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Localization of the membrane-associated thiol oxidase of rat kidney to the basal-lateral plasma membrane

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The localization of the membrane-associated thiol oxidase in rat kidney was investigated. Fractionation of the kidney cortex by differential centrifugation demonstrated that the enzyme is found in the plasma membrane. The crude plasma membrane was fractionated by density-gradient centrifugation on Percoll to obtain purified brush-border and basal-lateral membranes. γ-Glutamyltransferase, alkaline phosphatase and aminopeptidase M were assayed as brush-border marker enzymes, and $(Na^+ + K^+)$-stimulated ATPase was assayed as a basal-lateral-membrane marker enzyme. Thiol oxidase activity and distribution were determined and compared with those of the marker enzymes. Its specific activity was enriched 18-fold in the basal-lateral membrane fraction relative to its activity in the cortical homogenate, and its distribution paralleled that of $(Na^+ + K^+)$-stimulated ATPase. This association indicates that thiol oxidase is localized in the same fraction as $(Na^+ + K^+)$-stimulated ATPase, i.e. the basal-lateral region of the plasma membrane of the kidney tubular epithelium.

Renal thiol oxidase (this enzyme has been previously referred to as GSH oxidase or renal sulphydryl oxidase in the literature) is a membrane-associated enzyme that catalyses the rapid oxidation of extracellular GSH to GSSG. It has been shown to be dependent on O_2 (Jones *et al.*, 1979) and has the following stoichiometry of reaction (Ormstad *et al.*, 1979):

$$2\,GSH + O_2 \rightarrow GSSG + H_2O_2$$

In an attempt to clarify the function of the oxidase, Ormstad & Orrenius (1980) have examined the metabolism of extracellular GSH in the isolated perfused rat kidney. Their data indicated that the GSH-oxidizing activity is present on the contraluminal side of the tubular epithelium. To determine the subcellular localization of thiol oxidase in the kidney tubular epithelium, we have isolated the crude plasma membrane, fractionated the basal-lateral (contraluminal) and brush-border (luminal) membranes by density-gradient centrifugation, and compared the distribution of the oxidase with those of marker enzymes. The results of these studies demonstrate that the membrane-associated thiol oxidase is localized in the basal-lateral region of the plasma membrane.

Abbreviations used: GSH and GSSG, reduced and oxidized glutathione.

Materials and methods

Materials

Phenylmethanesulphonyl fluoride, γ-glutamyl *p*-nitroanilide, leucine *p*-nitroanilide, *p*-nitrophenyl phosphate, catalase, Percoll, NADH, phosphoenolpyruvate, ATP, pyruvate kinase, lactate dehydrogenase and ouabain were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). The dye-reagent concentrate for the determination of protein was obtained from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were of reagent grade. Doubly distilled deionized water was used for the fluorimetric assay of GSSG; deionized water was used for other assays and for the membrane preparation.

Membrane preparation

Male Charles River rats weighing 200–300 g were anaesthetized with diethyl ether and killed by cutting through the diaphragm. The kidneys were immediately removed and placed in ice-cold 10 mM-triethanolamine/HCl buffer, pH 7.6, containing 250 mM-sucrose and 0.1 mM-phenylmethanesulphonyl fluoride to inhibit proteolysis. The same buffer was used throughout, with additions as indicated. The brush-border and basal-lateral membranes were prepared by a modification of the method of Scalera *et al.* (1980) for intestinal

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epithelia. After the medulla was removed, the cortex was sliced and homogenized in 35ml of buffer with a glass/Teflon homogenizer (1200rev./min, 20 strokes, clearance 0.15–0.23mm; Arthur H. Thomas Co., Philadelphia, PA, U.S.A.) and then diluted 1:2 with buffer. Centrifugations were performed in a Sorvall SS34 rotor at 4°C in a Sorvall RC2B centrifuge. The homogenate was centrifuged at 2500*g* for 15min. The supernatant was decanted and then centrifuged at 20500*g* for 20min to obtain the fluffy layer (crude plasma membrane). The fluffy layer was resuspended in 17.5ml of buffer, from which 13.8ml was taken for the crude plasma-membrane fractionation. To this was added 1.2ml of the self-orienting modified colloidal-silica gradient medium, Percoll. This mixture was centrifuged at 48000*g* for 30min. The gradient was fractionated from the top by pumping a 60% (w/v) sucrose solution to the bottom of the centrifuge tube via a steel cannula. Each fraction collected was approx. 0.6ml.

