. The H^+ ATPase purified directly from brush border was analyzed for glycosylation by the periodate oxidation and hydrazide-marker enzyme method, and no evidence of glycosylation was detected (Hemken *et al.*, 1992). There was also no evidence for phosphorylation of the subunit, either from LLC-PK_1 cells labeled to equilibrium with ^{32}P-orthophosphate, or in primary cultures of rabbit kidney proximal tubules incubated with P^{32} orthophosphate (Hemken *et al.*, 1992). An analysis of the sequence of the M_r 31,000 subunit did reveal any potential site for myristoylation or polyisoprenylation, although there was a single internal cysteine that could, in principle, act as an acceptor for palmitoylation (Hirsch *et al.*, 1988). However, experiments conducted thus far have not revealed any evidence for post-translational modification of the subunit that could account for the different lower mobility forms, and the basis for the difference of structure in the subunit in the brush border remains unresolved.

The M_r 56,000 subunit also showed differences in structure among the different H^+ ATPases isolated by immunoaffinity purification (Wang and Gluck, 1990; Wang and Gluck, manuscript in preparation). In two-dimensional protein gels of both the brush border and microsomal enzymes a cluster of polypeptides was observed at M_r 58,000 and 56,000. In both H^+ ATPase preparations, the 58,000 appeared as a single spot, while the 56,000 appeared as a series of four to six spots. The number of spots in the microsomal enzyme was consistently greater than in the brush border, which may reflect the source of the microsomal ATPase, which was isolated from a more complicated mixture of membrane compartments than the brush border H^+ ATPase. The lysosomal H^+ ATPase also retained a 56,000 molecular weight polypeptide, detected as several spots on two dimensional gels, but the lysosomal H^+ ATPase did not contain the 58,000 molecular polypeptide (Wang and Gluck, manuscript in preparation). *c*DNA cloning from our laboratory (Nelson *et al.*, 1992) and others (Südhof *et al.*, 1989; Bernasconi *et al.*, 1990) has shown that at least two isoforms of the M_r 56,000 subunit exist. Comparison of the sequences of the human and bovine $M_r \sim$ 56,000 isoforms shows that the middle portion of the coding region is highly conserved, but both the amino and carboxy terminal sequences of the two isoforms are entirely different (Nelson *et al.*, 1992). These differences in sequence are probably not due to random mutations because they are highly conserved in the corresponding isoform of a different species.

RNA blots from different human tissues revealed that the kidney has the greatest levels of expression of the "kidney" (M_r 58,000) isoform (or isoform 1) of

any tissue. Moderate levels were detected in the placenta, and low levels were detected in the lung; hybridization was undetectable in poly-A^+ mRNA from other human tissues. Similar results were found on RNA hybridization studies performed on total RNA from various bovine tissues. In contrast, RNA hybridization blots performed on human poly-A^+ RNA with the "brain" (M_r 56,000) isoform (isoform 2) showed nearly equal levels expressed in all tissues, but with somewhat higher levels of expression in brain and adrenal medulla (Nelson *et al.*, 1992).

We performed immunoblots on microsomal membranes from various bovine tissues to examine the protein distribution of the "kidney" isoform of the subunit, which has a M_r of 58,000 on SDS gels. The results were similar to those of the RNA blots: the subunit isoform protein was detectable only in kidney. To examine the cellular distribution of the isoform in kidney, we performed immunocytochemistry on rat kidney sections using a polyclonal antibody to a peptide from the unique carboxy terminal sequence of the "kidney" isoform, the M_r 58,000 subunit. As a control, we examined the distribution of H^+ ATPase using a monoclonal antibody to the M_r 31,000 subunit of the H^+ ATPase. We showed previously that the antibody to the M_r 31,000 subunit (E11) stains a number of segments of the nephron. The E11 antibody exhibited heavy staining in the initial part of the proximal tubule and in the invaginations at the base of the microvilli throughout the entire proximal tubule. It showed moderate staining of the apical membrane and subapical vesicles in the thick ascending limb, and moderate staining in apical region of the distal convoluted tubule. In the collecting duct, the E11 monoclonal antibody to the M_r 31,000 subunit gave intense staining of the intercalated cells. In the cortex there are two functionally distinct types of intercalated cells: an acid-secreting intercalated cell, or A cell, and a bicarbonate-secreting intercalated cell or B cell. We showed previously that the A cells stained predominantly in the apical pole. In contrast, the B cells stained variously in the basolateral membrane, or diffusely throughout the cytoplasm, or, in some cells, in both the apical and basal poles of the cell. In the medullary collecting duct, only the A type intercalated cells are present, the E11 antibody showed apical staining only.

