Isolation and Immunolocalization of a Rat Renal Cortical Membrane Urate Transporter*

Barbara A. Knorr, Michael S. Lipkowitz, Barry J. Potter†, Sandra K. Masur‡, and Ruth G. Abramson

From the Renal Division, Department of Medicine and †Department of Ophthalmology, Mount Sinai School of Medicine, New York, New York 10029 and the ‡Department of Physiology, Louisiana State University Medical Center, New Orleans, Louisiana 70112-1393

Two modalities of urate transport have been reported in rat kidney, a urate/anion exchanger and a potential sensitive, uricase-like uniporter. As an initial attempt to isolate and characterize the responsible transport protein(s), rat renal cortical membranes were harvested, solubilized, and subjected to affinity chromatography with urate or xanthine as the affinity ligand. Pig liver uricase was purified with the same system, and the enzymatically active protein was used to generate polyclonal antibodies in rabbit. Silver stain of SDS-polyacrylamide gel electrophoresis gels of the eluted fraction containing the affinity-purified renal membrane protein(s) demonstrated bands at 25, 32, 36, and 41 kDa. On Western blot, two of these bands (32 and 36 kDa) were immunoreactive to the polyclonal antibody to pig liver uricase. In 6 of 10 studies, the affinity-purified renal membrane protein(s) also oxidized urate. Anti-pig liver uricase produced a selective and dose-dependent inhibition of the urate-like urate uniporter in renal membrane vesicles, but did not affect the urate/anion exchanger or the sodium-dependent glucose transporter. Immunocytochemical studies of rat renal cortex with the same antibody indicated that the immunoreactivity was localized to proximal tubules. These studies demonstrate that the renal cortical plasma membranes contain urate-binding proteins, which have some functional and immunological homology to the hepatic peroxisomal core protein, uricase. Within the renal cortex, these proteins are localized to proximal tubules, the site of urate transport. Since the antibody that reacts with the affinity-purified urate-binding proteins on Western blot selectively inhibits urate transport in intact membrane vesicles, it is concluded that at least one of the affinity-purified urate-binding proteins is a uricase-like urate transporter.

It is now generally accepted that urate is bidirectionally transported in the renal proximal tubule (reviewed in Ref. 1). Since little, if any, net urate flux has been detected at nephron sites distal to the pars recta of the proximal tubule (2, 3), it has been concluded that the vast majority of urate flux occurs within the renal cortex (1). As a consequence it has been possible to utilize brush border and basolateral membrane vesicles prepared from the renal cortex to physiologically characterize the transport mechanisms that are responsible for urate reabsorption and secretion. Two modalities of urate transport have been reported in rat renal proximal tubule brush-border and basolateral membranes. One, an anion exchanger, transports urate in exchange for a variety of organic and inorganic anions (4, 5). The second, described by our laboratory, is a voltage-sensitive urate uniporter with a number of characteristics similar to those of the hepatic peroxisomal enzyme uricase (6, 7); both the transporter and uricase are Cu²⁺-dependent, have virtually the same affinity for urate, oxidize urate to allantoin, and are inhibited by oxonate, a specific inhibitor of the oxidative activity of uricase (8). Although uricase that resides in pig liver peroxisomes functions solely as an oxidative enzyme (9–11), this protein was found to be capable of functioning as a saturable urate transporter when inserted in a lipid bilayer (12). Oxonate, which inhibited urate transport in the renal membranes, also inhibited transport in uricase-containing proteoliposomes (12). While these studies in proteoliposomes reinforced the hypothesis that the renal urate uniporter has some homology to hepatic uricase, to date uricase has not been identified in the rat kidney; in rat, uricase has only been localized to liver peroxisomes (9, 11, 13–17).

As a first approach to purifying and characterizing the proteins responsible for urate transport in renal cortical plasma membranes, an affinity chromatography system was used to isolate the urate transporter(s). Evidence has been obtained indicating that rat renal cortical plasma membranes contain urate-binding protein(s), which have some homology to hepatic peroxisomal uricase. Functional homology was revealed by uricase-like enzymatic activity of the purified renal membrane protein. Immunologic homology was demonstrated by immunoreactivity of the purified renal membrane protein to an antibody to hepatic uricase. This antibody also inhibited urate transport in intact membrane vesicles, suggesting that the immunoreactive purified proteins represent components of the uricase-like urate transporter. On the basis of immunocytochemical labeling with the antibody to hepatic uricase, this putative urate transporter has been localized in the renal cortex to proximal tubules, the site of urate transport.