Enzyme assays

High concentrations of Percoll were found to interfere with protein determination by the Folin phenol reagent. Therefore protein was measured by the method of Bradford (1976) with the protein-dye-reagent concentrate obtained from Bio-Rad and bovine serum albumin as a standard.

γ-Glutamyltransferase (Glenner & Folk, 1961; Glossman & Neville, 1972; George & Kenny, 1973; Liang & Sacktor, 1977), alkaline phosphatase (Mölbert *et al.*, 1961; Wilfong & Neville, 1970; Heidrich *et al.*, 1972; George & Kenny, 1973) and aminopeptidase M (Nachlas *et al.*, 1960; Holt & Miller, 1962; George & Kenny, 1973) activities were monitored as markers for the brush-border microvilli. γ-Glutamyltransferase (EC 2.3.2.2) activity was measured as described by Orlowski & Meister (1963), with γ-glutamyl *p*-nitroanilide as substrate and glycylglycine as γ-glutamyl acceptor. Alkaline phosphatase (EC 3.1.3.1) activity was measured by a modification of the method of Bessey *et al.* (1946), with *p*-nitrophenyl phosphate as substrate and 2-amino-2-methylpropan-1-ol as solvent. Aminopeptidase M (EC 3.4.11.2) activity was measured as described by Szasz (1967), with leucine *p*-nitroanilide as substrate.

$(Na^+ + K^+)$-stimulated ATPase activity was measured as a marker for the basal-lateral plasma membranes of the proximal tubule (Kinne *et al.*, 1971: Schmidt & Dubach, 1971; Heidrich *et al.*, 1972; Liang & Sacktor, 1977). $(Na^+ + K^+)$-stimulated ATPase (EC 3.6.1.3) activity was assayed as described by Schoner *et al.* (1967) and calculated as the difference in activity found in the presence and absence of ouabain.

To assay for thiol oxidase activity, samples were incubated in a solution containing 20mM-serine–borate, pH7.6, 1mM-GSH and 1μl of a 39mg/ml stock solution of catalase/ml. The serine–borate complex was added to inhibit γ-glutamyltransferase activity (Revel & Ball, 1959). GSSG formation was measured by a fluorimetric assay (Hissin & Hilf, 1976). GSH loss was measured by the method of Saville (1958).

Microsomal contamination was assessed by the measurement of glucose 6-phosphatase (EC 3.1.3.9) as a marker enzyme (Goldfischer *et al.*, 1965), as described by Swanson (1955), with the release of P_i measured by the method of Fiske & SubbaRow (1925).

Results

The cortical homogenate was centrifuged at 2500*g* for 15min and gave a precipitate (P1) and a supernatant (S1). Fraction S1 was centrifuged at 20500*g* for 20min, and gave a supernatant (S2) and a precipitate which was composed of two layers, namely a dark pellet on the botton (P2a) and a light layer called the fluffy layer (P2b). The fluffy layer contained the crude plasma membranes (Scalera *et al.*, 1980). These fractions were characterized by the distribution and recovery of marker enzymes for the brush-border and basal-lateral membranes.

