GLUTATHIONE PEROXIDASE: INHIBITION BY CYANIDE AND RELEASE OF SELENIUM[1]

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Summary: Treatment of highly purified ovine erythrocyte glutathione peroxidase with KCN resulted in loss of enzyme activity and release of selenium from the enzyme. The inactivation by cyanide was time-dependent and the rate was strongly influenced by temperature, concentration of cyanide, oxidation state of the enzyme, and pH. The pH effect could be explained on the basis of the increasing proportion of cyanide ion with increasing pH. Inhibition could be prevented by prior reduction of the purified enzyme with glutathione, dithiothreitol, or dithionite. Oxidation with cumene hydroperoxide was necessary to demonstrate cyanide inhibition of glutathione peroxidase activity in rat liver cytosol. These observations explain why cyanide inhibition of glutathione peroxidase has not been noted previously and provide new approaches for studying the chemical nature of the enzyme-selenium.

Glutathione peroxidase (glutathione: hydrogen peroxide oxidoreductase, EC 1.11.1.9) (GSH-Px) was discovered in 1957 by Mills (1). A unique feature of GSH-Px noted by Mills and by subsequent investigators (2) was its insensitivity to cyanide, in contrast to other peroxidases. This could be rationalized by the finding that GSH-Px lacked heme and various other prosthetic groups (3). More recently it was discovered that the enzyme required selenium for activity (4) and contained 4 g-atoms of Se/mole (84,000 g) of protein (5,6). Despite extensive investigations of the enzyme isolated from a variety of species and tissues (reviewed in 7,8), little is known about the nature of selenium in GSH-Px.

In an extension of previous studies (9) using proteolytic digestion of GSH-Px purified from sheep erythrocytes, one of us (S.-H. O.) discovered that the use of papain activated with cyanide (10) caused selenium to be released completely from GSH-Px in a low molecular weight form. It was then found that

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cyanide alone would bring about the same effect. The present paper describes this finding and demonstrates that, under the appropriate conditions, GSH-Px is readily inhibited by cyanide.

MATERIALS AND METHODS

^{75}Se-labeled GSH-Px was isolated (6) from erythrocytes of an ewe given [^{75}Se] sodium selenite (New England Nuclear, Boston, MA). Radioactivity was determined by means of a well-type gamma scintillation counter and total selenium by a fluorometric procedure (6).

The purified enzyme was stored at 4°C as a stock solution (0.04 mg/ml) in 5 mM sodium phosphate, pH 7, containing 10% ethanol. Enzyme activity was measured by a coupled spectrophotometric procedure using glutathione reductase and cumene hydroperoxide (ICN K & K Chemicals, Cleveland, OH) as described elsewhere (11) with the following changes: (1) KCN was omitted; (2) 0.1 M potassium (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate) (HEPES), pH 7.5, was used in place of pH 7 potassium phosphate buffer; (3) preincubation time with GSH was increased to 10 min. Under these conditions the purified enzyme oxidized 250-300 µmoles GSH/min/µg Se.

To study the release of selenium from GSH-Px, the enzyme was treated with KCN under conditions similar to those used in the initial study using cyanide-activated papain. One ml of a stock solution of [^{75}Se]-GSH-Px (40 µg containing 22,300 cpm) was diluted to 20 ml with water. KCN (65 mg) was added, without neutralization, to give a final concentration of 0.05 M, and the mixture incubated at 40°C for 16 hours. After incubation, the sample was subjected to ultrafiltration on a PM-10 membrane (Amicon Corp).

Unless otherwise indicated, studies of GSH-Px inactivation with cyanide and other compounds were carried out at 25°C in stoppered tubes containing 2×10^{-2} M HEPES (pH 7.5), 10^{-3} M KCN (Mallinkrodt, St. Louis, MO), and 2.5×10^{-8} M GSH-Px (10^{-7} M Se) and terminated by dilution (200x) into the assay medium. Controls containing KCl in place of KCN were run simultaneously.

