CHARACTERISTICS OF RENAL GLUTATHIONE OXIDASE ACTIVITY

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1. Introduction

Studies with freshly isolated renal cells and with perfused rat kidney have provided evidence for the existence of a renal glutathione oxidase activity catalyzing the O_2-dependent conversion of extracellular glutathione (GSH) to glutathione disulfide (GSSG) [1,2]. Whereas results obtained in our laboratory earlier suggested that this activity is catalyzed by an enzyme distinct from γ-glutamyltransferase (γGT) [1–3], subsequent studies attributed renal GSH oxidase activity to the function of γGT [4–6]. This discrepancy has now been resolved, since Ashkar and associates [7] have managed to solubilize and separate the two activities from rat kidney; a partially purified GSH oxidase completely devoid of γGT activity has been isolated and found to exhibit properties similar to those of the membrane-bound GSH oxidase activity of isolated renal cells.

The present report describes several characteristics of the GSH oxidase activity of rat kidney as investigated with suspensions of epithelial cells, the isolated plasma membrane fraction and a solubilized, partly purified GSH oxidase preparation. Based on inhibition and reconstitution experiments it is suggested that a copper-containing catalyst most probably is responsible for the bulk of the renal GSH oxidase activity. The physiological role of this enzyme is discussed.

2. Materials and methods

Unstarved, male Sprague-Dawley rats (180–250 g) were used for isolation of renal tubular cells [8] and for preparation of kidney plasma membrane fraction [9]; the later procedure was modified by the addition of 8.5% glycerol to all media. Glutathione oxidase was separated from γGT by the gel filtration technique described in [7]. The 100 000 × *g* supernatant of a whole-kidney homogenate was applied to a column of Ultrogel AcA 34 (LKB, Stockholm, Sweden), length 86 cm and width 1.6 cm and eluted with a Tris–HCl buffer, pH 7.6, containing 30% glycerol at room temperature at a flow rate of 10 ml h^{-1}. Fractions of 3.3 ml were collected and assayed for various enzyme activities.

The purity of the renal plasma membrane fraction and of the partly purified GSH oxidase preparation was estimated by assaying various marker enzymes including succinate-cytochrome *c* reductase [10], NADPH-cytochrome *c* reductase [11], Mg^{2+}-dependent(Na^+, K^+)-activated ATPase [12] and γGT activities [1,13]. Protein concentration was determined spectrophotometrically according to Bradford [14] or Peterson [15].

Incubations were performed at 37°C in a modified Krebs–Henseleit buffer, pH 7.4, supplemented with 25 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), in rotating round-bottom flasks under continuous gassing with 95% O_2, 5% CO_2, unless otherwise indicated. O_2 concentrations were assayed in a closed, thermostated glass cell supplied with a Clark-type oxygen electrode (Rank Brothers, Bottisham, England). H_2O_2 was determined spectrophotometrically [16]. In experiments where H_2O_2 was to be assayed, NaN_3 was added to a final concentration of 50 μM in order to inhibit any contaminating catalase; NaN_3 has previously been shown not to affect renal GSH oxidase activity [3].

GSH and GSSG were analyzed spectrofluorometrically [17]. When GSSG was to be assayed, a combination of serine and borate at 20 mM concentrations was included in the incubation medium to inhibit

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GSSG breakdown by γGT [18]; serine · borate has previously been shown not to affect GSH oxidase activity [1].

All chemicals were of at least reagent grade and were purchased from local commercial sources.

3. Results and discussion

As shown in table 1, the GSH oxidase activity of the crude kidney homogenate was concentrated more than 30-fold in the isolated plasma membrane fraction. As expected, γGT and Mg^{2+}-dependent(Na^+, K^+)-activated ATPase activities were also markedly enriched in this fraction, suggesting that it contained both brush-border and basolateral membrane fragments [cf 19,20]. A certain admixture of endoplasmic reticular membranes was evident from the NADPH-cytochrome *c* reductase activity of the plasma membrane fraction which, on the other hand, appeared to be relatively free from mitochondrial contamination as judged by low succinate-cytochrome *c* reductase activity. In a separate series of experiments, microsomal, mitochondrial and nuclear fractions were prepared from kidney homogenate; these fractions were either devoid of apparent GSH oxidase activity (mitochondrial and nuclear fractions), or exhibited a much lower specific activity than the plasma membrane fraction (microsomal fraction).