Approx. 5% of the total protein of the renal-cortical homogenate was recovered in the crude plasma-membrane fraction. As a representative brush-border marker enzyme, γ-glutamyltransferase recovery and distribution were studied (Table 1). Almost 91% of the total activity in the homogenate was recovered in fraction S1. On centrifugation of fraction S1, over 5-fold enrichment of activity was found in the fluffy layer (P2b), with recovery of 25% of the total activity. Approx. 20% of the total activity was recovered in both fractions P2a and S2, but the specific activity was much lower in these two fractions.

The distribution and recovery of $(Na^+ + K^+)$-stimulated ATPase activity was similar to that of γ-glutamyltransferase (Table 1). The recovery of $(Na^+ + K^+)$-stimulated ATPase activity in the crude plasma-membrane fraction was 26%, and only 7% of the total activity was recovered in fraction P2a and 10% in fraction S2.

Thiol oxidase activity was measured by either the loss of GSH or the appearance of GSSG. The formation of GSSG was more sensitive, inasmuch as none was present initially in the incubation solution. Accordingly, all data presented were obtained by measuring GSSG formation. Data from experiments measuring GSH loss were in agreement with those obtained by using GSSG formation. The

Table 1. *Recovery of γ-glutamyltransferase, $(Na^+ + K^+)$-stimulated ATPase and thiol oxidase in the fractions obtained during the isolation of the crude plasma membrane*

The results are expressed as means ± S.E.M. for three individual preparations. The abbreviations for the fractions are as follows: H, homogenate, P1, precipitate from centrifugation at 2500 *g* for 15 min; S1, supernatant from centrifugation at 2500 *g* for 15 min; P2a, dark precipitate from centrifugation at 20500 *g* for 20 min; S2, supernatant from centrifugation at 20500 *g* for 20 min; P2b, crude plasma membrane (see the Materials and methods section).

			γ-Glutamyltransferase		$(Na^+ + K^+)$-stimulated ATPase		Thiol oxidase	
Fraction	Volume (ml)	Protein (mg/ml)	Sp. activity (μmol/min per mg)	Activity recovered (% of total)	Sp. activity (nmol/min per mg)	Activity recovered (% of total)	Sp. activity (nmol/min per mg)	Activity recovered (% of total)
H	70	3.16 ± 0.57	1.47 ± 0.33	100	75.3 ± 17.8	100	3.30 ± 0.59	100
P1	13.6 ± 3.1	6.31 ± 1.39	0.31 ± 0.06	7.8 ± 1.9	52.0 ± 17.3	27.2 ± 7.0	1.55 ± 0.40	15.8 ± 3.8
S1	64.2 ± 1.2	1.53 ± 0.26	2.72 ± 0.47	90.8 ± 4.9	65.7 ± 29.4	38.7 ± 15.0	6.37 ± 1.75	77.6 ± 5.4
P2a	8.0 ± 2.1	1.87 ± 0.56	4.39 ± 0.28	19.3 ± 1.1	90.1 ± 34.8	7.6 ± 3.6	4.76 ± 1.41	7.1 ± 2.0
S2	60.5 ± 1.1	1.40 ± 0.16	0.80 ± 0.18	21.1 ± 4.1	21.1 ± 2.0	11.0 ± 1.3	4.95 ± 0.20	55.4 ± 1.8
P2b	17.5	0.63 ± 0.09	7.51 ± 2.69	25.0 ± 3.8	330 ± 75	26.2 ± 5.0	17.16 ± 1.86	26.9 ± 2.6

principal difference was a higher background when substrate loss was measured.

The distribution and recovery of thiol oxidase activity is shown in Table 1. The 26% recovery of the total thiol oxidase activity in the crude plasma-membrane fraction was similar to the recovery of the other enzymes in this fraction. The large recovery of activity in fraction S2, which contains the cytosol and the microsomal fraction, was investigated. Fraction S2 was centrifuged at 100000 *g* for 60 min to pellet the microsomal fraction. Less than 2% of the total thiol oxidase activity was recovered in the microsomal fraction, indicating that the activity found in fraction S2 is soluble. Formation of GSSG in the cytosolic fraction may be due to a portion of the membrane-associated thiol oxidase which was solubilized during the homogenization, to autoxidation of GSH owing to metal ions, to the action of GSH peroxidase, or to the numerous thiol–disulphide exchange reactions present in this fraction. Alternatively, this activity may be due to a cytosolic thiol oxidase. Because previous studies have shown an oxidase activity which utilizes extracellular thiols as substrates, we focused our studies on the membrane-associated activity.