RESULTS

Treatment of GSH-Px with cyanide released 92% of the ^{75}Se from the enzyme and 85% was recovered in the PM-10 ultrafiltrate[2]. The filtrate was concentrated 10-fold by evaporation in an open vessel at room temperature and chromatographed on Sephadex G-10 (Figure 1). A major ^{75}Se peak was located at 154 ml. Recovery of ^{75}Se off the column was 84%. Although the major ^{75}Se peak eluting near the position for GSH was not identified in this experiment, subsequent experiments have shown that selenite is eluted near GSH on G-10 columns under conditions of low ionic strength. We have identified selenite and selenocyanate

[2]Earlier experiments had shown that ^{75}Se was not released from heat (65°C) denatured GSH-Px (6) and only 12% of the enzyme ^{75}Se was ultrafiltrable after treatment with 1 N NH_4OH (9).

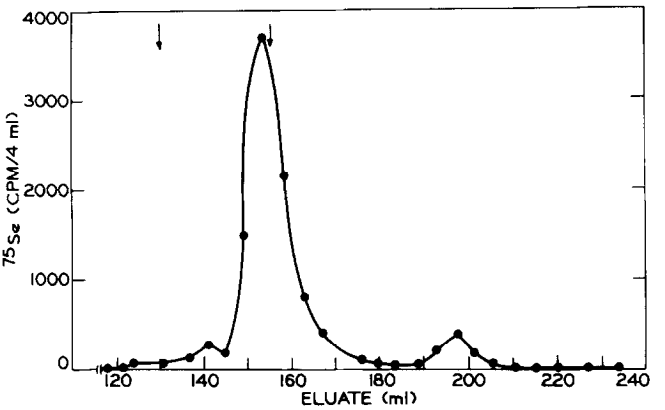

Figure 1. Sephadex G-10 chromatography of ultrafiltrable ^{75}Se released from glutathione peroxidase by treatment with cyanide. After ultrafiltration and concentration of the ^{75}Se released from GSH-Px by cyanide (see text) the sample was chromatographed at 25°C on a 1.8 x 130 cm Sephadex G-10 column (bed volume 330 ml) eluted with 10% ethanol and 4-ml fractions collected. Arrows indicate the elution positions for GSSG (centered at 130 ml) and GSH (155 ml) obtained in a calibration run.

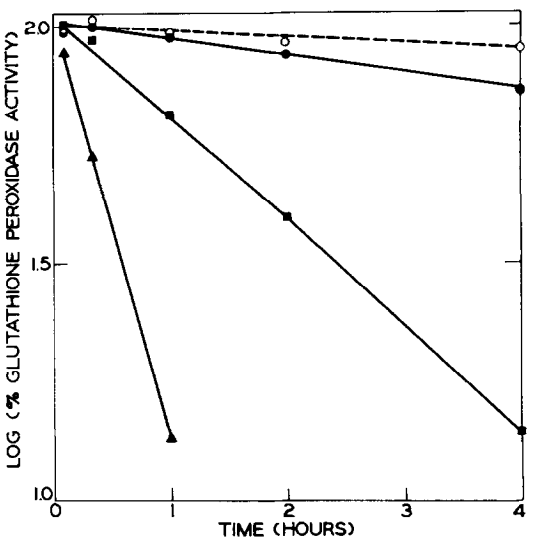

Figure 2. Effect of KCN concentration on inhibition of glutathione peroxidase activity. At the various times indicated aliquots were removed and assayed for residual enzyme activity. Incubations were at pH 7.5 in either 1 mM KCl (o) or in 0.1 mM (●), 1 mM (■), or 10 mM (▲) KCN which had been neutralized (pH 7.5) with HCl.

