The Na,K-ATPase comprises a catalytic α subunit and a glycosylated β subunit. Another membrane polypeptide, γ, first described by Forbush et al. (Forbush, B., III, Kaplan, J. H., and Hoffman, J. F. (1978) Biochemistry 17, 3667-3676) associates with α and β in purified kidney enzyme preparations. In this study, we have used a polyclonal anti-γ antisera to define the tissue specificity and topology of γ and to address the question of whether γ has a functional role. The trypsin sensitivity of the amino terminus of the γ subunit in intact right-side-out pig kidney microsomes has confirmed that it is a type I membrane protein with an extracellular amino terminus. Western blot analysis shows that γ subunit protein is present only in membranes from kidney tubules (rat, dog, pig) and not those from axolemma, heart, red blood cells, kidney glomeruli, cultured glomerular cells, α1-transfected HeLa cells, all derived from the same (rat) species, nor from three cultured cell lines derived from tubules of the kidney, namely NRK-52E (rat), LLC-PK1 (pig), or MDCK (dog). To gain insight into γ function, the effects of the anti-γ serum on the kinetic behavior of rat kidney sodium pumps was examined. The following evidence suggests that γ stabilizes E1 conformation(s) of the enzyme and that anti-γ counteracts this effect: (i) anti-γ inhibits Na,K-ATPase, and the inhibition increases at acidic pH under which condition the E2(K) → E1 phase of the reaction sequence becomes more rate-limiting, (ii) the oligomycin-stimulated increase in the level of phosphoenzyme was greater in the presence of anti-γ indicating that the antibody shifts the E1 ↔ E2P equilibria toward E2P, and (iii) when the Na+-ATPase reaction is assayed with the Na+ concentration reduced to levels (≤2 mM) which limit the rate of the E1 → E2P transition, anti-γ is stimulatory. These observations taken together with evidence that the pig γ subunit, which migrates as a doublet on polyacrylamide gels, is sensitive to digestion by trypsin, and that Rb+ ions partially protect it against this effect, indicate that the γ subunit is a tissue-specific regulator which shifts the steady-state equilibria toward E1. Accordingly, binding of anti-γ disrupts αγ-γ interactions and counteracts these modulatory effects of the γ subunit.

The Na,K-ATPase is a ubiquitous membrane protein complex that couples the exchange of three cytoplasmic sodium ions for two extracellular potassium ions to the hydrolysis of one molecule of ATP. The minimal functional enzyme consists of two subunits, α and β. Catalytic functions of the sodium pump including Na+- plus K+-activated ATP hydrolysis and the binding and occlusion of cations have been ascribed to the α subunit, whereas the role of β is mainly structural (for recent reviews, see Refs. 1–4). A third subunit, γ, was discovered nearly 20 years ago when an ~12-kDa peptide was specifically labeled by a photoactivatable derivative of ouabain, a cardiac glycoside which specifically binds to and inhibits the sodium pump (5). The γ subunit has since been found to co-immunoprecipitate with both the α and β subunits and the γ subunit of rat, mouse, cow, and sheep (6), and more recently, human (7) and Xenopus laevis (8) have been cloned. Although the γ subunit of the rat contains 58 residues with a predicted molecular size of 6.5 kDa (6), its mobility on SDS-PAGE1 corresponds to molecular masses as high as 12 kDa.

To date, little is known about the tissue distribution of the γ subunit, or whether, and in what way, γ has a role in Na,K-ATPase function. Hardwicke and Freytag (9) reported that detergent-mediated dissociation of the γ subunit did not affect ATPase activity, and Scheiner-Bobis and Farley (10) saw no difference in enzymatic or transport activities in yeast cells that had been transfected with all three subunits compared with only the α and β subunits. More recently, however, Beguin et al. (8) saw a small change in the apparent affinity for external cations in Xenopus oocytes transfected with the γ subunits of rat and Xenopus.

Although the γ subunit is not essential for function, several observations suggest that it may have an important role in sodium pump function. The high degree of similarity between γ subunits of different species (93%; see Ref. 6), the observation that γ subunit RNA is expressed in a tissue-specific fashion (6–8), and the recent reports of sequences homologous to γ (11–13) all point to an important function of the γ subunit. The sequence similarity between γ and a number of other small membrane peptides is particularly intriguing, as it raises the possibility that γ may belong to a family of ion transport modulators. In this paper, we have determined the topology of the γ subunit and determined its tissue distribution at the protein level. We also present evidence that the peptide may have a direct role in regulating Na,K-ATPase activity. A preliminary report of this work has been published in abstract form (14).

