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OXIDIZED FORMS OF OVINE ERYTHROCYTE GLUTATHIONE PEROXIDASE

CYANIDE INHIBITION OF A 4-GLUTATHIONE:4-SELENOENZYME

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Summary

[^{75}Se]Glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9) containing 4 mol selenium per mol was isolated in 33% yield using 10% ethanol to stabilize the purified enzyme. When reduced with GSH and rapidly separated from GSH by gel filtration chromatography, GSH peroxidase was eluted in a labile oxidized (iodoacetate-insensitive) form which was stable at 4°C but unstable at 25°C (form A). When GSH-reduced enzyme was allowed to oxidize in the course of dialysis a more stable oxidized form was obtained (form C) which was rapidly inactivated by cyanide. Using [^{35}S]GSH, form C was shown to contain tightly bound glutathione in approx. equimolar ratio with selenium. The cyanide sensitivity of GSH peroxidase is therefore correlated with the presence of a glutathione moiety in the enzyme. The isolation of GSH peroxidase containing bound glutathione suggests that intermediates containing glutathione bound to selenium may be formed during the catalytic cycle.

Glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9), isolated from erythrocytes and other tissues, was the first naturally occurring selenoprotein to be identified [1] and, thus far is the only protein to be isolated from animal tissues with a stoichiometric amount of selenium and a well established biological function [2—5]. Despite considerable evidence that the selenium in GSH peroxidase is directly involved in catalysis, little progress

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has been made in elucidating the chemical forms and redox states of selenium in the enzyme. The GSH-reduced form of the enzyme has been reported to contain a selenocystein residue in a selenol (—SeH) form which is alkylated by iodoacetate [6]. As isolated, the enzyme is in an 'oxidized' state and is not inhibited by iodoacetate, but is inhibited after reduction with GSH and other reducing agents [3]. Conversely, the 'reduced' enzyme is not inhibited by cyanide, whereas cyanide inhibits oxidized GSH peroxidase [7]. Although these studies serve to define at least two forms of the enzyme, we have noted variations in behavior of the enzyme during isolation and storage which indicate that a more complex situation exists in regard to the redox state of selenium in the enzyme.

Methods

Pure [^{75}Se]glutathione peroxidase was isolated from erythrocytes of a ram given [^{75}Se]sodium selenite (New England Nuclear) over 6 months earlier, plus blood from unlabeled rams. A 3800-fold purification and a yield of 33% were obtained using a modification of the method of Awasthi et al. [8]. GSH peroxidase activity was assayed in a 2 ml volume by a coupled method [7], at 25°C in 0.1 M potassium (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes)) buffer (pH 7.5) containing 3 mM EDTA, 1 mM GSH, 0.11 mM NADPH and 4 μg glutathione reductase. After a 10 min preincubation, cumene hydroperoxide (0.1 mM final concentration) was added to start the reaction (1 unit (U) of enzyme activity = 1 μmol GSH oxidized/min.)

The [^{35}S]GSH (27—44 mCi/mmol) was obtained from Schwarz-Mann and carrier-free solutions prepared in 50 mM potassium phosphate (pH 7.2). TLC on cellulose in isobutyric acid/H_2O/28% NH_4OH (66 : 33 : 1), followed by direct scanning of the plate showed that about 90% of the radioactivity was associated with a ninhydrin-positive spot corresponding to GSH and the remainder with GSSG. For assay of ^{35}S in solutions, samples were dissolved in 10 ml Aquasol (New England Nuclear) and counted by liquid scintillation spectrometry using the same settings as for ^{14}C, and corrected for efficiency (89%) and decay. Total selenium was assayed by a fluorimetric procedure [2], total protein by the Lowry method using bovine serum albumin as a standard [9], and ^{75}Se by means of a well-type scintillation counter. For samples containing both ^{35}S and ^{75}Se a small correction was made for the ^{75}Se contribution to the apparent ^{35}S counting rate.

Results

GSH peroxidase was isolated in a highly active form containing 3761 μg Se/mg of protein (4 mol Se per 84 000 g of protein), with a specific activity of 1670 units/mg protein, or 440 units/μg Se (Table I). The inclusion of 10% ethanol during final purification or storage has been an important advance, since the lability of oxidized GSH peroxidase is well known [10,11]. Yields of only 2—13% have been reported previously [8,12,13], whereas we obtained 33% yield.