The crude plasma membrane was fractionated by density-gradient centrifugation in Percoll to isolate the brush-border and basal-lateral regions of the plasma membrane. Protein was concentrated in the bottom of the gradient, and had a maximal concentration of 3.5 mg/ml in the next-to-last fraction (Fig. 1*a*). Activity recovery after gradient centrifugation was 70–90% for the various enzymes studied.

As shown in Figs. 1(*b*)–1(*d*), maximal specific activity of the brush-border marker enzymes γ-glutamyltransferase, alkaline phosphatase and aminopeptidase M corresponded to peak protein concentration, occurring in the 14.0–14.5 ml region of the gradient.

The central region of the protein-concentration curve (6.0–8.5 ml) contained the fractions of maximal specific activity of the basal-lateral-membrane marker enzyme $(Na^+ + K^+)$-stimulated ATPase (Fig. 1*e*). Maximal specific activity occurred at 6.5 ml on the gradient. Ouabain-sensitive ATPase activity accounted for approx. 60% of the total ATPase activity in the basal-lateral fraction and less than 5% of the total ATPase activity in the brush-border fraction.

Thiol oxidase distribution (Fig. 1*f*) paralleled that of $(Na^+ + K^+)$-stimulated ATPase, with maximal activity occurring at 6.5 ml on the gradient, showing that it is also located in the basal-lateral region of the plasma membrane.

The extent of recovery of the microsomal fraction in the basal-lateral membrane fraction was determined by the distribution of the microsomal marker enzyme glucose 6-phosphatase. The activity increased with density and showed no preferential distribution in the region of the basal-lateral membrane (results not shown). Scalera *et al.* (1980), using the same procedure as was followed here, also showed minimal contamination of the basal-lateral fraction with mitochondria, lysosomes or the cytosol.

Enzyme activities in the crude plasma membrane, brush-border and basal-lateral membrane fractions are summarized in Table 2. As judged by the specific activities of marker enzymes in the brush-border and basal-lateral membrane fractions, brush-border microvilli were purified approx. 9-fold and basal-lateral infoldings approx. 20-fold. Enrichment of enzyme specific activities relative to the homogenate in the crude and purified membrane fractions is