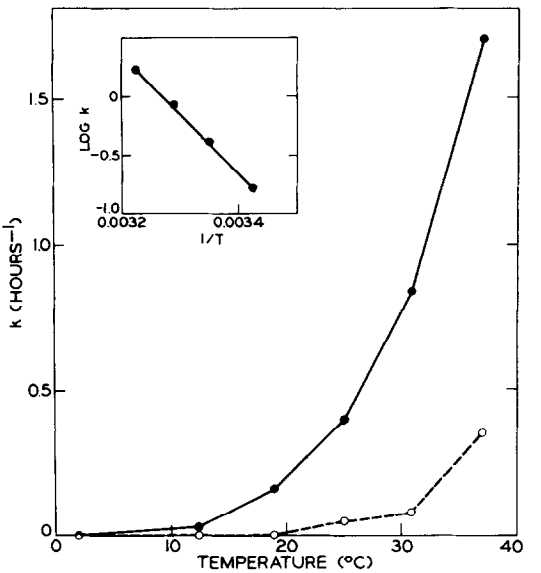

Figure 3. Effect of temperature on the rate of glutathione peroxidase inactivation by KCN. Reactions were at pH 7.5 with 1 mM KCN (●) or 1 mM KCl (○). Pseudo first-order rate constants were obtained from data graphed as in Figure 2. The inset shows an Arrhenius plot of the data from cyanide-treated enzyme between 19-37°C.

($SeCN^-$) as two of the products formed when GSH-Px is treated with cyanide, but it is not implied that either of these is necessarily the initial product of the cyanide reaction.

To determine whether cyanide could inhibit GSH-Px, the activity of the enzyme treated with 0.1-10 mM KCN was assayed after various time intervals (Figure 2). Plotting the log of the percent GSH-Px activity remaining vs. time gave straight lines; thus an apparent first-order kinetic behavior is observed when cyanide is present in large excess. From similar studies it was found that the rate of inactivation was proportional to KCN concentration up to 5 mM. About 1.5 hours were necessary to inhibit 50% of the GSH-Px activity when using KCN at 1 mM.

NaN_3, KOCN, KSCN, KSeCN, KBr, KI, and β-aminopropionitrile at concentrations of 10 mM did not inhibit GSH-Px when the enzyme was treated with these compounds for at least 2 hours at pH 7.5 and 25°C. However, the same enzyme

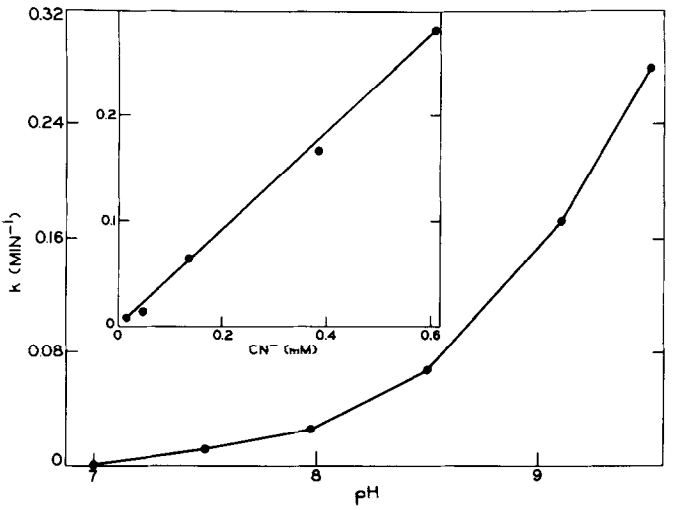

Figure 4. Effect of pH on the rate of glutathione peroxidase inactivation by 1 mM KCN. Incubations were carried out at 25°C in 0.05 M HEPES (pH 7, 7.5, 8) or glycylglycine (pH 8.5, 9, 9.5). Pseudo first-order rate constants were obtained from data graphed as in Figure 2 and plotted against the final pH. The inset shows the relation between these rate constants (pH 7.5 to 9.5) and the concentration of free cyanide ion calculated from the measured pH and using a pK of 9.31 for HCN (13).

preparation was reversibly inhibited by 1 mM Na_2SO_3, as reported by others (12).