It became apparent during the course of our studies that purified GSH per-

TABLE I

PURIFICATION OF GLUTATHIONE PEROXIDASE

A modification of the procedure of Awasthi et al. [8] was employed. All operations were at 5—10°C. Hydroxyapatite chromatography was used to concentrate and further purify the enzyme; ultrafiltration was not employed. GSH (0.5 mM) was included in all chromatographic steps, except final chromatography. Ethanol, at a concentration of 10%, was used in a buffer composed of 50 mM potassium phosphate (pH 7.2), to stabilize the enzyme [10] during final chromatography on Sephadex G-150 and storage.

Procedure	Protein (mg)	Enzyme activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Enzyme yield (%)
Hemolysate	269 625	118 165	0.44	1	100
1. $(NH_4)_2SO_4$ (20—55% satn.)	4 830	79 261	16.4	37	67.1
2. CM-cellulose	1 992	68 482	34.4	78	57.9
3. DEAE-cellulose	74	57 685	780	1773	48.8
4. Sephadex G-150	40	42 521	1064	2418	36.0
5. Hydroxyapatite *	28.1	43 159	1536	3491	36.5
6. Sephadex G-150 **	23.3	38 877	1670	3795	32.9

* The enzyme eluted from step 4 in 10 mM potassium phosphate (pH 6.9) containing 0.5 mM GSH and 1 μM EDTA was applied directly to a 2.5 × 3.5 cm column of hydroxyapatite and eluted with a linear gradient (100 ml each) of 10—200 mM potassium phosphate (pH 6.9) containing 0.5 mM GSH and 1 μM EDTA. The eluted enzyme was dialyzed against 50 mM potassium phosphate (pH 7.2) containing 5% ethanol and then against the same buffer containing 10% ethanol.

** The dialyzed enzyme from step 5 was applied to a 2.5 × 82 cm column of Sephadex G-150 and eluted with 50 mM potassium phosphate (pH 7.2) containing 10% ethanol.

oxidase underwent changes in its stability, as well as its susceptibility, to cyanide during handling. The results of a systematic investigation of these phenomena are summarized in Table II and Fig. 1. Freshly isolated GSH peroxidase was not inhibited (less than 10% loss of activity) by iodoacetate, but was rapidly inactivated by cyanide. After the addition of GSH, the reduced enzyme (sample I) was completely (over 90%) inhibited by iodoacetate (Table II), but was not inhibited by cyanide (Fig. 1, panel A, I). Aliquots of reduced enzyme were then treated so that oxidation took place under three different conditions. Sample I was immediately subjected to gel filtration chromatography so that GSH would be separated rapidly from the reduced enzyme and the enzyme would undergo autooxidation under conditions where little or no GSH was present. Sample II was oxidized with substrate levels of H_2O_2. Sample III was dialyzed against buffer containing 10% ethanol, so that GSH was gradually removed under aerobic conditions (this procedure is similar to that employed during isolation). Following these treatments all three samples of GSH peroxidase were insensitive to iodoacetate, indicating that oxidation had taken place (Table II). After the gel filtration treatment, sample I showed rapid loss of activity when incubated at 25°C with cyanide (Fig. 1, panel B, I); however, the sample I control incubated with potassium chloride at 25°C also showed rapid loss of activity (see below). Sample II (oxidized with H_2O_2) was inactivated slowly by incubation with cyanide (Fig. 1, panel A, II). Sample III (dialyzed) was rapidly and completely inactivated by incubation with cyanide (Fig. 1, panel A, III). Samples II and III were also subjected to gel filtration and then

TABLE II

PROPERTIES OF VARIOUS FORMS OF GLUTATHIONE PEROXIDASE

The inhibitions of GSH peroxidase by cyanide and iodoacetate was determined as follows. Samples of enzyme were diluted about 10 to 100-fold (to approx. $2 \cdot 10^{-7}$ M Se) in 20 mM Hepes buffer, pH 7.5, containing bovine serum albumin (50 μg/ml) and incubated at 25°C in sealed tubes with (a) 1 mM sodium iodoacetate, pH 7.5, or 1 mM sodium acetate (control) for 20 min; (b) 10 mM KCN, pH 7.5, or 10 mM KCl (control) for up to 250 min. Small aliquots taken at zero time and subsequent intervals were assayed for GSH peroxidase and the activity remaining in the samples calculated. Inhibition by iodoacetate was defined as a loss of 90% or more of the activity within 20 min. Inhibition by cyanide indicates 80% or more loss of activity within 2 h (see Fig. 1 for more complete information).