MATERIALS AND METHODS

Isolation of Plasma Membranes—Renal cortical membrane vesicles were prepared with minor modifications of methods previously described (6, 18). In brief, in each experiment 45–50 male rats (Charles River Breeding Laboratory, Wilmington, MA) were anesthetized with an intraperitoneal injection of pentobarbital (45 mg/kg body weight). The kidneys were harvested, placed in ice-cold 250 mM sucrose, 10 mM Tris buffered to pH 7.4 with HCl (ST buffer), decapsulated, and slices of renal cortices were obtained. The cortical slices were weighed (79.2±3.7 g, wet weights), placed in fresh ST buffer (2 mM/g tissue) containing 0.2 mM phenylmethylsulfonyl fluoride, finely minced with scissors, and ho-
mogenized. The homogenate was then subjected to differential centrifugation using a Sorvall model RC-5B refrigerated centrifuge with a SS-34 rotor (6, 19). The final membrane pellet was suspended in 280 mM sucrose, 8.5 mM Tris acetate buffered to pH 7.4 with NaOH in a volume estimated to yield a protein concentration of 8–10 mg/ml. Brush border and basolateral membranes were subsequently separated and isolated by free flow electrophoresis (model FF5, Garching Instruments, Bender and Hoeben, Munich, Germany) using methods identical to those previously described (6, 19). The electrophoretic fractions containing brush border and basolateral membranes were identified on the basis of the mannitol-phosphate buffer (determined with a Sigma test kit) and potassium-activated para-nitrophenylphosphate (20), respectively. Thereafter, the purified brush border and basolateral membrane fractions were recombined, diluted in an equal volume of 100 mM mannitol, 20 mM NaN3, 10 mM PEPES buffered with Tris to pH 7.4, homogenized, and centrifuged at 35,000 g for 30 min. The resulting pellet was solubilized in a volume of 0.1 ml, buffered at 0.2%, CHAPS. 1% glyceral at pH 9.0 estimated to yield a detergent-protein ratio of approximately 1:2 w/w. Following 30 min on ice the solubilized membranes were centrifuged at 32,000 g for 30 min. The supernatant was aspirated and subsequently subjected to affinity chromatography.

Pilot studies demonstrated that identical results were obtained when either solubilized, electrophoretically purified membrane fractions or less pure, combined cortical membrane suspensions (pre-electrophoresis) were applied to the affinity gel. Thus, in subsequent studies the membrane suspensions were not subjected to free-flow electrophoresis. In lieu of the mannitol-phosphate buffer, the 10,000 g supernatant was dialyzed against the same buffer suspension to yield approximately 70% Cu++/mg protein. After 30 min on ice, the membranes were centrifuged at 32,000 g for 30 min, the supernatant was discarded, and the pellet was solubilized and subsequently handled in a manner identical to that detailed above. In all studies the protein concentrations of the membrane suspensions and solubilized membranes were determined by the method of Lowry et al. (21) using bovine serum albumin as the standard.

Affinity Chromatography—An affinity gel, in which the ligand urease was linked to epoxy-activated Sepharose 6B (Pharmacia LKB Biotechnology Inc.), was prepared by a modification of the method of Batista-Vieira et al. (19). The affinity gel was linked to epoxy-activated Sepharose (Sigma) was substituted for the urate-Sepharose. All other reagents were purchased from Sigma Chemical Company (St. Louis, MO). Medium K+ was 10 mM HEPES buffered with Tris to pH 7.4 or 204 mM NaCl, 10 mEq Na+/mM, Amphersam, CA) was added to 10 mM HEPES, 10 mEq Na+/mM, 10 mEq MgSO4, pH 7.5 or 204 mM NaCl, 10 mM Tris-Hepes, pH 7.5. In a separate experiment the uptake of [6-3H]glucose (6.4 pCi/mM; DuPont, NEN) was measured by separating urate and allantoin with a previously described column chromatographic technique (28).

Transport Studies—Membrane vesicles were isolated and treated with 0.1 mM EDTA as described earlier. Uptake was calculated from the disintegrations per minute of each filter was measured in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). Uptake was calculated from the disintegrations per minute of each filter was measured in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). Uptake was calculated from the disintegrations per minute of each filter was measured in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). Uptake was calculated from the disintegrations per minute of each filter was measured in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

The abbreviations used are: CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DIDS, 4,4'diisothiocyanostilbene-2,2'-disulfonic acid.
Kidney were then dehydrated, cleared, and embedded in paraffin. Sections (3 μm) were cut and placed on polylysine-coated slides. After deparaffinization, immunoperoxidase staining was performed using a Super Sensitive Multilink Immunodetection System (BioGenex, San Ramon, CA) in which the antibody is localized via a biotin-streptavidin hors eradish peroxidase complex using diaminobenzidine as substrate.