γGT activity is known to be restricted to the brush-border membranes of tubular epithelial cells [20], whereas kidney perfusion experiments have suggested a different localization of GSH oxidase activity, i.e. in the basolateral regions of the tubular epithelium [2]. It is therefore of interest to note that the relative enrichment of the two activities in the plasma membrane fraction differed appreciably in the present study. This was probably due to a similar difference in enrichment of brush-border and basolateral membrane fragments in the total renal plasma membrane fraction.

As further shown in table 1, partial purification of GSH oxidase activity according to the procedure described by Ashkar and associates [7], resulted in an ~75-fold enrichment to the activity present in the crude kidney homogenate. Although not pure, the GSH oxidase fraction was completely devoid of γGT activity and therefore suitable as an enzyme source in further experiments. However, as previously observed by Ashkar and collaborators [7] the solubilized GSH oxidase preparation was partly inactivated under oxidizing conditions. Studies of the kinetics and stoichiometry of the GSH oxidase reaction were therefore performed with the renal plasma membrane fraction.

Maximal rate of GSH oxidation by the renal plasma membrane fraction was observed at GSH and O_2 concentrations of about 2 mM and 0.5 mM, respectively (fig.1). The calculated, apparent K_m values were

Table 1
Some enzyme activities present in rat kidney plasma membrane and solubilized GSH oxidase fractions

Enzyme activity	Crude homogenate	Plasma membrane fraction	Solubilized GSH oxidase fraction
Protein (mg g^{-1} kidney wet weight)	68.0 ± 6.5	0.6 ± 0.1	0.8 ± 0.2
Succinate-cytochrome *c* reductase activity (nmol mg^{-1} protein per min)	141 ± 16.2	6.0 ± 2.0	3.9
NADPH-cytochrome *c* reductase activity (nmol mg^{-1} protein per min)	10.2 ± 1.8	15.6 ± 5.4	9.1
γ-Glutamyltransferase activity (nmol GSSG metabolized mg^{-1} protein per min)	13.2 ± 3.0	228 ± 54	0
GSH oxidase activity (nmol GSH oxidized mg^{-1} protein per min)	21.0 ± 6.0	720 ± 168	1560 ± 250
Mg^{2+}-dependent(Na^+, K^+)-activated ATPase activity (μg P_i formed mg^{-1} protein per min)	1.7 ± 0.3	39.5 ± 6.1	0

GSH oxidase activity was assayed with 2 mM GSH, and γGT activity with 2 mM GSSG, as substrate. Results are given as mean ± S.D. of three experiments

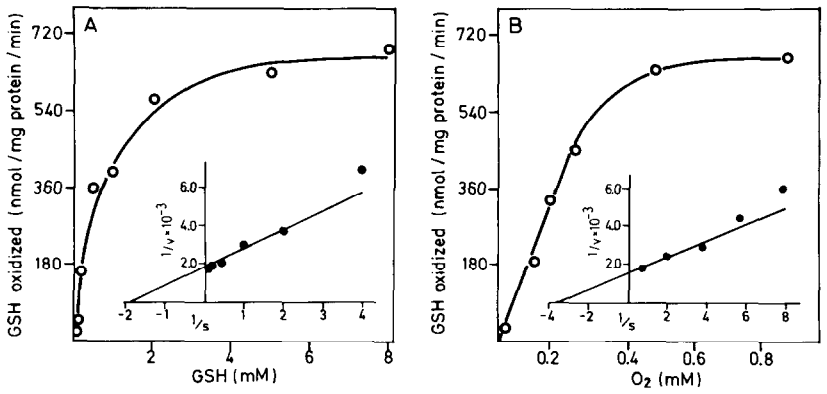

Fig.1. Influence of GSH (A) and O_2 (B) concentrations on GSH oxidase activity of isolated renal plasma membrane fraction. A: GSH was incubated with renal plasma membrane fraction (25 μg protein ml^{-1}) under continuous carbogen (95% O_2, 5% CO_2) gassing (O_2 concentration in incubate was ~0.5 mM). B: GSH (2 mM) was incubated with renal plasma membrane fraction under continuous gassing with various mixtures of carbogen and N_2.