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Membrane Preparations**—Rat α1-transfected HeLa and MDCK cells were grown in Dulbecco’s modified Eagle’s medium

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MDCK, Madin-Darby canine kidney; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid.

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This paper is available on line at http://www.jbc.org
supplemented with 10% fetal bovine serum, LLC-PK cells, in a minimal essential medium containing 10% fetal bovine serum, normal rat kidney cells (NRK-52E), in Dulbecco’s modified Eagle’s medium containing 5% calf serum, and cultured glomerular epithelial cells, as described by Cybulsky et al. (15), and generously supplied by Dr. A. V. Cybulsky, McGill University. Membranes from HeLa cells, kidney medulla and axolemma, were prepared and stored as described previously (16) and from other cultured cells by the same procedure used for transfected HeLa cells. Rat glomeruli were isolated according to the procedure described by Kreisberg and Wilson (17) and membranes prepared from them by the same procedure as that used for membranes of kidney. Rat red blood cell membranes were prepared from sodium citrate-treated blood as described by Blostein (18).

Western Blots and Enzyme-linked Immunosorbent Assays (ELISA)—Unless otherwise indicated, intact pig kidney microsomes were purified on a metrizamide gradient, incubated (2 mg/ml) overnight at 0 °C in the presence of 100 μM MgCl₂ and 2 mM MgCl₂, and incubated first with tosylphenylalanyl chloromethyl ketone-treated trypsin (1:4 w/w) for 2 h at 37 °C, with trypsin inhibitor added (5:1 w/w) to stop the digestion. The microsomes were washed three times by centrifugation at 250,000 × g for 1 h and resuspension in 0.25 M sucrose, 0.03 M histidine, 1.0 mM Tris-EDTA, pH 7.5, with trypsin inhibitor (10 μg/ml) present in the first two washes. The final pellet was dissolved in 2% SDS, and the protein precipitated with methanol (4:1 v/v) overnight at −20 °C. Sequencing was carried out as described previously (22).

**Results**

γ subunit protein distribution in various tissues and cell lines expressing the rat α₁ enzyme was assessed using a polyclonal rabbit antisera raised against SDS-PAGE-purified rat γ subunit. Fig. 1A is a representative Western blot of seven tissues expressing the rat α₁ subunit. Aliquots comprising similar amounts of Na₉,K-ATPase activity were applied to each lane. Following transfer to polyvinylidene difluoride membranes, the upper half of the blot was probed with an anti-α₁ antisera, the lower half with the anti-γ antisera. The intensities of bands reactive with anti-α₁ antisera (upper lanes) indicate also that similar amounts of the catalytic α subunit from each tissue were analyzed. Since the specific activity of the red blood cell enzyme was lower than that of other tissues and to ensure that equivalent activities were loaded on the gel, an excess of red blood cell membranes were analyzed. Accordingly, the anomalous migration of the red blood cell α₁ band is likely due to protein overloading. Since γ subunit mRNA is relatively abundant in human pancreas as well as kidney (7), it would have been interesting to analyze pancreas for the presence of γ subunit protein. However, the specific activity of Na₉,K-ATPase in this tissue is extremely low (23), and in our preparation, it was less than 5% that of red blood cells, precluding meaningful analysis by Western blotting. Bands reac-
tive with anti-γ antiserum and appearing as doublets as reported previously (6) were detected only in tubules of the kidney; none could be visualized in axolemma, heart, kidney glomerulus, cultured glomerular cells, red blood cells, or rat α₁-transfected HeLa cells, even following longer exposure (not shown). Furthermore, as indicated in Fig. 1B, although the γ subunit is detected in kidney tubules of rat, pig, and dog, none could be detected in cultured epithelial cells of tubular origin from the same species (NRK-52E, LLC-PK, MDCK).

