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REACTION OF CYANIDE WITH GLUTATHIONE PEROXIDASE[1]

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SUMMARY: An oxidized form of ovine erythrocyte GSH peroxidase (Form C) that contains bound glutathione in equimolar ratio to the enzyme selenium is inactivated by cyanide. When Form C was treated with 1 or 10 mM KCN at pH 7.5, there was a rapid increase in ultraviolet absorption at 250 nm, S-cyanoglutathione was released, and the enzyme was reduced, as shown by inactivation with iodoacetate (1 mM, pH 7.5) and uptake of label from [^{14}C]iodoacetate in equimolar ratio to enzyme selenium. These observations suggest that glutathione is bound to enzyme selenium by a selenenyl-sulfide linkage (E-Se-SG) which is cleaved by cyanide to release a selenol and S-cyanoglutathione; spontaneous oxidation of the selenol to a labile oxidized form of GSH peroxidase leads to irreversible inactivation.

INTRODUCTION: GSH peroxidase (GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) is a tetrameric enzyme containing 4 g-atoms of selenium per mole and no heme. Although originally thought to be insensitive to cyanide, an oxidized form of the enzyme was later shown to be inactivated by cyanide, whereas the reduced enzyme was not (1). We have recently discovered (2) that cyanide sensitivity is correlated with the presence of a glutathione moiety in the oxidized enzyme (1 mole/g-atom Se) which may be bound covalently to Se in a selenenylsulfide form such as E-Se-SG. The glutathionyl derivative of the enzyme (designated Form C) is fairly stable and is readily inactivated by 10 mM cyanide under mild conditions (pH 7.5, 25°), whereas other oxidized forms of the enzyme are either unstable or are less sensitive to cyanide (2). The cyanide susceptibility of GSH peroxidase thus provides a useful tool

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[2]Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate; GSCN, S-cyanoglutathione.

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for differentiating oxidized forms of this selenoenzyme, and a better understanding of this reaction may be helpful in further characterization of the enzyme and its selenium moiety.

We have observed that if GSH peroxidase is reduced with GSH and separated from GSH by gel filtration at 4°, an oxidized form of the enzyme (Form A) is obtained which undergoes spontaneous inactivation when diluted and incubated at 25° (2). We decided to investigate whether cyanide might also reduce the enzyme, leading to indirect inactivation of the enzyme by a similar process.

METHODS: Pure GSH peroxidase was isolated from ovine erythrocytes as previously described (2) and stored at 4° as a stock solution in buffer (50 mM potassium phosphate, pH 7.2, containing 10% ethanol). [^{35}S]GSH (1.3 mCi/mmol) was obtained from Schwarz-Mann and purified by thin layer electrophoresis at 10° on cellulose plates at pH 1.3 (formic acid:acetic acid:water, 100:150:750) and at pH 5.6 (pyridine:acetic acid:water, 20:5:2000). [^{14}C]iodoacetate (12.6 mCi/mmol) was obtained from New England Nuclear. S-Cyanoglutathione was synthesized by reacting GSH with 2-nitro-5-thiocyanobenzoic acid (3). Procedures for assay of ^{14}C and ^{35}S by liquid scintillation spectrometry, total Se by a fluorometric procedure, protein by a Lowry procedure, and GSH peroxidase by a coupled assay were described previously (2).

For preparation of oxidized Form C of GSH peroxidase, a desired volume of the stock enzyme solution was treated with either unlabeled or [^{35}S]GSH (SH/Se = 4) for 20 min at 25°. After the enzyme was reduced (iodoacetate-sensitive), the mixture was exhaustively dialyzed at 4° against buffer (10% ethanol in 50 mM KPO_4, pH 7.2) to give oxidized Form C. Sensitivity to iodoacetate and to cyanide was monitored as previously described (2) to verify the oxidation state of the enzyme. The molar S/Se ratio in the dialyzed enzyme prepared with [^{35}S]GSH ranged between 0.9 and 1.18.

