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PARTIAL CHARACTERIZATION OF A GLUTATHIONE OXIDASE PRESENT IN RAT KIDNEY PLASMA MEMBRANE FRACTION

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Summary: The previously reported glutathione oxidizing activity of isolated renal cells was recovered in the plasma membrane fraction of a rat kidney homogenate. Glutathione disulfide and hydrogen peroxide were formed during the reaction which was dependent on the access to molecular oxygen and inhibited by KCN and EDTA, but not by NaN_3. The EDTA-inhibited activity could be restored by addition of $CuSO_4$, but not $FeCl_3$, to the plasma membrane fraction after dialysis. The results strongly suggest that a Cu-protein present in the plasma membrane is responsible for the glutathione oxidase activity of renal cells.

In previous communications we have reported that freshly isolated rat kidney cells catalyze the oxidation of extracellular glutathione (GSH) to glutathione disulfide (GSSG) (1,2). The reaction was found to be O_2-dependent and to represent the initial step in the renal degradation of extracellular GSH to the constitutive amino acids, the subsequent reactions being catalyzed by γ-glutamyltransferase and cysteinylglycine didpeptidase (3,4).

In the present investigation we have characterized the renal GSH-oxidizing activity further and provide evidence that a Cu-containing protein present in the plasma membrane fraction of a rat kidney homogenate acts as a GSH oxidase in renal metabolism of extracellular glutathione.

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Methods

Male Sprague-Dawley rats, weighing 200-250 g and fed *ad lib.*, were used. Under ether anaesthesia, kidneys were perfused *in situ* with 0.9% NaCl at 37°, removed and stripped from fat and connective tissue. A crude homogenate was prepared in 0.3 M sucrose and diluted to a concentration of 1 g kidney wet-weight/8 ml. The plasma membrane fraction was isolated as described for rat liver by Coleman *et al.* (5). This procedure yielded about 1 mg membrane protein per g kidney wet-weight.

Incubations were performed at a final membrane protein concentration of 0.1 mg/ml in rotating, round-bottom flasks at 37°. Unless otherwise stated, the incubates were gassed with 95% O_2-5% CO_2. The incubation medium was 50 mM Tris-HCl buffer, pH 7.4, with additions as indicated.

GSH and GSSG were assayed in deproteinated samples according to Saville (6) or Hissin & Hilf (7). H_2O_2 was measured as described by Ovenston & Parker (8). To inhibit catalase-mediated metabolism of H_2O_2, NaN_3 was added to the incubate at a final concentration of 50 μM; at this concentration, NaN_3 did not affect GSH oxidation rate. To inhibit γ-glutamyltransferase-mediated metabolism of GSSG, L-serine and boric acid, 20 mM each, were added to the incubate (9). Neither this addition affected GSH oxidation rate.

Mg^{2+}-dependent, Na^+, K^+-activated ATPase activity was assayed according to LeBel *et al.* (10). Succinate-cytochrome *c* reductase activity was measured as described by Sottocasa *et al.* (11), and NADPH-cytochrome *c* reductase activity according to Dallner (12).

To characterize the properties of the GSH-oxidizing activity various inhibitors were added to the plasma membrane fraction and preincubation performed for 30 minutes before addition of GSH. Plasma membrane suspension treated with EDTA (ethylenediamine tetraacetic acid) was dialyzed for 2 hours against 0.15 M KCl to remove EDTA-metal complexes and excess EDTA. Subsequently, 1 mM $FeCl_3$ or 1 mM $CuSO_4$ was added to the suspension 10 minutes before incubation with GSH was started. At this concentration, neither Fe^{3+} or Cu^{2+} exerted any GSH-oxidizing activity *per se*.

Results and Discussion

Some characteristics of the isolated rat kidney plasma membrane fraction are shown in Table I. Comparison of succinate-cytochrome *c* reductase and NADPH-cytochrome *c* reductase activities in crude homogenate and plasma membrane fraction revealed little contamination of the latter with mitochondria but a certain admixture of microsomal membranes. γ-Glutamyltransferase activity was about 40 times higher in the plasma membrane fraction as compared to the crude homogenate and a similar enrichment in

TABLE I. Comparison of various enzyme activities in rat kidney homogenate and plasma membrane fraction

Enzyme activity	Plasma membrane fraction	Crude homogenate	Relative specific activity ratio
Succinate-cytochrome c reductase (nmol/mg protein/min)	10	125	0.08
NADPH-cytochrome c reductase (nmol/mg protein/min)	18	9	2
γ-Glutamyltransferase (nmol GSSG metabolized/mg protein/min)	76	2	38
GSH oxidation (nmol GSH oxidized/mg protein/min)	24	0.5	48
Mg^{2+}-dependent, Na^+, K^+-activated ATPase (μg P_i formed/mg protein/min)	17		

plasma membrane fraction was observed for GSH-oxidizing activity. The plasma membrane fraction also exhibited high Mg^{2+}-dependent, Na^+, K^+-activated ATPase activity.

Figure 1 shows the rate of GSH disappearance catalyzed by the isolated rat kidney plasma membrane fraction as a function of either GSH concentration in incubate (A) or oxygen concentration in gas phase (B). Maximal activity was obtained at ∿2 mM GSH and ∿25% O_2, respectively. These results indicate that the enzyme is more readily saturated with oxygen than previous experiments with isolated renal cells have suggested (2), the discrepancy most probably being due to competition for oxygen between GSH oxidation and other O_2-requiring metabolic processes in isolated cells.