Unbound protein were too numerous to count. In contrast, silane stain of the protein fraction that was specifically eluted from the affinity gel.

Immunocytochemistry—Rat kidneys were perfused in situ with normal saline followed by 4% paraformaldehyde. Slices (2–3 mm thick) of kidney were then dehydrated, cleared, and embedded in paraffin. Sections (3 μm) were cut and placed on polylysine-coated slides. After deparaffinization, immunoperoxidase staining was performed using a Super Sensitive Multilink Immunodetection System (BioGenex, San Ramon, CA) in which the antibody is localized via a biotin-streptavidin horseradish peroxidase complex using diaminobenzidine as substrate. Thereafter the tissue was counterstained with Harris hematoxylin. The primary antibody was a 1/6400 dilution of the IgG fraction of anti-porcine uricase or undiluted antigen-purified antibody. A 1/6400 dilution of non-immune IgG or undiluted antigen-purified preimmune serum served as the control. The tissue was examined with a Zeiss Axiomat using light and differential interference contrast microscopy.

RESULTS

Purification of Urate-binding Proteins—The extent of purification of renal urate-binding proteins that was achieved with the above described affinity chromatography system was evaluated by SDS-PAGE (Fig. 1). As anticipated, the number of protein bands in both the sample of solubilized membranes that was applied to the affinity gel and in the fraction containing unbound protein were too numerous to count. In contrast, silver stain of the protein fraction that was specifically eluted with hypoxanthine revealed two to four protein bands. In virtually all studies bands were seen at 25 and 36 kDa; less frequently bands were detected at 32 and 41 kDa. Protein bands of comparable size were obtained when xanthine was substituted for urate as the affinity ligand. SDS-PAGE of hepatic uricase that was purified with the same affinity system yielded a protein band that approximated 33 kDa. Despite the difference in size in the purified renal proteins and hepatic uricase, these proteins bond to the same ligands and eluted with the same substrate. Additional studies were therefore performed to assess whether these proteins displayed any immunologic and/or functional homology.

Immunoreactivity of Urate-binding Proteins—Western blot analysis with rabbit anti-porcine hepatic uricase revealed that the solubilized renal membranes and the affinity-purified protein were highly reactive to the antibody (Fig. 2), but non-reactive to preimmune serum and non-immune rabbit IgG (not depicted). Identical immunoreactive bands were detected independent of whether immune serum, the purified IgG fraction of immune serum, or an antigen-purified antibody was used. Although innumerable protein bands were evident on the silver stain of solubilized renal membranes (Fig. 1), only three bands at 27, 32, and 36 kDa were immunoreactive (the 36-kDa band may represent a doublet) (Fig. 2). The sizes of these immunoreactive bands were indistinguishable before and after treatment with peptide-N-glycosidase F, suggesting that the different size bands do not reflect different degrees of glycosylation of the same protein(s). Two of the immunoreactive bands (32 and 36 kDa) that were present in solubilized membranes were detected in the purified protein fraction (Fig. 2); the 27-kDa immunoreactive band that was seen in solubilized membranes was not detected in the purified protein fraction. Since the smallest of the immunoreactive membrane proteins was not detected in the purified protein fraction, it may have been degraded or denatured after solubilization, obviating purification via the affinity gels employed.

Functional Assessment of Urate-binding Proteins—The uricase-like activity of the purified proteins and solubilized membranes was assessed by measuring the ability of these proteins to oxidize urate. In 6 of 10 experiments, the fraction containing the purified protein oxidized 8.0 ± 2.6% of 2 μM \([14C]urate\); activity was not detected in the remaining 4 studies. In the same six experiments in which the purified proteins oxidized urate, the solubilized membranes oxidized a similar percent (9.25 ± 2.9%). It is of note that the assays with solubilized membranes contained 0.74 ± 0.18 mg of protein, yielding a specific activity of 161 ± 64 pmol of urate oxidized/mg of protein/h, whereas assays with the purified protein contained an amount of protein that was below the lower limit (1.0 μg) of detection with the Pierce Micro BSA protein assay. Although the specific activity could not be determined, the finding that a comparable amount of urate was oxidized by such disparate amounts of purified protein and solubilized membrane protein indicates that the specific activity of the purified urate-binding protein was markedly enriched (at least 740-fold) relative to that in the membrane from which it derived. Since uricase functions as an enzyme that oxidizes urate, the finding that the purified renal urate-binding protein(s) also oxidized urate indicates that this protein has some functional homology to hepatic peroxisomal uricase.