~0.5 mM for GSH and ~0.25 mM for O_2. The pH optimum of the reaction was at ~7.4 (not documented). The relatively low affinity of renal GSH oxidase for both GSH and molecular oxygen makes it important to measure this activity under well oxygenated conditions and in the presence of high concentrations of GSH to achieve optimal reaction velocity. However, also at non-saturating GSH concentrations, the oxidase activity of renal plasma membrane fragments was appreciable (cf fig.1).

Fig.2 compares GSH and O_2 consumption with GSSG and H_2O_2 production during GSH oxidation catalyzed by renal plasma membrane fraction. For technical reasons O_2 consumption and H_2O_2 production were assayed in parallel experiments. From these data it appears that the renal GSH oxidase reaction has the overall stoichiometry: $2GSH + O_2 \rightarrow GSSG + H_2O_2$. A several-fold increase in the rate of reduction of acetylated or succinylated cytochrome *c* by GSH observed in the presence of renal plasma membrane

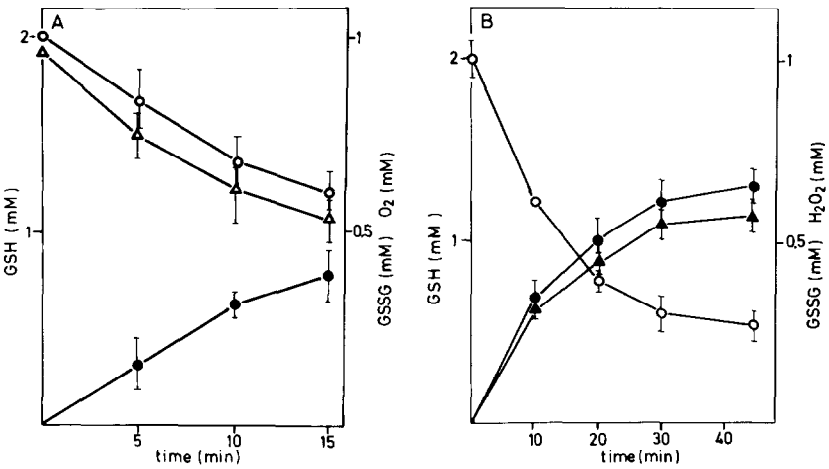

Fig.2. O_2 consumption (A) and H_2O_2 production (B) during GSH oxidation by renal plasma membrane fraction. A: Renal plasma membrane fraction (100 μg protein ml^{-1}) was incubated with GSH (2 mM) and NaN_3 (50 μM) in a thermostated closed cell supplied with an oxygen electrode. Before the reaction was started the incubate was saturated with carbogen gas (95% O_2, 5% CO_2), and no further oxygenation was performed during the incubation. Results are given as mean ± S.D. of three assays. B: Renal plasma membrane fraction (100 μg protein ml^{-1}) was incubated with GSH (2 mM) and NaN_3 (50 μM) under continuous gassing with carbogen (95% O_2, 5% CO_2). Results are given as mean ± S.D. of three experiments. (○), GSH; (●), GSSG; (△), O_2; (▲), H_2O_2.

fraction, or solubilized GSH oxidase preparation, suggests that H_2O_2 generation may be preceded by formation and release of superoxide anion radicals during the oxidase reaction. In an experiment with 2 mM GSH and solubilized GSH oxidase present at a concentration of 150 μg protein ml^{-1}, GSH oxidation rate was 204 nmol min^{-1}, whereas the rate of reduction of acetylated cytochrome *c* was 115 nmol min^{-1}.

