RESOLUTION OF A RENAL SULFHYDRYL (GLUTATHIONE) OXIDASE FROM γ-GLUTAMYLTRANSFERASE

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Received 2 January 1981

1. Introduction

Rapid oxidation of GSH by kidney extracts was recognized by Ames and Elvehem 30 years ago [1], but further investigation of this process did not proceed until it was recently rediscovered in studies of GSH metabolism by isolated kidney cells [2,3]. The reaction requires molecular oxygen [3] and has the stoichiometry:

$$2\ GSH + O_2 \rightarrow GSSG + H_2O_2\ [4]$$

The reaction was shown to be insensitive to inhibition by 20 mM serine–borate [3], a complex that gives essentially complete inhibition of γ-glutamyl transferase (γ-GT) [5]. However, an apparent GSH oxidase activity can be obtained with purified rat renal γ-GT [6], that both GSH oxidase activity and γ-GT are released in parallel during membrane digestion with bromelian [6], and that both activities are inhibited by the anti-tumor agent L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125) [7,8]. These results indicate that both GSH oxidase activity and γ-GT activity are due to the same enzyme. We undertook fractionation of the enzyme activities in order to determine whether the GSH oxidase activity of plasma membrane fragments was due to γ-GT. The results demonstrate resolution of these activities and describe certain properties of the oxidase that will facilitate further purification and characterization.

2. Materials and methods

Male Sprague-Dawley rats (200–250 g) were sacrificed by CO_2 suffocation. The kidneys were perfused with 0.9% NaCl (w/v) at 0°C, decapsulated and homogenized in cold 0.08 M Tris–HCl (pH 7.6) containing 1 mM EDTA. The homogenate was centrifuged at 800 × *g* for 15 min. The supernatant was recentrifuged at 100 000 × *g* for 2 h and the supernatant discarded. The precipitate was resuspended in 0.08 M Tris–HCl (pH 7.6) containing 1% Triton X-100 (v/v) and 1 mM DTT, and allowed to stand overnight at 4°C. Samples (1 ml) were applied to a descending gel filtration column (Bio-Rad P-200, 1.5 × 35 cm) preequilibrated with 0.08 M Tris–HCl (pH 7.6) containing 30% glycerol (v/v) at room temperature and eluted with the same buffer; active fractions were pooled.

Sulfhydryl oxidation was measured as disappearance of free sulfhydryl according to [9] as modified [10] or by the fluorometric method [11] for GSH. GSSG was determined enzymatically using glutathione reductase [12]. γ-GT was assayed using γ-glutamyl *p*-nitroanilide [13] with glycylglycine as an acceptor or by the Sullivan and Hess method as modified [14]. Cysteinylglycine was determined by the modified Sullivan and Hess method [14]. Protein was determined as in [15] with bovine serum albumin as a standard.

3. Results and discussion

3.1. *Resolution of a glutathione oxidase activity from γ-glutamyl transferase*

Fractionation of plasma membranes that had been treated with 1% Triton X-100, was obtained by gel filtration (fig.1). Most of the protein and γ-GT activity eluted with the void volume. Two GSH oxidase activity peaks were present, one associated with the trailing edge of the void volume protein and one

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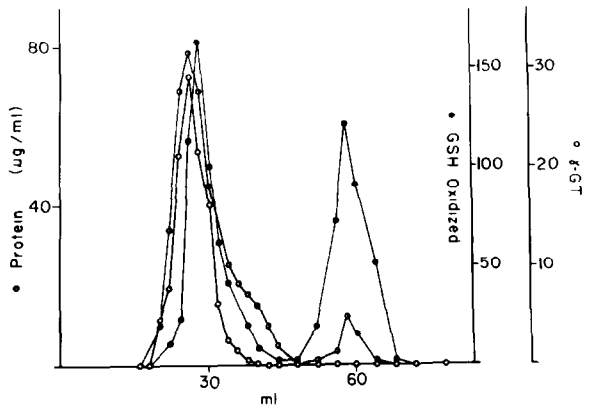

Fig.1. Gel filtration elution profile of GSH oxidase activity and γ-glutamyltransferase activity. Sample (1.0 ml 0.6 mg protein/ml Tris–HCl (pH 7.6) containing 1% Triton X-100 (v/v)) was applied to a 1.5 × 40 cm P-200 (Bio-Rad) column equilibrated with Tris–HCl (pH 7.6) containing 30% glycerol (v/v) at room temperature and run at 3.8 ml/h. GSH oxidation was measured as GSH loss by the colorimetric assay [9], and γ-GT activity was measured by the *p*-nitroanilide method [13]. Data are given as µmol . min^{-1} . ml^{-1} sample for γ-GT and nmol . min^{-1} . ml^{-1} for oxidase. Protein determination was as in [15].

associated with a ~60 ml elution vol. This latter peak was completely free from γ-GT activity as measured by either the Sullivan and Hess method or the *p*-nitroanilide method with glycylglycine as an acceptor. These results demonstrate the resolution of a GSH oxidase activity from γ-GT.

3.2. *Characteristics of the oxidase activity*

GSH oxidase activity from peak II (elution vol. 50–65 ml) was used for all the characterizations that follow. The purification by this procedure was ~4000-fold; the resultant activity was heat labile and non-dializable. No difference in oxidase activity was detected by addition of 20 mM serine–borate. The stoichiometry of the reaction described in [4] was confirmed. Experiments with added *t*-butylhydroperoxide showed that this fraction did not support a GSH peroxidase reaction; instead, *t*-butylhydroperoxide is a potent inhibitor of the activity (table 1).