RESULTS: To test whether a reduced form of GSH peroxidase might be the initial product of the cyanide treatment, we decided to trap the reaction product with iodoacetate, which is known to react rapidly with reduced GSH peroxidase (4), but does not inhibit the oxidized enzyme. Oxidized Form C of the enzyme labeled with ^{35}S from GSH was treated with cyanide in the presence of iodoacetate at 15°, to minimize thermal inactivation of any enzyme that underwent autooxidation to Form A (2). GSH peroxidase was inactivated rapidly when treated with KCN plus iodoacetate (Fig. 1), whereas little or no activity was lost in controls treated with KCl plus iodoacetate (or with KCN alone). When the reaction mixture was chromatographed

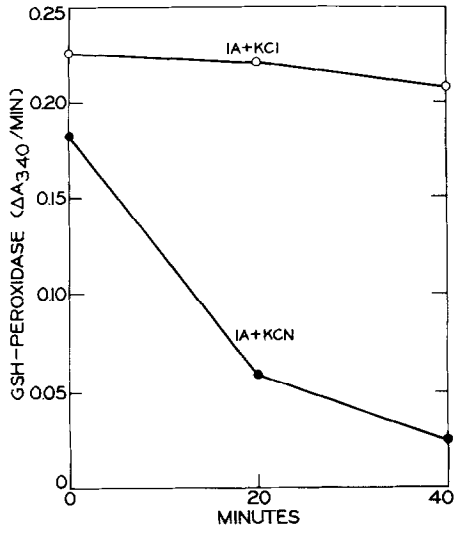


Fig. 1: Inhibition of cyanide-treated GSH peroxidase by iodoacetate. [^{35}S]GSH-labeled oxidized Form C (Se = 15.4 μM) in 1.5 ml of 20 mM HEPES (pH 7.5) containing 10% ethanol and 1 mM iodoacetate was treated at 15° with 10 mM KCN (pH 7.5) (●) or 10 mM KCl (○) (control). Aliquots were taken immediately after mixing (zero time), 20, and 40 min, diluted 1:40 in 20 mM HEPES (pH 7.5) plus 10% ethanol, and 10 μl aliquots assayed for GSH peroxidase activity.

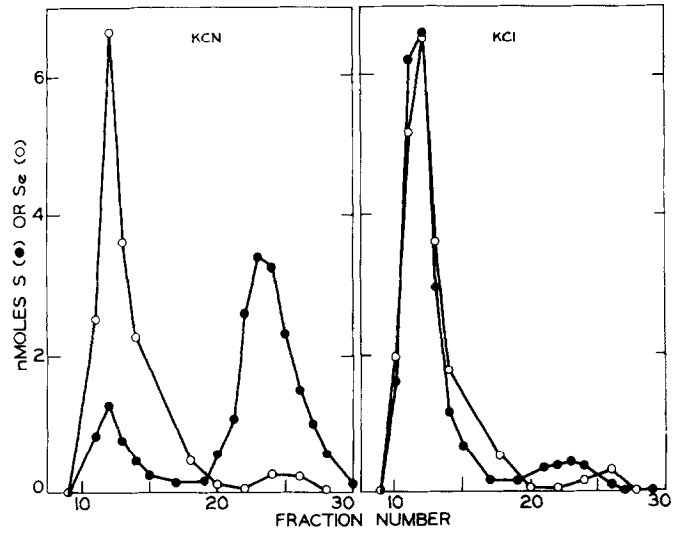


Fig. 2: Release of ^{35}S from [^{35}S]GSH peroxidase (oxidized Form C) treated with cyanide. The reaction mixture from Fig. 1 was chromatographed on Sephadex G-25 (1.5 x 17 cm), equilibrated and eluted with 50 mM potassium phosphate (pH 7.2) plus 10% ethanol at 4°. Flow rate was 20 ml/h, and 1 ml fractions were collected.

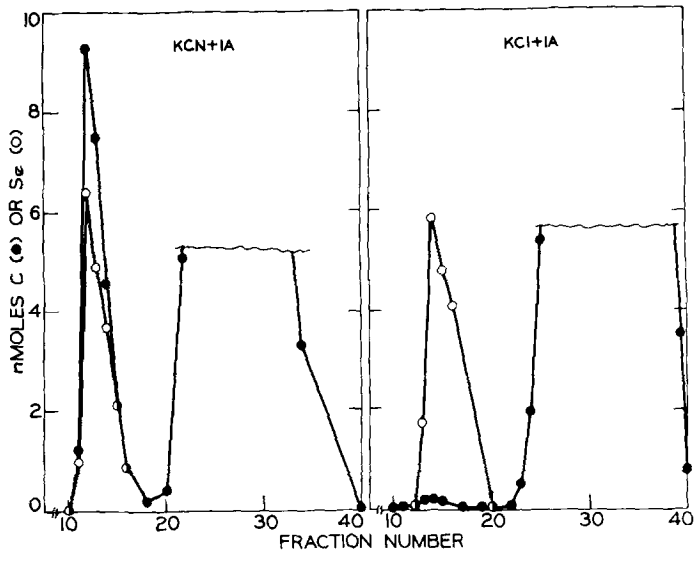
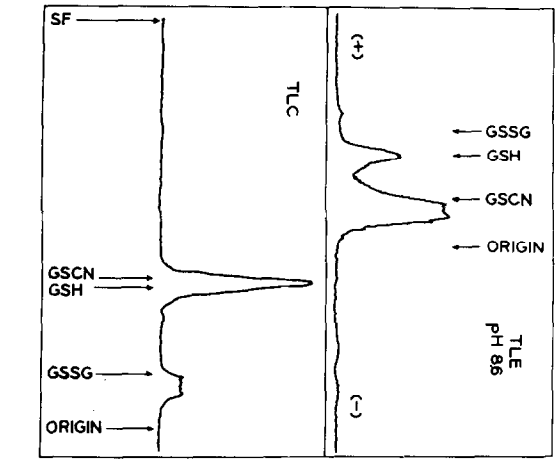