The effect of temperature on the pseudo first-order rate constants, determined from the slopes of lines as in Figure 2, is presented in Figure 3. The inset shows that the rates of inactivation for the cyanide-treated enzyme between 19-37°C gave a straight line in an Arrhenius plot, from which an apparent energy of activation (ΔH_a) of 20 kcal/mole was calculated, after correction for inactivation in the absence of cyanide.

Figure 4 shows that the rate of inactivation increased with increasing pH; a close correspondence between the rate of inactivation and the calculated cyanide ion concentration at each pH is evident. In the absence of KCN no loss of GSH-Px activity was detected between pH 7 and 9.5, but was evident above pH 10 and below pH 6.

Table I demonstrates that GSH-Px was protected against cyanide inactiva-

Table I

EFFECT OF PRETREATMENT OF GSH-Px ON INACTIVATION BY CYANIDE[a]

	% GSH-Px Remaining	
Pretreatment	Control	Cyanide
None	99	21
Glutathione	105	100
Cumene hydroperoxide	96	22
Dithiothreitol	99	104
Sodium arsenite	102	21
Sodium arsenite[b]	104	30
Sodium dithionite ($Na_2S_2O_4$)	96	105

[a]Glutathione peroxidase was preincubated with 2×10^{-4} M of the above compounds for 5 min. prior to the addition of either 10 mM KCN or KCl (control). Aliquots were assayed for glutathione peroxidase as described in Methods immediately following CN^- addition (zero time) compared to 60 min. later.

[b]Sodium arsenite tested at 2×10^{-3} M.

tion by its substrate, GSH, and also by other reducing agents such as dithiothreitol and sodium dithionite ($Na_2S_2O_4$). The oxidizing substrate, cumene hydroperoxide, neither protected nor accelerated the rate of loss of activity, suggesting that GSH-Px was fully susceptible as isolated.

In contrast to purified enzyme, inhibition of the GSH-Px activity of rat liver cytosol (which had been dialyzed for 42 hours against dilute phosphate buffer, pH 6.5, containing 10% ethanol) required preincubation with cumene hydroperoxide and the use of 40 mM KCN at pH 9. This suggests that crude enzyme samples (often assayed in the presence of cyanide to inhibit catalase and other peroxidases) are not sensitive to cyanide under normal assay procedures; others probably failed to observe cyanide inhibition with partially

purified GSH-Px because the reactions were run in the presence of GSH (1) or used enzyme that was in a nonsusceptible form (2).

DISCUSSION

This study demonstrates that cyanide can release selenium from GSH-Px and causes loss of enzyme activity. The rate of inactivation is proportional to the calculated concentration of free CN^- and is accelerated by increasing temperature. This inhibition was demonstrated at rather low cyanide levels; inactivation was readily demonstrated at 10^{-4} M KCN, which, at pH 7.5, is equivalent to 1.5×10^{-6} M CN^- (the apparent reactive species). The inactivation by cyanide was completely blocked by treating GSH-Px with reducing agents; however, activity was not recovered when cyanide-inactivated enzyme was diluted and assayed after a 10 minute preincubation with 1 mM glutathione.

At physiological concentrations of GSH and peroxides, GSH-Px is in a reduced form (14). However, when GSH-Px is isolated in highly purified form from either bovine or ovine red blood cells it is presumably in a more oxidized state because it can be inhibited by cyanide (Table I) and is not inhibited by iodoacetate (15); after reduction GSH-Px is not inhibited by cyanide (Table I) but is inhibited by iodoacetate (15). These studies with inhibitors clearly support the likelihood that the Se in GSH-Px can exist in at least two forms or oxidation states, as suggested on the basis of kinetic studies (14) and x-ray photoelectron spectroscopy (16). It is possible that oxidized GSH-Px contains Se in a form related to a selenenic acid (8), since cyanide reacts with sulfenic acid derivatives of certain enzymes (17, 18). Further studies are in progress on the chemical nature of the selenium in GSH-Px.

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