Membrane Topology of γ and the Side Specificity of Its Interaction with Anti-γ Antibodies—The transmembrane orientation of the putative single transmembrane γ subunit chain was determined by treating intact right-side-out pig kidney microsomes with trypsin and then analyzing the products following SDS-PAGE and amino-terminal sequencing. As shown in Fig. 2B, trypsinization led to the appearance of two distinct bands, a 8-kDa band and a 16-kDa band. Either the cleavage results in loss of the amino-terminal sequence of the 8-kDa fragment has been previously reported (22) and as shown in Fig. 2B, the yields (pmol per cycle) of residues obtained in the sequences are as follows. For the 16-kDa band: A, 36.6; B, 3; G, 8.2; K, 7.5; A, 10.7; K, 6; E, 3.3; E, 2.5; G, 10.8; and for the 8-kDa band: G, 9; D, 2.5; V, 3.5; D, 1.4; P, 2.8; F, 4.9; Y, 3.1; Y, 3.3; D, 1; Y, 1.7.

As described below, when intact microsomes are digested with trypsin, the higher molecular species of the γ subunit doublet disappears. Either the cleavage results in loss of the entire polypeptide, presumably by a process of internalization and degradation, or more likely, the product is of lower molecular size and becomes indistinguishable from the lower band. The evidence for this is that the epitope(s) recognized by the antiserum requires permeabilization of the microsomes. Thus, the reactivities with anti-γ antiserum of microsomes which were either untreated or permeabilized by SDS in the presence of bovine serum albumin according to the method of Forbush (20) were compared in an ELISA. As shown in Fig. 3, the relative absorbance (A₄₀₅) for intact compared with permeabilized microsomes was similar with either anti-γ antiserum or an anti-α antiserum (antiserum 754; see Ref. 16) which is known to react with the amino terminus of the α subunit (15.1 ± 1.3% and 11.7 ± 0.4% for anti-γ and anti-α, respectively). In addition, the data are consistent with experiments showing that the unpermeabilized microsomal Na,K-ATPase activity (presumably due to the presence of fragmented membranes) represents 15.4 ± 1.4% of the total activity (not shown). That the increase in antibody binding is secondary to different amounts of protein in wells containing intact versus SDS-treated microsomes is unlikely since: (i) antibody reactivity with intact and SDS-treated microsomes was similar using an antiserum that recognizes an epitope on the extracellular side, namely amino acids 290 to 302 of the β₁ subunit (antiserum 940; see Ref. 25), and (ii) A₄₀₅ values obtained with equivalent dilutions of non-immune serum are not significantly different between permeabilized and intact microsomes (see legend to Fig. 3).

Effect of Anti-γ Antiserum on Na,K-ATPase Activity—Based on evidence of a close association of the γ subunit with the Na,K-ATPase α-β dimer (5, 6), experiments were carried out to determine whether anti-γ antibodies can perturb the association in a manner that would have functional ramifications. Accordingly, we tested the effect of anti-γ antiserum on the
microsomes, 0.20 bars were coated with SDS-permeabilized (solid bars) or intact (open bars) rat kidney microsomes and analyzed using either anti-γ subunit antisera, anti-α-amino terminus antisera, or anti-β₁-carboxyl terminus antisera as described under "Experimental Procedures." Values shown are expressed as percentages, each representing the difference in A₄₀₅ obtained with immune and non-immune rabbit serum. With immune serum, A₄₀₅ values for permeabilized microsomes were 1.39 ± 0.06, 1.13 ± 0.01, and 1.21 ± 0.08, with γ, α, and β-specific antisera, respectively. With non-immune serum at dilutions equivalent to those with the respective antisera, values for permeabilized microsomes were 0.19 ± 0.01, 0.17 ± 0.01, and 0.51 ± 0.01, and for intact microsomes, 0.20 ± 0.02, 0.17 ± 0.01, and 0.53 ± 0.01.

following: (i) Na,K-ATPase activity under V₀ max conditions as a function of pH, (ii) Na-ATPase activity with sufficient Na⁺ to substitute for K⁺ at extracellular sites, as well as with Na⁺ reduced to bind at only high affinity cytoplasmic sites, and (iii) the level of phosphoenzyme measured at 0 °C in the absence of K⁺, without and with oligomycin present.