Fig. 3: Alkylation of cyanide-treated GSH peroxidase by [^{14}C]-labeled iodoacetate. Stock [^{14}C]-iodoacetate was prepared by mixing 200 µl of 10 mM [^{14}C]iodoacetate with 800 µl of 10 mM unlabeled iodoacetate. Oxidized Form C (15.8 µM Se) prepared with unlabeled GSH in 1.5 ml of 20 mM HEPES (pH 7.5) containing 10% ethanol was treated at 15° with 10 mM KCN (pH 7.5) or KCl in the presence of 1 mM [^{14}C]iodoacetate for 40 min. The reaction mixture was then chromatographed on Sephadex G-25 as described in Fig. 2.

on Sephadex G-25, ^{35}S was nearly completely released from the enzyme, whereas enzyme treated with KCl plus iodoacetate was eluted with ^{35}S and Se in a 1:1 molar ratio (Fig. 2).

The trapping experiment was repeated using [^{14}C]iodoacetate. As shown in Fig. 3, the enzyme treated with KCN plus labeled iodoacetate incorporated ^{14}C in slightly over equimolar ratio with Se, whereas the control treated with KCl in place of KCN incorporated only a trace of radioactivity.

The low molecular weight ^{35}S released from [^{35}S]GSH peroxidase (see Fig.2) was concentrated under vacuum by rotoevaporation at 40° , then subjected to thin layer chromatography or to electrophoresis. The major ^{35}S peak co-chromatographed with GSCN and GSH at R_f = 0.37 (with the remainder in the area of GSSG); electrophoresis at pH 8.6 separated GSCN from GSH and showed that ^{35}S was mainly associated with GSCN (Fig. 4).



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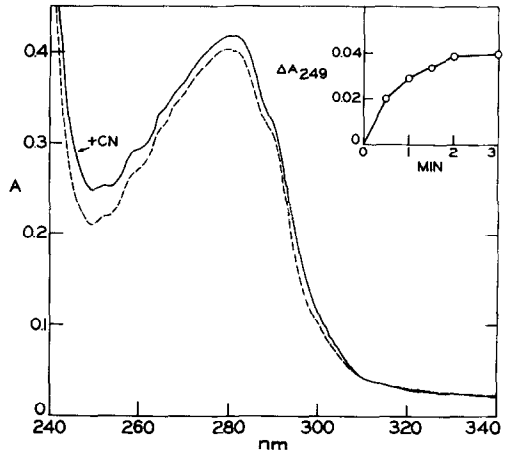

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Fig. 4: Properties of ^{35}S released from Form C of GSH peroxidase by cyanide. Fractions 22-25 of CN-treated GSH peroxidase (see Fig. 2) were lyophilized, then chromatographed (lower panel) on cellulose plates in n-butanol:acetic acid:water (12:3:5) or electrophoresed (upper panel) in barbital buffer, pH 8.6, for 50 min at 10°, 20 V/cm. The plates were scanned with a windowless proportional counter. Arrows show position of known compounds.

Fig. 5: Spectrum of oxidized Form C of GSH peroxidase before and after treatment with cyanide. The sample cell (path length 1 cm) contained 2 ml of oxidized GSH peroxidase (Form C) in 50 mM potassium phosphate (pH 7.2) containing 10% ethanol, to which was added 0.985 ml of 61 mM HEPES (pH 7.5) containing 10% ethanol (14.5 µM Se final concentration) The reference cell contained everything except enzyme. The temperature was maintained at 15°; gas phase = air. The spectrum was recorded in an Aminco DW 2 spectrophotometer (1.0 nm band pass, 0.5 A full scale), then 15 µl of 0.2 M KCN (pH 7.5) was added to bring the cyanide concentration to 1 mM. The absorbance change was monitored at 249 nm (inset), and a final spectrum (solid line) was recorded after A_{249} had stabilized. A similar sample of enzyme treated with 1 mM KCl (control) showed no spectral changes.