The oxidase activity was also inhibited by one of its products, hydrogen peroxide (fig.2). The reaction was linear only up to 20 min, but upon the addition of catalase, the linearity continued to 40 min. Moreover, the enzymatic activity was lost upon storage but was fully recovered upon the incubation with 1 mM DTT. Glutathione was also capable of reactivating the

Table 1
Effects of inhibitors on partially purified sulfydryl oxidase

Inhibitor	GSH oxidation rate (µmol . min^{-1} . mg $protein^{-1}$)		% inhibition
None	11.7	±0.5	–
EDTA (1 mM)	8.3	±0.2	31
(3 mM)	<0.10		>99
Serine–borate (20 mM)	11.5	±0.2	2
t-Butylhydroperoxide (0.1 mM)	<0.10		>99
Streptozotocin (1.0 mM)	<0.10		>99
AT-125 (0.25 mM)	<0.10		>99

Values are expressed as mean ± standard error (n = 3)

enzyme (fig.2) as was apparent from a lag phase in the time course of the reaction. The results indicate that the enzyme undergoes facile oxidation (auto-oxidation) to an inactive form that can be reactivated by reduction under mild conditions.

Substrate specificity studies (table 2) revealed that although GSH and cysteine are good substrates, the oxidase is capable of oxidizing other thiol compounds; hence, the enzyme activity is that of a general sulfhydryl oxidase rather than a specific GSH oxidase. Other sulfhydryl oxidases have been characterized from

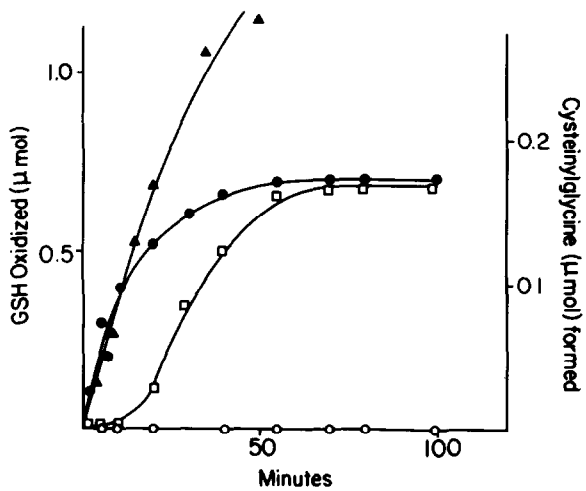

Fig.2. Time course for GSH oxidation. Incubations were performed at 18–20°C under air with 0.08 M Tris–HCl (pH 7.6) and 2.6 mM GSH with 1 µg protein/ml. GSH oxidized was measured as loss of GSH [9]: (□) untreated, without catalase; (●) DTT-treated, without catalase; (▲) DTT-treated, with catalase. No cysteinylglycine was formed in these incubations (○).

Table 2
Characteristics of sulfhydryl oxidation by partially purified sulfhydryl oxidase

Compound	K_m (mM)	V_{max} (μmol . min^{-1} . mg protein^{-1})
GSH	2.2	0.033
Cysteine	1.0	0.045
N-Acetylcysteine	0.6	0.015
DTT	0.2	0.008
Glycylglycylcysteine	0.8	0.020

bovine milk [16] and seminal vesicles [17]. In [4] the renal oxidase was found sensitive to EDTA and can be reconstituted by addition of $CuSO_4$, whereas in [16] the milk oxidase was reconstituted with $FeSO_4$, and in [17] the seminal vesicle oxidase was found to be a flavoprotein and insensitive to EDTA. As found in [4] with the crude plasma membrane fraction, we have found that the peak II oxidase activity is inhibited by EDTA (table 1) and can be reconstituted by copper but not iron (not shown). Recent studies by Ormstad and Orrenius using purified plasma membrane fragments has allowed detection of Cu^{2+} in parallel to loss of activity following addition of H_2O_2 (S. Orrenius, personal communication). These results corroborate the characteristics described herein and provide the first strong evidence that the renal sulfhydryl oxidase is a cuproenzyme.

Incubation of the active oxidase fraction with the anti-tumor agent AT-125 resulted in loss of the activity (table 1). Thus, although the activity is separable from γ-GT, both activities are susceptible to inhibition by the same alkylating agent. Streptozotocin, another alkylating agent that inhibits γ-GT (F. B., unpublished), also inhibits the oxidase activity (table 1).

The results presented here demonstrate that a renal sulfhydryl oxidase that accepts GSH as substrate can be resolved from γ-GT. Moreover, the rapid loss of activity during purification can be overcome by reactivating the enzyme with a reducing agent. Inclusion of catalase in the assay medium improves the linearity of the assay by eliminating product inactivation. Finally, the inhibition of the activity of EDTA and recovery of activity with copper suggests that this enzyme activity may be distinct from the milk and seminal vesicle sulfhydryl oxidases.

Acknowledgements

The authors thank Professor Donald J. Reed for the AT-125 for these experiments, and Professors Reed and Alton Meister for helpful comments concerning the manuscript. This research was supported in part by NIH grants HD 11431 to F. B. and GM 28176 to D. P. J..

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