GSH-peroxidase sample	Inhibitor		Stability	Designated form of enzyme
	Iodoacetate (1 mM)	KCN (10 mM)		
Freshly isolated enzyme [a]	No inhibition	Inhibition	Stable	Oxidized C
Reduced with GSH (Sample I, Fig. 1)				
Before gel filtration	Inhibition	No inhibition	Stable	Reduced
After gel filtration	No inhibition [b]	Inhibition [c]	Unstable	Oxidized A
Reduced with GSH, oxidized with HOOH (Sample II, Fig. 1)				
Before and after gel filtration	No inhibition	Partial inhibition	Stable [d]	Oxidized B
Reduced with GSH and dialyzed (Sample III, Fig. 1)				
Before and after gel filtration	No inhibition	Inhibition	Stable [d]	Oxidized C
Stored in 10% ethanol [e]	No inhibition	No inhibition	Stable	—

[a] Eluted from Sephadex G-150 column (Table I), treated with 0.5 mM GSH, then dialyzed against 10% ethanol in 50 mM potassium phosphate (pH 7) and stored at 4°C.

[b] No difference between treatment with sodium iodoacetate vs. sodium acetate; however, this form of the enzyme is unstable at 25°C.

[c] Controls incubated with 10 mM KCl also lost activity, but treatment with cyanide resulted in a somewhat more rapid loss of activity.

[d] Some loss of activity after long incubation but not exceeding, 20% of the initial activity (See Fig. 1).

[e] Freshly isolated enzyme (see above) was stored for 5 weeks at 4°C in 50 mM potassium phosphate (pH 7) plus 10% ethanol; 70% of the activity was retained.

assayed for cyanide sensitivity as a control for sample I; gel filtration did not alter the cyanide sensitivity of these samples (Fig. 1, panels B, II and III).

The control samples of GSH peroxidase (incubated with KCl or sodium acetate rather than KCN or sodium iodoacetate) revealed marked differences in the stability of GSH peroxidase following the various treatments. The enzyme reduced with GSH was very stable (Fig. 1, panel A, I). However, after gel filtration, the same sample of enzyme rapidly lost activity under the same conditions (Fig. 1, panel B, I), and retained only about 30% of its activity after 150 min incubation at 25°C. This indicates that autooxidation of reduced GSH peroxidase in the absence of GSH tends to produce a temperature-labile form. This form, however, could be stored for several days at 4°C without appreciable loss of activity. Sample II showed excellent stability (Fig. 1, panels A, II and B, II). Sample III showed some loss of activity at 25°C after long incubation times (Fig. 1, panel A, III and B, III), but showed little loss of activity during the first 30 min during which cyanide inactivation took place.

During purification of GSH peroxidase by hydroxyapatite chromatography in the presence of 0.5 mM GSH, the eluted enzyme was found to have a relatively high A_{255}/A_{280} ratio. The elevated A_{255} was due in part to GSSG (assayed