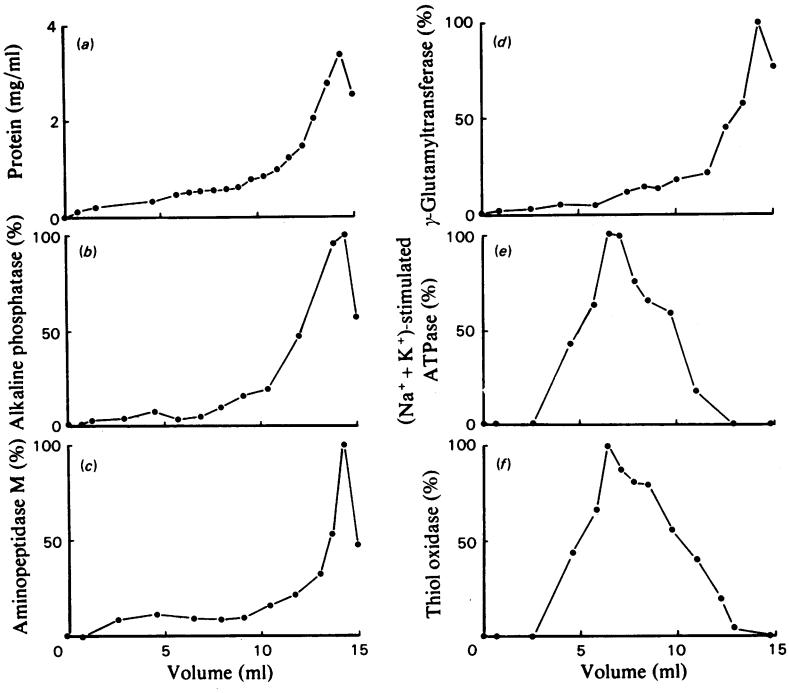

Fig. 1. *Protein concentration and enzyme-activity profiles of membrane fractions from the Percoll gradient*
Results from one representative preparation are given. The top of the gradient is 0ml and the bottom is 15ml. Activity is expressed as a percentage of maximal specific activity. Fractions were collected and assayed for protein or enzyme activities as described in the Materials and methods section. (*a*), Protein; (*b*), alkaline phosphatase; (*c*), aminopeptidase M; (*d*), γ-glutamyltransferase; (*e*), $(Na^+ + K^+)$-stimulated ATPase; (*f*), thiol oxidase.

Table 2. *Enzyme activities in crude plasma-membrane, brush-border and basal-lateral membrane fractions*
Fractions were collected as described in the Materials and methods section. For γ-glutamyltransferase, aminopeptidase M and alkaline phosphatase, activities are expressed in μmol/min per ml and specific activities in μmol/min per mg of protein. For $(Na^+ + K^+)$-stimulated ATPase and thiol oxidase, activities are expressed in nmol/min per ml and specific activities in nmol/min per mg of protein. Means ± S.E.M. for at least four preparations are given. Enrichment is expressed as the ratio of the specific activities in the various fractions to that of the cortical homogenate.

Fraction	γ-Glutamyltransferase*	Aminopeptidase M	Alkaline phosphatase	$(Na^+ + K^+)$-stimulated ATPase*	Thiol oxidase
Crude membrane					
Protein (mg/ml)	0.63 ± 0.09	0.78 ± 0.05	0.80 ± 0.50	0.63 ± 0.09	0.63 ± 0.09
Activity	4.53 ± 1.22	0.19 ± 0.03	0.34 ± 0.13	228 ± 53	11.6 ± 1.9
Sp. activity	7.51 ± 2.69	0.23 ± 0.04	0.44 ± 0.20	330 ± 75	17.2 ± 1.9
Enrichment	5.11			4.38	5.20
Brush-border					
Protein (mg/ml)	2.73 ± 0.18	2.86 ± 0.40	2.81 ± 0.30	2.12 ± 0.58	2.22 ± 0.34
Activity	44.1 ± 0.6	1.38 ± 0.50	2.28 ± 0.58	73.5 ± 24.5	3.57 ± 0.61
Sp. activity	16.2 ± 0.4	0.48 ± 0.15	0.84 ± 0.28	46.3 ± 27.6	2.41 ± 0.29
Enrichment	11.1			0.62	0.73
Basal-lateral					
Protein (mg/ml)	0.51 ± 0.05	0.56 ± 0.01	0.52 ± 0.05	0.54 ± 0.06	0.49 ± 0.05
Activity	0.83 ± 0.46	0.04 ± 0.02	0.05 ± 0.03	494 ± 34	29.3 ± 7.1
Sp. activity	1.65 ± 0.77	0.07 ± 0.02	0.09 ± 0.05	918 ± 49	61.1 ± 18.5
Enrichment	1.12			12.2	18.5

* Activities and specific activities for the brush-border and basal-lateral membrane fractions are corrected for 20–30% losses in activity when the fluffy layer was put on the Percoll gradient.

given in Table 2. Thiol oxidase activity was enriched 18-fold in the basal-lateral membrane fraction and was diminished in the brush-border fraction.

