Vol. 93, No. 4, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1980 Pages 1181-1188

INCORPORATION OF SELENIUM FROM SELENITE AND SELENOCYSTINE INTO GLUTATHIONE PEROXIDASE IN THE ISOLATED PERFUSED RAT LIVER[1]

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Received March 13, 1980

SUMMARY: The erythrocyte-free, isolated perfused rat liver was used to study the incorporation of selenium into glutathione peroxidase. Gel filtration and ion exchange chromatography of liver supernatant demonstrated ^{75}Se incorporation into glutathione peroxidase. A 9-fold excess of unlabelled selenium as selenite or selenide very effectively reduced ^{75}Se incorporation from L[^{75}Se]-selenocystine, but a 100-fold excess of unlabelled selenium as selenocystine was relatively ineffective as compared to selenite or selenide in diluting ^{75}Se incorporation from [^{75}Se]selenite. These results indicate that selenide and selenite are more readily metabolized than is selenocysteine to the immediate selenium precursor used for glutathione peroxidase synthesis, and suggest a posttranslational modification at another amino acid residue, rather than direct incorporation of selenocysteine, as the mechanism for formation of the presumed selenocysteine moiety of the enzyme.

INTRODUCTION: The essentiality of selenium in animals was demonstrated by Schwarz and Foltz (1), who found that selenite, selenocystine and seleno-methionine had equivalent abilities to protect rats against liver necrosis. This protective effect of Se[2] can be explained biochemically by its role as an essential component of glutathione peroxidase (GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) (2). Liver GSH-Px activity falls dramatically when rats are fed a Se-deficient diet (3). Repletion of Se-deficient rats with large single doses of selenite or Se-Met results in equal increases in liver GSH-Px activity (4); the ability of Se-Cys to increase GSH-Px in Se-deficient rats has not been reported. Preliminary reports of isotope dilution experiments indicate that

[1] Research supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by United States Public Health Service Program Grant no. AM 14881.

[2] Abbreviations: Se, selenium; Se-Met, selenomethionine; GSH, glutathione; Se-Cys, selenocysteine; $Se\text{-}Cys_2$, selenocystine; GSH-Px, glutathione peroxidase.

0006-291X/80/081181-08$01.00/0

Se-Cys, Se-Met and selenide decrease the specific activity of ^{75}Se incorporated into GSH-Px from [^{75}Se]selenite to about the same extent (5). Thus Se-Cys and selenite would appear to be equivalent substrates for GSH-Px synthesis.

Forstrom et al. (6) have reported that the Se moiety in the reduced form of GSH-Px is Se-Cys. Furthermore, preliminary results have suggested that this amino acid is incorporated directly into the peptide backbone of the enzyme using a Se-Cys-specific tRNA (7,8). Thus it is of interest to better define the metabolic pathways of Se leading to GSH-Px synthesis.

This study compares the effectiveness of excess selenite, selenide or $Se\text{-}Cys_2$ in diluting ^{75}Se incorporation into GSH-Px from either [^{75}Se]selenite or [^{75}Se]selenocystine. The purpose was to evaluate which compound is more readily metabolized to the immediate Se precursor of GSH-Px. The isolated perfused rat liver was chosen for this study to gain better control over the form of Se actually entering the liver.

METHODS: Male Holtzman 250 g rats were fed Ralston Purina stock diet until use. Liver perfusions were carried out using the methods and apparatus of Miller (9). The perfusate and supplement were those described by John and Miller (10) consisting of glucose, an amino acid mixture reflecting the composition of rat fibrinogen, insulin, cortisol, antibiotics and heparin in Krebs-Ringer-bicarbonate buffer (pH 7.4) containing 3% albumin. Growth hormone was not used and erythrocytes were not included as they rapidly take up and metabolize selenite (11). Erythrocytes were removed from the liver by discarding the first 50 ml of perfusate passing through the liver. The volume of circulating perfusate was 100 ml. Selenium compounds were premixed with 10 ml of perfusate withdrawn from the apparatus and added back after 15 minutes of perfusate circulation. Bile flow was determined hourly.