As shown in Fig. 4A, pretreatment of permeabilized kidney tubule microsomes with anti-γ antisera results in partial (31.2% ± 3.2%) inhibition of Na,K-ATPase activity. The anti-serum had no effect on non-Na,K-ATPase (baseline) activity, and increasing its concentration (to 1:25 v:v) had no further effect (not shown). In addition, as shown in Fig. 4B, the inhibition was not observed in cells which lack γ as shown in experiments with transfected HeLa cells, axolemma, and red blood cells. Fig. 4A also shows that a significant decrease in the maximal level of phosphoenzyme (EP max) measured in the absence of K⁺ with oligomycin present to trap the enzyme in the E₁P conformation could not be detected. In the absence of oligomycin, antibody treatment caused a significant decrease (32.1% ± 7.6%) in phosphoenzyme. The decrease in ratio of V₀ max/EP max indicates that antibody binding decreases the catalytic turnover of the enzyme. The observation (Fig. 4A) that oligomycin increases the level of phosphoenzyme to a greater extent in the anti-γ compared with the control non-immune serum-treated enzyme also suggests that anti-γ alters the steady-state equilibrium between dephospho- and phosphoenzyme in favor of the latter. In support of this conclusion is the observation (Fig. 5) that anti-γ inhibition of Na-ATPase is reduced when the Na⁺ concentration is decreased to levels (<2 mM) that are considered to limit the rate of the E₁P → E₂P transition (26). In fact, at the lowest concentrations used (<0.6 mM), a moderate but significant activation is observed. Comparison of Figs. 5 and 4A also shows that at high Na⁺ concentration, Na-ATPase is less inhibited than Na,K-ATPase.

To gain further insight into the part of the reaction cycle affected by anti-γ treatment, we tested the effect of antisera pretreatment on Na,K-ATPase activity measured at acidic versus alkaline pH. According to Forbush and Klodos (27), the pH dependence of Na,K-ATPase is limited partly by the rate of K⁺ deocclusion at acidic pH, by the rate of the E₁P → E₂P transi-

![Fig. 3. Sidedness of antibody reactivity with γ subunit, ELISA plates were coated with SDS-permeabilized (solid bars) or intact (open bars) rat kidney microsomes and analyzed using either anti-γ subunit antisera, anti-α-amino terminus antisera, or anti-β₁-carboxyl terminus antisera as described under "Experimental Procedures." Values shown are expressed as percentages, each representing the difference in A₄₀₅ obtained with immune and non-immune rabbit serum. With immune serum, A₄₀₅ values for permeabilized microsomes were 1.39 ± 0.06, 1.13 ± 0.01, and 1.21 ± 0.08, with γ, α, and β-specific antisera, respectively. With non-immune serum at dilutions equivalent to those with the respective antisera, values for permeabilized microsomes were 0.19 ± 0.01, 0.17 ± 0.01, and 0.51 ± 0.01, and for intact microsomes, 0.20 ± 0.02, 0.17 ± 0.01, and 0.53 ± 0.01.](Image 62x573 to 294x729)

![Fig. 4. Effect of anti-γ antibody binding on Na,K-ATPase and phosphoenzyme. Saponin-permeabilized membranes were pretreated with 0.01 volume of either non-immune serum (control; solid bars) or anti-γ antisera (open bars), and Na,K-ATPase activity or phosphoenzyme levels were measured as described under "Experimental Procedures." Results are averages ± S.D. of at least 3 separate experiments. A, Na,K-ATPase and phosphoenzyme without and with oligomycin as indicated. Fold increases in phosphoenzyme effected by oligomycin were 1.7 in non-immune serum-treated samples and 2.4 in anti-γ-treated samples; B, Na,K-ATPase of kidney, axolemma, α₁-transfected HeLa, and red blood cells. Differences between non-immune- (control) and anti-γ-treated kidney membranes are significant. p < 0.01 (*) using the Student's t test.](Image 314x379 to 557x729)
ences between pH values are statistically significant. ATPase assays were carried out at pH 6.2, 7.4, or 8.9 using 30 mM Na,K-ATPase. Rat kidney microsomes were pretreated at pH 7.4 with non-immune serum or anti-

gamma antibody binding on Na-ATPase as a function of Na\(^+\) concentration. Na-ATPase measurements were carried out at 1 \(\mu\)M ATP and varying Na\(^+\) concentrations on non-immune serum-treated (control) and anti-gamma serum-treated membranes as described under "Experimental Procedures." Concentrations of Na\(^+\) take into account the amount of Na\(^+\) (150 mM) present in serum. Results are averages ± S.D. of at least three separate experiments. Differences between non-immune (control) and anti-gamma-treated membranes are significant. \(p < 0.01 (*)\) and \(p < 0.02 (**)\) using the Student's \(t\) test.

preincubated in the presence of Rb\(^+\) were partially protected against digestion of the upper band.