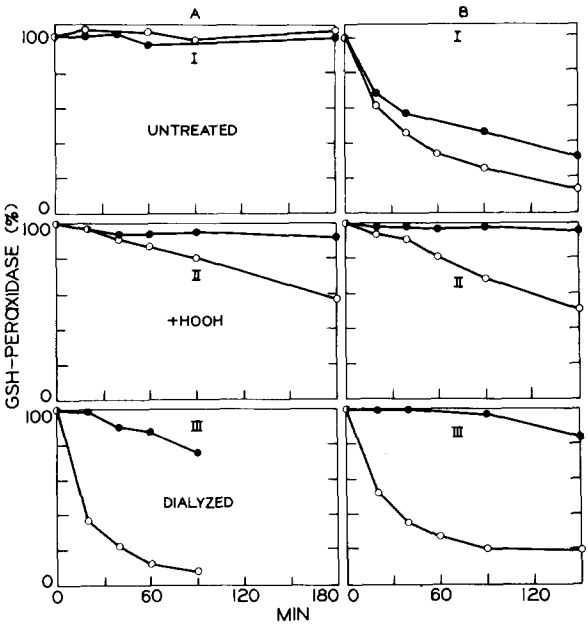

Fig. 1. Effect of 10 mM cyanide on GSH peroxidase activity of samples assayed before or after chromatography on Sephadex G-150. GSH peroxidase (4.5 ml, 0.2 μmol Se) in buffer (50 mM potassium phosphate (pH 7.2) plus 10% ethanol) was treated with 40 μl of 20 mM [^{35}S]GSH (0.8 μmol) for 20 min at 25°C and assayed for iodoacetate and cyanide sensitivity, then divided into three parts: one aliquot of the reduced enzyme (sample I) was chromatographed immediately (see Fig. 2) without further treatment; sample II (0.044 μmol Se) was treated with excess peroxide (5 μl of 0.5 M H_2O_2) (2.5 μmol) for 20 min at 25°C and then chromatographed on a second column; sample III was exhaustively dialyzed for 3 days at 4°C against the buffer (see above), then chromatographed. Samples were incubated with 10 mM KCN (○———○) or KCl (●———●), as well as iodoacetate (see Table II), before (A) and after (B) chromatography (See Fig. 2).

with glutathione reductase) that was coeluted with the enzyme, but dialyzed enzyme still had a higher A_{255}/A_{280} ratio compared to reduced enzyme. Studies were therefore undertaken with [^{35}S]GSH, in conjunction with the studies described above, to determine if a form of GSH peroxidase containing bound glutathione might be isolated. When enzyme was reduced with [^{35}S]GSH and immediately chromatographed on Sephadex G-150 (sample I), only a little ^{35}S was eluted with the enzyme (S/Se = 0.128 ± 0.004), and most was eluted as a peak of low molecular weight (Fig. 2). Similarly, enzyme reduced with GSH and then oxidized with excess H_2O_2 (sample II) contained little enzyme-bound ^{35}S (S/Se = 0.123 ± 0.10). In contrast, sample III, which had been reduced with [^{35}S]GSH, then dialyzed prior to chromatography, showed a major peak of ^{35}S eluting with the ^{75}Se and enzyme activity (S/Se = 1.188 ± 0.028) and only a minor ^{35}S peak of lower molecular weight. Subtracting the S/Se ratio of sample I from sample III gives a net value of 1.06 mol S bound per mol Se.

In other studies (data not shown) it was found that the ^{35}S bound to GSH peroxidase could be removed almost completely (S/Se of the enzyme = 0.077) in a low molecular weight form by rechromatographing the labeled enzyme peak in the presence of 0.5 mM unlabeled GSH; when the low molecular weight ^{35}S peak was lyophilized and desalted by thin-layer electrophoresis, followed