If inactivation by cyanide at pH 7.5 occurred by the indirect mechanism described, the initial step of reduction might be experimentally separated from the subsequent steps of oxidation and inactivation. Upon reduction there should be a spectral change corresponding to the appearance of the selenolate chromaphore, $E\text{-}Se^-$ (assuming this is the reduced form of the enzyme). Treatment of Form C of GSH peroxidase with 1 mM KCN at 15° did result in a rapid increase in ultraviolet absorption that was complete within 2 minutes (Fig. 5), without loss of enzyme activity. An aliquot of the enzyme treated with 1 mM iodoacetate was 87% inhibited, showing

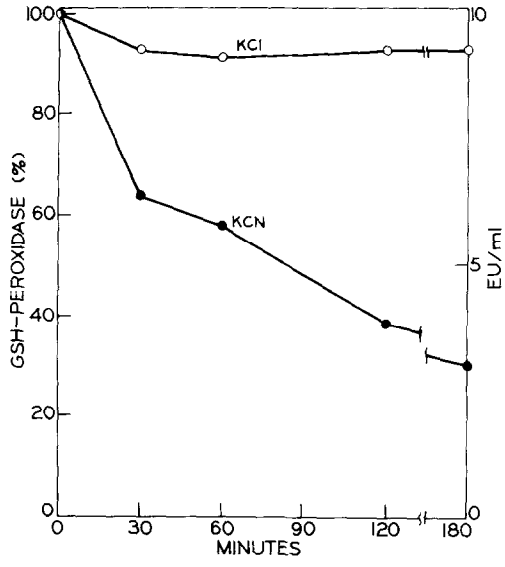

Fig. 6: Inactivation of cyanide-treated GSH peroxidase. Aliquots of the enzyme which had been treated with 1 mM KCN at 15° (see Fig. 5) and the control sample treated with 1 mM KCl were diluted 1:40 with the buffer from the reference cell, incubated at 25°, and assayed at zero time and subsequent intervals for GSH peroxidase activity. The KCN-treated enzyme was also tested for inhibition by 1 mM iodoacetate (87% inhibited at zero time); KCl-treated enzyme was not inhibited by iodoacetate.

reduction had taken place. Upon dilution and incubation at 25°, the cyanide-treated enzyme was inactivated, whereas a control treated with KCl retained nearly all of its activity (Fig. 6).

DISCUSSION: There are two possible ways that a Se-S bond in E-Se-SG can be cleaved by cyanide: $E\text{-}SeSG + CN^- \longrightarrow E\text{-}SeCN + GS^-$ (reaction 1); $E\text{-}SeSG + CN^- \longrightarrow E\text{-}Se^- + GSCN$ (reaction 2). The irreversible inactivation of GSH peroxidase by cyanide at pH 7.5 could be explained if cyanide reacted with Se as in reaction 1, followed by β-elimination of $SeCN^-$ (5), unless the E-SeCN derivative would be repaired during the preincubation with GSH prior to assay of enzyme activity, as occurs with the thiocyanate derivative of papain (6): $E\text{-}SeCN + GS^- \longrightarrow E\text{-}Se^- + GSCN$ (reaction 3).

However, it appears that cyanide treatment at neutral pH can cause inactivation of GSH peroxidase without removing selenium, by an indirect

mechanism involving rapid formation of a reduced form of the enzyme (reaction 2) which then oxidizes to an unstable form that is inactivated during the incubation at 25°. By following the spectral changes induced by 1 mM KCN at 15°, it was possible to show that a reaction with cyanide did occur very rapidly, and this reaction did not cause loss of enzyme activity. The cyanide-treated enzyme was sensitive to iodoacetate, indicating the enzyme was in a reduced form. The reaction of cyanide with E-SeSG would be expected on thermodynamic grounds to liberate an ionized selenol (RSe^-) rather than the thiol, by analogy with the cleavage of unsymmetrical disulfides by cyanide, because the selenol has a lower pK than the thiol group. The observation that [^{35}S]GSCN is released upon treatment of the [^{35}S]glutathione-labeled enzyme is further evidence that the cleavage takes place by reaction 2.

A number of different mechanisms for cyanide inactivation of GSH peroxidase are possible. Although the present work provides a reasonable mechanism for inactivation that does not involve the direct formation of a selenocyanate derivative of the enzyme, such a form could arise by the further reaction of cyanide with an oxidation product such as a selenenic acid (E-SeOH), formed by oxidation of the reduced selenium generated in the initial reaction with cyanide.

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