DISCUSSION

Since its discovery nearly 20 years ago, the tissue distribution and function of the gamma subunit of the Na,K-ATPase have remained unknown. That it has an important role in enzyme function is an attractive possibility since its sequence is so highly conserved between species (6). Until recently, there was no clear evidence to support this notion. In this study, we show, for the first time, that the gamma chain is not ubiquitous, at least in the rat. Moreover, among the tissues and cell lines examined, it is apparent in only kidney tubules. In this tissue, we have defined unequivocally its topology and obtained evidence of its involvement in Na,K-ATPase activity, consistent with the recent findings of Beuguin et al. (8).

Initially, the sequence of the gamma subunit could not be deter-
mined since its amino terminus is apparently blocked, preventing the use of the Edman degradation method of sequencing (24, 29). In 1987, Collins and Leszyk (29) succeeded in sequencing a tryptic fragment of the sheep gamma subunit, and more recently, Shainskaya and Karlsh (22) determined the amino-terminal sequence of an 8.1-kDa tryptic fragment which we now know is identical to part of the human gamma subunit (7) that follows a trypsin cleavage site (Fig. 2; see also Table III, band c of Ref. 22). The sequence homology between the tryptic fragment and human gamma subunit (Fig. 2B) as well as the removal of the blocked NH\(_2\) terminus after trypsin treatment of intact right-side-out microsomes shows that the amino terminus of the gamma subunit is on the extracellular side of the membrane. The same orientation was reported recently (8) and is not unexpected, since it is consistent with studies showing that positive residues of transmembrane proteins are on the cytoplasmic side (30). Phospholemman, another small membrane protein with homology to the gamma subunit, has also been shown to have this topology (11).

Although the close association of gamma with the alpha and beta subunits of the purified kidney Na,K-ATPase and its presence in a roughly 1:1 stoichiometry (8, 9, 31) with these subunits has led to the general belief that gamma is an obligatory component of the Na,K-ATPase, its function appears to be more subtle in nature. The present study not only confirms the earlier conclusion (8, 10) that the gamma subunit is not a necessary subunit, but shows that it is not widely distributed and that its expression is not maintained during culture of renal tubular cells.

It could be argued that the gamma subunit is, in fact, present in all tissues and that Western blotting is too insensitive or that the protein is digested by proteases during membrane preparation. These possibilities are unlikely for the following reasons. Gamma subunit protein remained undetected when (i) gels were overloaded and Western blots were overexposed, and (ii) intact cells (HeLa, MDCK, LLC-PK) were dissolved in sample buffer and applied directly (not shown). In addition, the measured alpha-gamma molar ratio of ~1 (8, 9, 31) indicates that if gamma is present in other tissues, its molar ratio in these tissues would be a small fraction of the ~1:1 ratio of kidney.

The tissue specificity of gamma subunit protein expression is consistent with the recent reports that gamma mRNA is expressed only in the kidney and pancreas of human (7) and predominantly in
The γ Subunit Is a Tissue-specific Modulator of Na,K-ATPase

32633

The kidney of *Xenopus* (8). On the other hand, Mercer et al. (6) reported the presence in several rat tissues of a 1.5-kilobase γ-cDNA-reactive mRNA species, with an additional, smaller (0.8-kilobase) species predominant in the kidney, suggesting either that protein expression is translationally regulated in the rat, or that only the smaller mRNA leads to the mature protein.

Physiologically, this tissue distribution of γ has interesting implications. While in most tissues the α1 isoform of the Na,K-ATPase has a housekeeping function, its role in the kidney is also specialized. In the nephron, the sodium pump is involved in Na\(^+\) reabsorption, which in turn regulates reabsorption of solutes such as glucose and amino acids (extensively reviewed in Ref. 32). The kidney must therefore have multiple ways of regulating sodium pump activity, which implies kidney-specific modulators of Na,K-ATPase. Although there is extensive literature on the subject and the effects of some of these modulators is partly understood (reviewed in Ref. 33), it is also clear that many pathways of regulation have yet to be discovered and/or defined. The γ subunit may be a candidate for such regulation, as evidenced by its high degree of homology with several other membrane peptides, namely PLM (phospholemman), CHIF (channel inducing factor), and Mat-8 (mammary tumor, 8 kDa). These proteins have been shown to induce ion currents across oocyte membranes (12, 13, 34) and are believed to be members of a family of ion channels or ion channel regulators. In light of this, it seemed reasonable to expect that the γ subunit might have a role in regulating the Na,K-ATPase in the kidney. Evidence in support of this notion has been obtained in studies of the effects of anti-γ antisera on kinetic behavior.