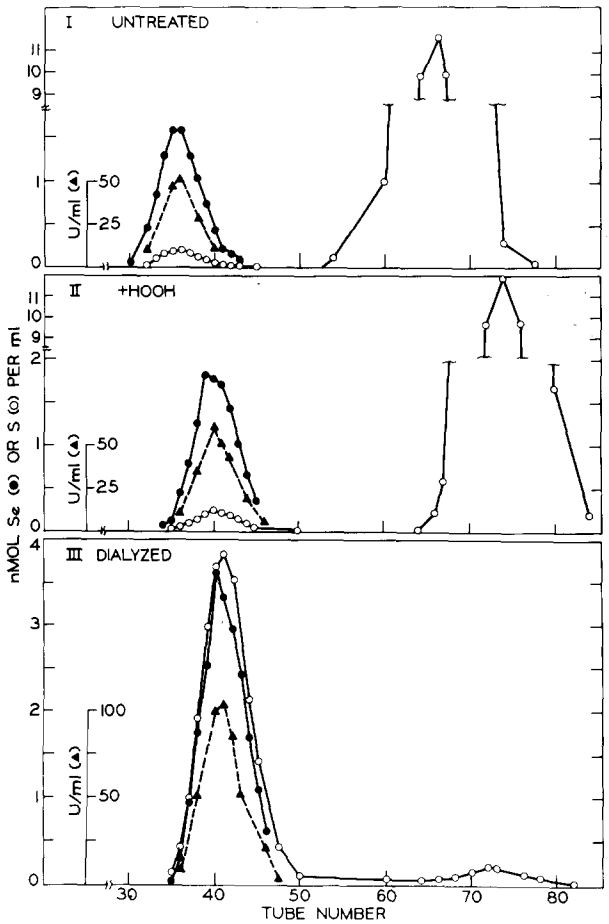

Fig. 2. Binding of [^{35}S]glutathione to [^{75}Se]glutathione peroxidase. Samples treated in various ways after reduction of the enzyme with labeled glutathione (see Fig. 1) were chromatographed on 2.5 X 42 or 45 cm columns of Sephadex G-150, and eluted with 50 mM potassium phosphate (pH 7.2) containing 10% ethanol, at 4°C, 30 ml/h. Fractions of approx. 3 ml were collected and assayed for ^{75}Se (●———●), ^{35}S (○———○), and GSH peroxidase activity (▲———▲). The five fractions having the highest Se content were averaged for the GSH peroxidase peak and the molar S/Se ratio reported in the text as the mean ± S.D.

by TLC and scanning, the ^{35}S was associated with ninhydrin-positive spots having the same mobility as GSH and GSSG. This result indicates that the ^{35}S bound is a glutathione moiety, not a radioactive impurity. The bound ^{35}S was also released by treatment with cyanide.

Discussion

The major significance of the work reported here is the clear demonstration that GSH peroxidase can be prepared in a number of different, oxidized forms, of widely varying properties. The description of procedures for preparing these forms and differentiating them by means of their temperature stability, or their reactivity with cyanide and iodoacetate, opens up opportunities for detailed

investigation of the form of selenium in the enzyme in various oxidation states.

Another important finding is the correlation of cyanide sensitivity with the presence of bound glutathione in GSH peroxidase. We had previously reported that cyanide inactivated the oxidized enzyme [7], and one other laboratory has recently confirmed this finding using a similar protocol [11]. However, we have observed (Table II) that oxidized (iodoacetate-insensitive) GSH peroxidase sometimes showed no reactivity with cyanide, as in the case of enzyme stored for long periods. Our discovery that cyanide sensitivity is correlated with the presence of bound glutathione in GSH peroxidase (form C) suggests that the mechanism of cyanide sensitivity may be related to the binding of glutathione to the active site of the enzyme, and that upon storage the enzyme releases the bound glutathione and becomes insensitive to cyanide.

The observation that the glutathione-GSH peroxidase complex survives gel filtration chromatography indicates that the binding is fairly strong. Our study and that of Flohe et al. [14] show that no complex is formed when GSH peroxidase is treated with labeled GSH and immediately subjected to gel filtration chromatography. This does not support the possibility that electrostatic interactions are the basis for the binding. We prefer to believe that binding of glutathione to GSH peroxidase involves the formation of a covalent Se-S or Se(O)-S bond at the active site of the enzyme.

The fact that an oxidized form of GSH peroxidase can be isolated with glutathione bound to it provides support for a proposed mechanism [5,15] in which intermediates containing glutathione bound to Se, such as E-Se-SG or E-Se(O)SG, would be formed during the catalytic cycle. Also, formation of a glutathione complex may clarify some previously reported physicochemical properties of GSH peroxidase, such as divergent X-ray photoelectron spectroscopy measurements [16,17], ultraviolet spectra and electrophoretic mobility.

The existence of multiple oxidized forms of GSH peroxidase emphasizes that the common practice of describing selenoenzymes as being 'oxidized' (or 'reduced') is an oversimplification that may confuse experimentation and interpretation of results. The use of inhibitors such as iodoacetate and cyanide as chemical probes to help define the form of selenium, in conjunction with physicochemical and chemical studies of selenoenzymes. is strongly recommended.

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