In the kidney sections stained with the antiserum to the "kidney" isoform, the M_r 58,000 subunit, intense staining was observed only in the plasma membrane of the intercalated cells. No staining was observed in any part of the proximal tubule or in the thick ascending limb. Weak to moderate staining was observed in the apical membrane of the distal convoluted tubule, and weak staining was found in the apical membrane of the principal cells of the inner medullary collecting duct. In the cortical collecting duct, staining in the apical or basolateral membranes of A and B intercalated cells was similar to that observed with E11 antibody, suggesting that the "kidney" isoform of the subunit does not contain structural determinants for selective apical or basolateral polarization. The kidney isoform, the M_r 58,000 subunit, is amplified selectively in the intercalated cells but is expressed in other tissues. We have found that both the "kidney" and "brain" isoforms of the $M_r \sim 56{,}000$ subunits are expressed in three different cell lines, including the LLC-PK_1 and MDBK cell lines. Immunocytochemistry using the antibody to the carboxy terminal peptide of the "kidney" isoform (described above) of these cells revealed staining of a vacuolar membrane compartment which is currently unidentified. Thus, the current evidence supports the premise that the proton-transporting apparatus of the intercalated cell represents an amplification of a vacuolar compartment present in general in eukaryotic cells, and that has been modified to serve in physiologically regulated transcellular hydrogen ion secretion. The specialized compartment of intercalated cells may be analogous to the amplified endoplasmic reticulum-like compartment observed in the UT-1 cell line (Chin *et al.*, 1982) that overexpresses HMG-CoA reductase.

REGULATION OF THE VACUOLAR H^+ ATPase IN RENAL EPITHELIA

Net renal hydrogen ion excretion is regulated with precision, such that the amount of acid excreted by the kidney is equal to the rate of generation of nonvolatile metabolic protons. In principle, regulation could occur by three different mechanisms: (1) an increase in the kinetic activity of the H^+ ATPase without a change in the quantity or distribution,(2) a change in the distribution of the H^+ ATPase such that the enzyme is recruited to the plasma membrane from an intercellular pool, or retrieved from the plasma membrane into an intercellular compartment, and (3) an overall increase in the quantity of H^+ ATPase without a change in its kinetics or relative distribution between different membrane compartments. Several

lines of investigation from our laboratory have focused recently on these mechanisms for regulation.

Prior studies in kidney (McKinney and Davidson, 1987; Mays and Alpern, 1991) and in model proton transporting epithelia such as the turtle urinary bladder (Steinmetz, 1986) provided evidence that kinetic changes in the rate of ATPase activity on the rate of proton pump ATPase activity contribute to the overall regulation of transepithelial hydrogen ion transport. Our studies on the properties of the isolated enzyme showed that the ATPase activity is affected somewhat by pH changes in the normal range of intracellular pH (Gluck and Caldwell, 1987; Wang and Gluck, 1990; Wang and Gluck, manuscript in preparation), and this may account in part for kinetic changes. However, the H^+ ATPase is not sufficiently sensitive to pH conditions likely to be encountered in the cytosol for this to be a principal direct regulator of activity in the intact cell. The effects of concentrations of other cations and anions likely to be encountered in the cytosol also did not have a significant effect on activity (Gluck and Caldwell, 1987; Wang and Gluck, 1990; Wang and Gluck, manuscript in preparation). We therefore sought to examine whether the cytosol might contain additional regulatory factors, not isolated with the H^+ ATPase by the immunoaffinity purification procedure, that may interact with the ATPase under certain conditions.

We found that bovine kidney cytosol contained both inhibitory and activating factors that directly modified the ATPase activity of the immunoaffinity purified enzyme (Zhang and Gluck, 1992a,b). To assay for the activator and inhibitor, we quantified H^+ ATPase activity, immunoaffinity-purified from different kidney membrane fractions and assayed on monoclonal antibody beads, with and without added cytosolic fractions. The difference with and without cytosol was used to determine the degree of inhibition or activation.

The inhibitor was purified from cytosol by sequential ultracentrifugation, ammonium sulfate fractionation, acid precipitation, cation exchange chromatography, anion exchange chromatography, and HPLC anion exchange chromatography. We could not determine the precise degree of purification because the starting cytosol contained activator activity which masked the inhibitor activity. Starting from the anion exchange column fractionation step, a 118-fold purification was achieved. The isolated inhibitor was a heat-labile protein that appeared on SDS gels as a M_r 6300 polypeptide (Zhang and Gluck, 1992a). The active fraction had an approximate relative mass of 12,000 by gel filtration, suggesting that the active form of the inhibitor is a dimer. Inhibition was concentration dependent and saturable, with a Hill coefficient of 1.46, consistent with a requirement for dimerization. The mode of action was nonenzymatic, and not reversible when the beads are washed. The inhibitor attenuated immunoaffinity-purified H^+ ATPase activity and inhibited ATP-dependent proton transport in bovine kidney microsomal vesicles. The percent of ATPase activity inhibited increased above pH 7.2. Thus, under conditions representing an increase in cytosolic pH in the physiologic range, the inhibitor was more efficient in suppressing H^+ ATPase activity. The inhibitor was highly specific for the vacuolar H^+ ATPases with little or no effect on Na^+, K^+ or Ca^{2+} ATPases, but did have a modest inhibitory effect on the mitochondrial $F_0F_1H^+$ ATPase. The isolated inhibitor had equal effects on the isolated microsomal, brush border, and lysosomal vacuolar H^+ ATPases from kidney, and therefore has a potential role in controlling H^+ transport in several cellular compartments (Zhang and Gluck, 1992a).