There are several points of evidence supporting the conclusion that γ shifts the equilibrium between E\(_1\) and E\(_2\) in favor of E\(_2\). For the former such that anti-γ counteracts this effect. One is that anti-γ displaces E\(_2\) (K\(^+\)) ↔ E\(_1\) in favor of E\(_2\) (K\(^+\)) since inhibition of Na,K-ATPase activity by anti-γ is greater at acidic pH under which condition the E\(_2\) (K\(^+\)) ↔ E\(_1\) reaction sequence becomes more rate-limiting (27). Second, the oligomycin experiment suggests that the E\(_2\) (K\(^+\)) ↔ E\(_1\) reaction sequence becomes more rate-limiting (27). Fourth, when the reaction is carried out in the absence of K\(^+\) (Na,ATPase activity), inhibition is observed only with sufficient Na\(^+\) ions to act as K\(^+\) congeners at extracellular sites. As the Na\(^+\) concentration is reduced so that mainly cytoplasmic sites are occupied, inhibition is no longer observed; with Na\(^+\) further reduced to levels (<2 mM) which limit the E\(_2\) ↔ ↔ E\(_1\) transition (26), anti-γ becomes stimulatory. Relevant to the notion that γ alters the poise in E\(_2\) ↔ E\(_1\) equilibria is in favor of E\(_2\) is a recent experiment (Table II of Ref. 35) showing that, under the same conditions as those used in this study, the oligomycin-stimulated increase in steady-state level of phosphoenzyme in the rat α\(_1\) enzyme transfected into HeLa cells (which lack γ) is 2.3-fold, which is similar to that of anti-γ-treated kidney enzyme (see legend to Fig. 4).

The aforementioned effect of γ on the E\(_1\) ↔ → E\(_2\) process may be relevant to the γ-mediated changes in voltage dependence of apparent affinity for extracellular K\(^+\) reported recently in experiments with *Xenopus* (8) since (i) E\(_1\) → E\(_2\) is the major voltage-sensitive phase of the reaction cycle (36), and (ii) an increase in rate of E\(_2\) formation would alter the apparent affinity for K\(^+\)-activated dephosphorylation.

It should be noted also that the effects of the anti-γ antisera are highly specific. First, the inhibitory effect was not observed with membranes isolated from tissues where γ protein is not expressed (Fig. 1A). Second, an effect on V\(_{\text{max}}\) was not observed with anti-α\(_1\)-amino-terminal serum. Third, a dose-response curve (not shown) showed that inhibition increases with antiseraum concentration and reaches a maximum of about 30% at 1:100, consistent with saturation of the binding sites with antibody rather than a nonspecific effect.

The role of the γ chain may be more complex than evident from studies with anti-γ. In particular, anti-γ inhibition of Na,K-ATPase at high sodium concentration has two possible explanations: (i) anti-γ acts synergistically with cytoplasmic Na\(^+\) to stabilize the (Na\(_2\)E\(_2\))P-form of the enzyme since, as shown by Appel et al. (37), Na-ATPase becomes inhibited by Na\(^+\) acting at high concentration on the cytoplasmic side to stabilize this E\(_2\) form or (ii) anti-γ inhibits the E\(_2\)P → E\(_2\) step which is activated by Na\(^+\) acting at high concentration as a congener of K\(^+\) at the extracellular side. If the latter holds true, an effect of γ, other than stabilization of E\(_1\) state(s), is invoked and may cast doubt on the physiological significance of the kinetic effects. The observation (38) that the phospho-enzyme form of the kidney enzyme is predominantly in the K\(^+\)-sensitive (E\(_2\)P) conformation compared with the red blood cell enzyme also appears to contradict an E\(_1\)-stabilizing role of the γ subunit. However, those studies were carried out with enzyme from two different species (human red blood cells *versus* guinea pig kidney) and, more importantly, were performed at 0 °C. Therefore it remains to be determined whether other as yet unknown tissue-specific regulatory functions of γ are more important. At the very least, however, our results suggest that the γ subunit is in very close proximity to the α subunit and intimately involved with the catalytic function of the enzyme.