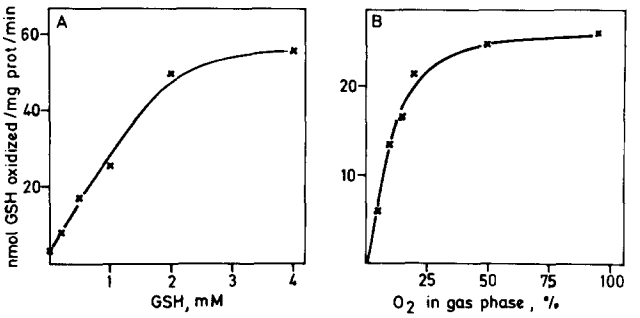

Fig. 1. GSH metabolism catalyzed by the plasma membrane fraction isolated from rat kidneys.
A: GSH disappearance rate as a function of initial GSH concentration. The incubate was gassed with 95% O_2 - 5% CO_2. B: GSH disappearance rate as function of O_2 concentration in gas phase. Initial concentration of GSH was 1 mM.

The metabolism of GSH by the kidney plasma membrane fraction was associated with the accumulation of GSSG and H_2O_2 in the incubate (Fig. 2). This could be demonstrated only when further metabolism of GSSG by plasma membrane γ-glutamyltransferase, and of H_2O_2 by contaminating catalase, was inhibited by the addition to the incubate of serine·borate and NaN_3, respectively. These inhibitors did not affect the rate of GSH oxidation. The fact that H_2O_2 accumulation in the incubate was almost stoichiometric to GSH disappearance and GSSG formation, and our inability to find spectrophotometric evidence for the participation of an intermediate electron acceptor in the reaction, suggest that molecular oxygen may in fact serve as the immediate electron acceptor in the GSH-oxidizing reaction.

Our previous finding that the GSH-oxidizing activity of isolated renal cells and crude plasma membrane fraction is efficiently inhibited by CN^- suggested the involvement of a metalloprotein in the reaction (2). This preliminary indication has been further substantiated in the present investigation.

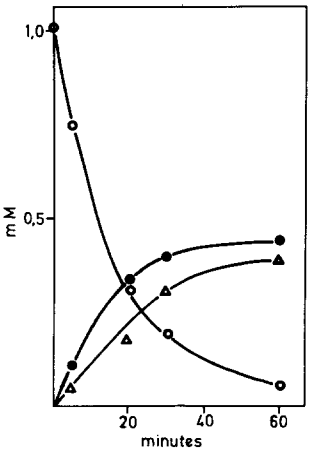

Fig. 2. GSH disappearance and concomitant GSSG and H_2O_2 formation in plasma membrane fraction isolated from rat kidneys.
o——o, GSH; ●——●, GSSG; Δ——Δ, H_2O_2. Serine·borate, 20 mM, was added to the incubate to inhibit γ-glutamyltransferase, and NaN_3, 50 μM, to inhibit contaminating catalase.

As shown in Table II, GSH oxidation catalyzed by kidney plasma membrane fraction was inhibited by KCN, EDTA and 1,10-ortophenanthroline. KCN was the most efficient of these inhibitors, producing ∿70% inhibition at 10 μM concentration, but also with EDTA pronounced inhibition of the reaction was obtained. In contrast, there was no effect of low concentrations ($\leq$50 μM) of NaN_3 on GSH oxidation rate.

To further investigate the nature of the GSH oxidase, the EDTA-treated plasma membrane fraction was subjected to dialysis and subsequent addition of either $CuSO_4$ or $FeCl_3$. As shown in Figure 3 the addition of $CuSO_4$ to the EDTA-inactivated system resulted in almost complete restoration of GSH oxidase activity, whereas $FeCl_3$ had no such effect. Thus, it appears that a Cu-containing protein is reponsible for the GSH oxidase activity of the rat kidney plasma membrane fraction.

TABLE II. Effect of inhibitors on GSH oxidation activity in rat kidney plasma membrane fraction

Inhibitor		Oxidation rate, nmol GSH ox/mg prot/min	Inhibition, %
None		26.3	
KCN,	0.01 mM	8.4	68
	0.10 mM	2.2	92
EDTA,	0.1 mM	6.0	77
	1.0 mM	4.1	84
1,10-ortho-phenanthro-line,	0.1 mM	11.8	55
	1.0 mM	7.9	70

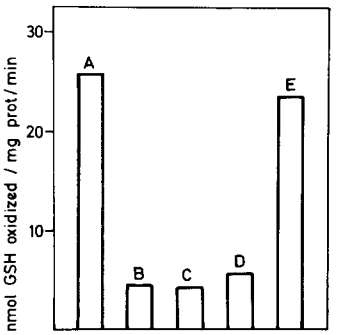

Fig. 3. Effect of various pretreatments on GSH oxidation in plasma membrane fraction isolated from rat kidneys. Initial GSH concentration was 1 mM.
A: No pretreatment; B: Fraction incubated with 1 mM EDTA before addition of GSH; C: EDTA-treated fraction dialyzed against 0.15 M KCl for 2 hours; D: 1 mM $FeCl_3$ added to fraction after dialysis; E: 1 mM $CuSO_4$ added to fraction after dialysis.

In summary, the present investigation has provided evidence that a Cu-containing protein located in the plasma membrane fraction is responsible for the GSH-oxidizing activity previously observed with isolated renal cells (1,2). The enzyme appears to

function as a GSH oxidase utilizing molecular oxygen as the immediate electron acceptor. Whether it is specific for GSH remains to be elucidated; cysteine was unable to serve as a substrate in our experiments. Further investigation is also required to define the role of this enzyme in renal glutathione metabolism more closely.

Acknowledgement

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