The inhibition of renal GSH oxidase activity by EDTA and *o*-phenanthroline has previously been taken to indicate the involvement of metal in this activity [3,7]. In the present study we have therefore investigated the effect of metal chelating agents on GSH oxidase in further detail. Table 2 shows that the copper chelators diethyldithiocarbamate and bathocuproine sulfonate both inhibited the reaction and that the latter agent, which is a potent Cu^I chelator, was the most efficient of the inhibitors tested. In an attempt to elucidate further the possible involvement of copper in the renal GSH oxidase activity, $CuSO_4$ was added to an EDTA-treated and dialyzed plasma membrane fraction. As shown by the titration curve in fig.3, a stimulatory effect of $CuSO_4$ on GSH oxidase activity of the EDTA-treated plasma membrane fraction was already detectable below 1 μM, whereas half-saturation of the activity occurred at about 2 μM

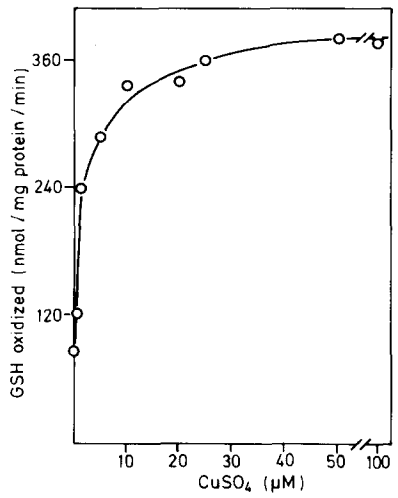

Fig.3. Effect of $CuSO_4$ on GSH oxidation in EDTA-treated, dialyzed renal plasma membrane fraction. The plasma membrane fraction was incubated with EDTA (1 mM) for 10 min, dialyzed overnight against 0.15 M KCl and subsequently incubated with GSH (1 mM) at a protein concentration of 100 μg ml^{-1} and addition of $CuSO_4$ as indicated. The results are given as mean of four experiments.

Table 2
GSH oxidation catalyzed by isolated rat kidney cells and solubilized GSH oxidase fraction in the absence or presence of various metal chelating agents

Addition	GSH oxidation rate	
	Cells (nmol GSH oxidized 10^{-6} cells per min)	Solubilized fraction (nmol GSH oxidized mg^{-1} protein per min)
None	40.2 ± 4.2	1560 ± 250
EDTA		
0.1 mM	39.8 ± 5.9	42.5 ± 11.0
0.5 mM	29.3 ± 3.0	13.1 ± 0.9
5.0 mM	7.0 ± 0.8	0
Diethyldithiocarbamate		
0.1 mM	28.7 ± 5.1	34.0 ± 3.9
0.5 mM	21.6 ± 1.8	8.3 ± 1.5
5.0 mM	10.2 ± 1.3	0
Bathocuproine sulfonate		
0.1 mM	21.5 ± 4.8	11.3 ± 2.5
0.5 mM	10.3 ± 1.2	2.0 ± 1.0
5.0 mM	4.7 ± 0.6	0

Incubations were performed with 2 mM GSH in the presence of 20 mM serine borate to prevent GSSG degradation by γGT and the results are given as mean ± S.D. of experiments with three separate preparations

$CuSO_4$. It should be noted that there was a small increase also in the rate of spontaneous GSH oxidation in the presence of $CuSO_4$. In comparison to the effect of copper on the enzymatic reaction, the stimulation of spontaneous GSH oxidation was, however, negligible ($\simeq$10% at 50 μM $CuSO_4$). The specificity and function of copper in the GSH oxidase reaction are presently under detailed investigation.

It has recently been reported that other small molecular thiols, including cysteine and dithiothreitol, can act as substrates for solubilized renal GSH oxidase [7]. Thus, it appears that the observed activity is due to a thiol oxidase that accepts GSH as a substrate rather than to a specific GSH oxidase. Other thiol oxidases have previously been characterized from bovine milk [21] and murine seminal vesicles [22]. However, certain properties of these enzymes differ significantly from those of the renal catalyst and there are presently no indications of catalytic identity between these activities.

At present, the physiological function of the renal GSH (thiol) oxidase is unknown. Provided that our earlier findings with respect to the localization of this enzyme on the contraluminal side of the tubular epithelium are correct, its activity would probably be restricted to thiols present in plasma. However, both glutathione and cysteine are present at very low concentrations in plasma, and predominantly in the disulfide form [23,24]. The significance of a renal activity functioning primarily in the oxidation of these thiols is therefore difficult to visualize. On the other hand, thiol group oxidation is a basal mechanism for local activation or inactivation of certain enzymes or peptide hormones in plasma, some of which act directly on the tubular epithelium. Further speculation on the possible involvement of the renal GSH oxidase must, however, await a more detailed analysis of the substrate specificity of this enzyme.

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