The H^+ ATPase activator was partially purified from bovine kidney cytosol by sequential ultracentrifugation, ammonium sulfate precipitation, acid precipitation, QAE anion exchange chromatography, and aminohexylagarose chromatography, yielding a 75-fold purification with 27% recovery of activity (Zhang and Gluck, 1992b). The activator was heat-stable, retaining 77% of its activity when heated to 75°C for 10 min, but retaining only 4% of its activity when boiled for 10 min; it was sensitive to trypsin and therefore is likely to be a protein. The activator inhibited the immunoaffinity-purified H^+ ATPase, and thus, like the inhibitor, exerted a direct effect on the enzyme. Activation was concentration dependent and saturable, with a Hill coefficient of 1.0 (Zhang and Gluck, 1992b). 70–80% of the activating effect occurred within 1 min, and the time course for activation was not affected by the concentration of activator, hence it probably works by a nonenzymatic mechanism, likely by binding to the H^+ ATPase. The activation was reversible when the beads are washed, but the binding affinity of the activator was pH dependent. At pH values below 7.5, the activator washed off the beads with a slower time course than at pH values of 7.5 or above. This property may be physiologically important, as a drop in cytosolic pH could promote binding of the activator to the H^+ ATPase and stimulate proton extrusion from the cell. The activator was highly

specific for the vacuolar H^+ ATPase, with no effect on the Na^+, K^+ or Ca^{2+} ATPases, and with only a slight effect on the mitochondrial $F_0F_1H^+$ ATPase. Unlike the inhibitor, the activator had a much greater effect on the isolated brush border and microsomal H^+ ATPases than it did on the lysosomal H^+ ATPase. Consequently, it may have a selective function in controlling vacuolar H^+ ATPases residing on the plasma membrane involved in transcellular proton transport (Zhang and Gluck, 1992b).

Changes in the distribution of H^+ ATPase within the intercalated cell also play a role in the physiologic regulation of hydrogen ion secretion. To examine this property, we studied a model of chronic acid administration in rats (Bastani *et al.*, 1991). Rats given ammonium chloride in their drinking water developed an acidified blood pH (metabolic acidosis) within a day, and their urine pH decreased 0.5 to 0.7 pH units. Over a period of two weeks of chronic acid administration, the kidney showed physiologic adaptive changes, increasing hydrogen ion secretion such that the animals were restored back to normal acid–base status. We examined the role of the H^+ ATPase in the nephron in this adaptational response. We first examined whether any changes occurred in the quantity of vacuolar H^+ ATPase protein, or in the steady-state levels of mRNA for the enzyme. These studies were conducted using an immunoassay for the M_r 31,000 subunit and the *c*DNA clone for the rat 31,000 subunit. Kidneys from the rats were examined at five different time points over the two-week period of acid administration. No change in either the quantity of immunoreactive H^+ ATPase or in the levels of steady-state message for the M_r 31,000 subunit were detected over the two-week period (Bastani *et al.*, 1991). Since these results suggested that changes in the quantity of enzyme did not have a role in adaptation, we used anti-H^+ ATPase immunocytochemistry in the kidney to examine whether a change in the distribution of the enzyme occurred with acid loading. As means for quantitating changes in the distribution of the H^+ ATPase, we counted the percent of intercalated cells with plasma membrane staining. In control rats or rats subjected to chronic administration of bicarbonate, H^+ ATPase in intercalated cells of the collecting tubule from kidney medulla was distributed predominantly in intercellular vesicles in the cytoplasm. In contrast, in rats subjected to chronic acid administration over two weeks, H^+ ATPase in the majority of medullary intercalated cells was detected mostly in the plasma membrane. There was a time-dependent increase in recruitment of H^+ ATPase from cytoplasm to the plasma membrane over the two-week period (Bastani *et al.*, 1991). Changes in intercalated cells in the cortical collecting duct were more complex because of the multitude of different morphologic types of H^+ ATPase staining. Nevertheless, an increase in polarization to the apical membrane was also observed in the cortical intercalated cells. These results suggest that the principal means for adaptation in the kidney is not change in the overall quantity of the H^+ ATPase, but rather a redistribution from an intercellular pool of vesicles with H^+ ATPase to the plasma membrane. We have subsequently found that similar changes occur in other models for renal adaptation. These results do not exclude a role for changes in the kinetic properties of the H^+ ATPase, and the potential for a role of the activator and inhibitor in adaptation is currently under investigation.

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