Discussion

The technique of density-gradient centrifugation as applied to renal-cortical plasma membranes takes advantage of the difference in content and morphology, and thus density, between the luminal (brush-border) and contraluminal (basal-lateral) surface of rat kidney proximal tubules. An examination of the high recovery of thiol oxidase activity in fraction S2 showed that the enzyme is not found in the microsomal fraction. Therefore the only membrane with which the enzyme is associated is the plasma membrane.

Renal thiol oxidase activity was measured and found to be localized in the same membrane fraction as $(Na^+ + K^+)$-stimulated ATPase, i.e. the basal-lateral region of the plasma membrane. Although the data indicate that thiol oxidase is not localized in the brush border, the limits of experimentation do not allow the conclusion that this fraction is devoid of activity. Although no GSSG formation in this fraction was detected, the high activity of γ-glutamyltransferase in the brush border may preclude the detection of formation of a small amount of GSSG, even in the presence of the inhibitory serine–borate complex.

Other reports have claimed that the oxidation of GSH to GSSG is a consequence of the catalytic function of γ-glutamyltransferase (Tate & Orlando, 1979; Griffith & Tate, 1980). Both activities were released in parallel during membrane digestion with bromelain and were both inhibited by the antitumour agent L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125). Other studies have confirmed the inhibition with AT-125 and have shown that AT-125 is a general inhibitor of GSH metabolism in isolated rat kidney cells (Reed *et al.*, 1980). However, this inhibition of GSH metabolism cannot be considered to be due to a single enzyme, since AT-125 is an alkylating agent that also inhibits the partially purified thiol oxidase (Ashkar *et al.*, 1981) in addition to γ-glutamyltransferase.

Ormstad *et al.* (1979) showed the oxidation of GSH in the kidney to be accompanied by the production of H_2O_2 in nearly stoichiometric amounts compared with the expected GSH loss and GSSG formation, and provided reconstitution studies which suggested that copper is a necessary cofactor. Other thiol oxidase activities have been described in other organs and have been shown to possess the same reaction stoichiometry as the kidney enzyme, but appear to have different cofactors. In contrast, the thiol oxidase recognized by the Enzyme Commission (EC 1.8.3.2) shows few similarities to the enzyme described in the present study. It catalyses a four-electron transfer reducing O_2 to $2H_2O$, rather than a two-electron transfer as shown for the renal thiol oxidase. In addition, GSH and cysteine are not substrates for the enzyme, but rather inhibit it. The functional relationship of the renal enzyme to these other enzymes is currently unknown.

The kidney is thought to play a major role in GSH metabolism in the body, largely as a consequence of high constitutive activities of GSH-metabolizing enzymes. In spite of extensive investigation, the physiological significance of many of these enzymes is unresolved. Two of these enzymes, γ-glutamyltransferase and thiol oxidase, are oriented in the kidney tubular epithelium such that extracellular GSH is accessible to their active sites. The finding that renal thiol oxidase is present on the basal-lateral region of the plasma membrane has important implications concerning its possible physiological function. Some specific functions have been ascribed to other thiol oxidases. For example, the bovine milk enzyme reoxidizes reductively denatured ribonuclease and fully re-activates it in a far shorter period of time than is possible in the absence of the oxidase (Janolino & Swaisgood, 1975). Ostrowski & Kistler (1980) showed the seminal-vesicle enzyme to be reactive towards protein-bound cysteine residues. Ziegler & Poulsen (1977) proposed that the mixed-function amine oxidase of hog liver endoplasmic reticulum that oxidizes cysteamine to cystamine functions in intracellular synthesis of protein disulphide bonds. The renal enzyme, having a contraluminal location in the proximal-tubular epithelium, may function in a similar manner in the maintenance of plasma proteins such as immunoglobulins and peptide hormones in the disulphide form. It may also function in the regulation of the plasma GSH/GSSG ratio and consequently, through thiol-exchange reactions, the thiol/disulphide ratio in a large number of plasma constituents. Additional studies are necessary to examine this possibility.

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