Since a very limited amount of purified urate-binding protein was recovered, it was not possible to assess transport function by the technique of reconstituting the purified proteins in liposomes. The strategy that was used to assess the role of these proteins in urate transport was based on the assumption that transport might be inhibited by an interaction between anti-urate and proteins in the intact plasma membrane if one or more of the immunoreactive membrane protein(s) (Fig. 2) is involved in urate transport. However, two modalities of urate transport have been detected in rat renal brush border membranes; the uricase-like uniporter has been observed in membranes exposed to trace amounts of \(Cu^{2+}\) (6, 7), while the anion exchanger has been evident in membranes prepared with \(Mg^{2+}\) (4, 5).

To assess the effect of anti-urate on each mode of urate transport, vesicles were prepared and urate uptake was examined under conditions in which either the uniporter or ex-

![Fig. 1. Silver stain of a 8-25% SDS-PAGE gel. Lane 1, molecular weight markers; lane 2, 1.5 μg of solubilized membranes; lane 3, 1.0 μg of nonspecifically eluted protein; lane 4, purified protein specifically eluted from the affinity gel.](image)

![Fig. 2. Western blot using rabbit anti-pig liver uricase as the first antibody and a peroxidase-labeled goat anti-rabbit IgG as the second antibody. Lane 1, 500 μg of solubilized renal membranes; lane 2, less than 0.3 μg of purified protein specifically eluted from the affinity gel.](image)
Isolation and Immunolocalization of a Urate Transporter

The present studies have demonstrated that urate-binding proteins that were purified from brush border and basolateral membrane vesicles of rat renal cortex display some homology to the hepatic peroxisomal protein uricase. Immunologic homology was revealed by the immunoreactivity of two of the purified proteins with anti-porcine uricase on Western blot (Fig. 2). Functional homology was evidenced by uricase-like enzymatic activity. In addition to demonstrating shared properties of the purified renal proteins and hepatic uricase, the present studies also suggest that one or more of the purified renal cortical membrane urate-binding proteins is a component of a urate transporter. This conclusion is based on the finding that anti-porcine uricase not only reacts with immobilized purified protein on Western blot (Fig. 2), it also reacts with intact membrane proteins to act as a potent and specific inhibitor of urate transport under conditions in which the uricase-like transporter is functional (Fig. 3). Finally, the immunoreactive urate-binding protein(s), the putative urate unipporter, has been immunolocalized within the renal cortex to proximal convoluted and straight tubules (Fig. 4, B-F).

In addition to the two immunoreactive proteins that were purified from the renal membranes, two other proteins (25 and 41 kDa) were purified that were non-reactive to anti-urate. As all of the assays were performed under reduced conditions, it is possible that the four protein bands represent subunits of a single urate-binding protein in which some subunits contain, but others lack, epitopes recognized by anti-urate. Alternatively, two distinct modalities of urate transport have been described in rat kidney, an anion exchanger (4, 5) and a potential sensitive uricase-like unipporter (6, 7), the non-reactive and immunoreactive proteins may represent different urate-binding proteins with each responsible for one mode of urate transport. Based on the immunologic and functional homology between the purified renal proteins and porcine liver uricase, as well as the fact that anti-urate inhibited urate uptake in intact membranes under conditions in which only the urate unipporter is detected (Fig. 3), it is concluded that the purified immunoreactive proteins are components of the uricase-like transporter. The failure for anti-urate to inhibit transport under conditions in which only the urate/anion exchanger is observed implies that urate/anion exchange occurs on a protein that is different from the uricase-like urate unipporter. If such is the case, then the purified urate-binding proteins that do not react to anti-urate are likely to be components of the urate/anion exchanger. However, the present studies do not totally exclude the possibility that the two modes of transport occur on a single protein whose structural configuration is altered when experimental conditions are varied; a change in the configuration of a single urate transport protein that interferes with binding of the antibody could obviate an effect of the antibody on urate transport via the exchanger. A definitive conclusion regarding this issue must await comparison of the protein sequences of the exchanger and unipporter.