There are precedents for effects of specific antisera on membrane transport systems. Well-documented examples are effects of specific antibodies on cation transport of genetically low-K\(^+\) (LK) red blood cells of sheep and goats (reviewed in Ref. 39). In these cells, one antigen, L\(_i\), renders the Na,K-ATPase susceptible to non-competitive inhibition by intracellular K\(^+\), while another, the L\(_o\) antigen, stimulates the KCl cotransport of LK red cells. The evidence for the effects of these antigens rests largely on the effects of isimmune antisera. Thus, antisera to these antigens have been shown to either stimulate Na,K-ATPase activity (anti-L\(_i\)) or inhibit K\(^+\)/Cl\(^-\) cotransport (anti-L\(_o\)), presumably through the disruption of L antigen: transporter interactions. Similarly, the effects observed using our anti-γ serum may be the result of disruption of αβ-γ interactions secondary to antibody binding.

One of the more puzzling aspects of the γ subunit is that it migrates as a doublet on SDS-PAGE (6). This doublet is not a consequence of multiple isoforms or differential splicing, since it is formed in rabbit reticulocyte lysate in the presence of a single mRNA species (6). As in the kidney of rat, dog, and pig (Fig. 1B), two polypeptide species are also observed in amphibian (*Xenopus laevis*) Na,K-ATPase (8). It seems fortuitous, however, that in *Xenopus* the two species are products of translation initiated at the two initiator methionines identified in the cDNA; in the rat, a single initiator methionine is present (6), leaving open the possibility of post-translational modification. Interestingly, the upper band of γ is trypsin-sensitive while the lower is not (Fig. 7). Complete trypsin digestion of the upper band requires Mg\(^2+\), but a partial decrease in immunological reactivity is also seen in the absence of the cation. Since both bands are the products of a single mRNA message (6), this differential trypsin effect may reflect a post-translational modification at or near the cleavage site. Since trypsin cleavage occurs at lysine residues which can be hydroxylated, acetylated, or ribosylated post-translationally (40), this is an attractive possibility. Supporting this conclusion is the observation that the difference in mobility is retained after the bands are
excised and eluted from a polyacrylamide gel and re-electrophoresed (19). Different modifications of the amino terminus, whether through proteolysis or covalent alteration, seem unlikely since the amino termini of both bands are similarly blocked to sequencing. Covalent modification of the γ subunit could also explain the discrepancy between the observed migration of the protein on SDS-PAGE and the predicted molecular weight, and why the bands migrate at different rates depending on the percent acrylamide in the gel. Since the tryptic fragment resulting from cleavage of the γ subunit (Fig. 2A) migrates at a molecular mass of 8 kDa as discussed above, it is expected to be indistinguishable from the intact lower band, which migrates at 7.9 kDa. Although sometimes difficult to discern and quantify, an increase in antiserum reactivity of the lower band was observed in some experiments (not shown), particularly when rat microsomes were used. Fig. 7 also shows that membranes incubated in the presence of 10 mM Rb⁺ (a congener of K⁺) are protected from the Mg²⁺-independent hydrolysis. This result is reminiscent of the Rb⁺-imported protection from tryptic digestion of both the α (28) and β (24) subunits and presumably indicates that the γ subunit is protected from tryptic digestion when the pump is in the E₂(K) state and therefore involved in occlusion of K⁺. Relevant to this conclusion is the observation (19) that the γ subunit is an integral part of the complex of peptide fragments found in so-called 19-kDa membranes.

Taken together, these results indicate that γ may have a role in regulating the renal Na,K-ATPase, possibly by stabilizing E₁ conformations. With γ-specific antisera, it is shown for the first time that the γ subunit protein is not ubiquitous. The absence of the γ subunit in most tissues and its similarity to putative ion channel modulators show that its presence is not required for normal function of the Na,K-ATPase, but rather that it may act as a modulator of activity. Furthermore, the protective effect of Rb⁺ ions on tryptic digestion of the γ subunit suggests that γ may be involved in K⁺ occlusion/deocclusion, which is intimately linked to and dependent upon the E₂ ↔ E₁ conformational equilibrium.

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2 S. J. D. Karlish, unpublished observation.