It is of interest that prior studies failed to detect immunoreactivity to an antibody to uricase in the rat kidney (13, 15). The marked immunoreactivity that was demonstrated in the current studies may, in part, be consequent to the fortuitous generation of a high titer, high affinity antibody. It may also be consequent to the fact that the protein that was used to generate antibody was not denatured as demonstrated by its ability to oxidize urate. However, the relevance of using an intact protein is uncertain since the biological activity of the antigens used to generate antibodies in previous studies (13, 15) was not commented upon. Perhaps more significantly, polyclonal or monoclonal antibodies were raised to rat uricase in prior studies (13, 15) whereas the present studies employed a polyclonal antibody that was raised to porcine uricase. Porcine uricase was selected for two reasons. First, studies in proteoliposomes demonstrated that porcine uricase, like the renal membrane protein, is capable of transporting urate when inserted into a lipid bilayer (12). Second, when antibodies to uricase have been raised in rabbit, porcine uricase has been shown to be more antigenic than dog, cow, horse, house musk shrew, and guinea pig uricase (29). Thus, in choosing the source of antigen the possibility was considered that porcine protein may also be more antigenic than the rat preparation. Regardless of the reason(s) for the difference in immunoreactivity of the antibody used in this and prior studies, the finding that anti-porcine uricase produced a profound inhibition of urate uptake in rat renal cortical membrane vesicles (Fig. 3) provides strong support for the use of this antibody as a marker of a urate transport protein.

Since the antibody that was employed was a selective and...
Isolation and Immunolocalization of a Urate Transporter

FIG. 4. Immunoperoxidase labeling of rat renal cortex examined with differential interference microscopy using non-immune IgG (A) and rabbit anti-pig liver uricase IgG (B-F). A, the glomerulus and all cortical nephron segments are non-reactive to non-immune IgG. B, brush-border (arrow) and subapical regions (arrowhead) of outer cortical proximal tubules are immunolabeled in the presence of immune IgG. Glomerulus (g) and distal tubule (*) are nonreactive. Note immunostaining of brush-border (arrow) in initial segment of proximal tubule (S1) at its origin in right lower corner of glomerulus. C, higher magnification of proximal tubule that has immunostaining in the brush-border membrane (arrow). D, higher magnification of proximal tubule that has immunostaining in subapical region of cell (arrowhead), but not in brush-border membrane (arrow). E, proximal tubules in inner cortex that have diffuse cytosolic immunostaining (arrowhead). F, higher magnification of inner cortical proximal tubule that has diffuse cellular immunostaining (arrowhead) and clear brush-border membrane (arrow). Magnification is the same in B and E; magnification is the same in C, D, and F.

potent inhibitor of urate transport, immunocytochemistry was used to localize the nephron sites of the urate transporter within the renal cortex. In previous studies in rat, it has been suggested that the majority of urate transport occurs within the proximal tubule (1-3). The finding that immunoperoxidase labeling was confined to proximal tubules (Fig. 4, B-F) is thus consistent with the physiologic data. At the cellular level, the demonstration of immunolabeling of the brush border (Fig. 4, B and C) is in accord with the fact that urate is transported across this membrane. Since this luminal membrane transporter must be delivered to the brush border from intracellular organelles, the subapical staining pattern (Fig. 4, B and D) may represent protein in vesicles involved in membrane trafficking or protein free within the cytoplasm. However, the precise intracellular localization that is represented by subapical labeling cannot be determined at the light microscopic level. Similarly, light microscopy is inadequate to determine the intracellular constituents that are labeled in the diffusely immunostained cells in S3 segments of the proximal tubule (Fig. 4, E and F). Since urate is also transported across the basolateral membrane, it would not be surprising if some of the diffuse staining was localized within this membrane, but the present study does not reveal enhanced staining of the basolateral membrane relative to the remainder of the cell. The failure to detect specific labeling of the basolateral membrane may indicate that reactive antigenic sites in the basolateral membrane to the uricase-like urate transporter are masked or urate is transported across this membrane by a different protein. Resolution of these issues will require further analysis with immunoelectron microscopy.

Although the current studies indicate that the urate uniporter has homology to hepatic peroxisomal uricase, it seems clear that the transporter and uricase are not identical proteins. First, pig hepatic uricase and the immunoreactive renal membrane urate-binding proteins have slightly different molecular weights. Second, the uricase-like enzyme activity of the purified membrane proteins appears to be quite weak relative to that of hepatic uricase. Finally, as Northern blot analysis of rat kidney RNA probed with the cDNA for rat hepatic uricase failed to detect transcripts (15, 30), it can be concluded
that the mRNA for uricase, per se, is absent or present in very low copy number. However, some epitopes must be conserved between hepatic uricase and the renal membrane transport protein; anti-porcine uricase not only recognizes urate-binding proteins purified from renal membranes, but specifically inhibits the urate uniporter without affecting other transporters (i.e. Na+–dependent glucose transporter and the urate/anion exchanger). A determination of the actual extent of homology between these proteins must await cloning the urate uniporter without affecting other transporters and subsequent comparison of the nucleotide sequences of the renal and hepatic proteins. The availability of a high titer polyclonal antibody to hepatic uricase may facilitate the cloning of the renal transporter.

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