After 4 hours of perfusion with the Se compounds, the liver was homogenized in 3 volumes of 0.05 M sodium phosphate buffer (pH 6.8), containing 0.5 mM GSH and 0.25 mM EDTA. The homogenate was centrifuged at 105,000 x g x 60 minutes to obtain the liver supernatant, which was used for chromatography. GSH-Px was assayed using H_2O_2 (12). ^{75}Se was measured on a Packard model 5220 gamma counter with an efficiency of 85%. Protein was estimated by absorbance at 280 nm. $H_2{}^{75}SeO_3$ was obtained from ICN Chemical and Radioisotope Division (37.7 mCi/mg Se), and L-[^{75}Se]selenocystine was obtained from Amersham (1.7 mCi/mg Se). Na_2SeO_3 was purchased from ICN K&K Laboratories. D,L-selenocystine from Sigma Biochemicals was dissolved in 1 N HCl and passed throught a 0.45 μm micropore filter to remove elemental Se. Na_2Se from Pfaltz and Bauer, Inc. was dissolved in water within 15 minutes of use. Selenium content of the stock solutions was confirmed by fluorometric analysis (13).

The quantity of ^{75}Se added to the perfusate was calculated according to body weight. In experiments using [^{75}Se]selenite, addition of 28 ng Se/100 g rat resulted in approximately 6.6 ng Se/g liver. Excess unlabelled Se compounds were added at 2.8 μg Se/100 g rat. In experiments using $^{75}Se\text{-}Cys_2$, addition of 330 ng/100 g rat resulted in approximately 78 ng Se/g liver; larger $^{75}Se\text{-}Cys_2$ additions were used because of lower specific activity.

Excess unlabelled Se compounds were added at 3.0 μg Se/100 g rat. Two livers were perfused with each combination of Se additions to provide replicate chromatograms.

RESULTS: Sephadex G-150 chromatography of liver supernatant from a rat liver perfused for 4 hours with [^{75}Se]selenite resulted in a ^{75}Se peak co-eluting with the GSH-Px peak (not shown). When the GSH-Px containing fractions were pooled and further purified by CM-cellulose chromatography, a single peak of GSH-Px activity and ^{75}Se resulted (Figure 1). A near constant ratio of enzyme units per cpm across the peak further demonstrated that essentially all of the ^{75}Se was in GSH-Px. A portion of the supernatant was dialyzed for 24 hours against 400 volumes of 0.05 M sodium phosphate solution, pH 11, containing 1 ppm Se as Na_2SeO_3, followed by dialysis against an identical solution adjusted to pH 6.85; this procedure is similar to that of Cummins and Martin (14). The filtered dialysate still retained 25% of the GSH-Px activity after this harsh treatment; gel filtration (not shown) of the dialysate resulted in only one peak of ^{75}Se which co-eluted with GSH-Px. The ratio of GSH-Px enzyme units/cpm ^{75}Se in this peak remained the same as in the GSH-Px peak of the undialyzed-supernatant chromatogram. These results demonstrated that ^{75}Se was incorporated into GSH-Px and that gel filtration alone was sufficient to determine the level of such ^{75}Se incorporation.

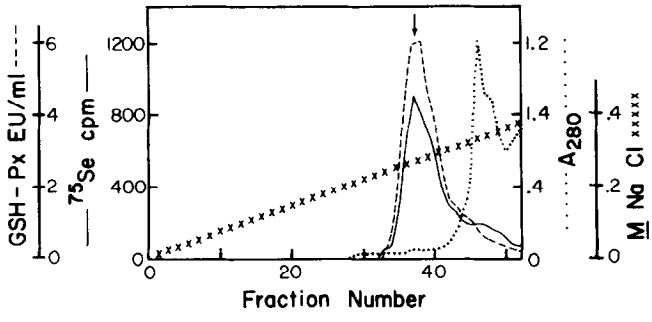

Fig. 1: CM-cellulose ion exchange chromatography. Pooled GSH-Px-containing fractions from Sephadex G-150 chromatography were dialyzed against buffer (5 mM acetic acid, 5 mM sodium phosphate, 0.5 mM GSH, 0.25 mM EDTA, pH 5.0) and then applied to 1.5 x 27 cm CM-cellulose column. After elution with 150 ml of buffer, a 400 ml linear gradient (0 to 0.5 M NaCl in buffer) eluted the ^{75}Se. Fractions contained 5 ml. The GSH-Px peak is indicated with an arrow.

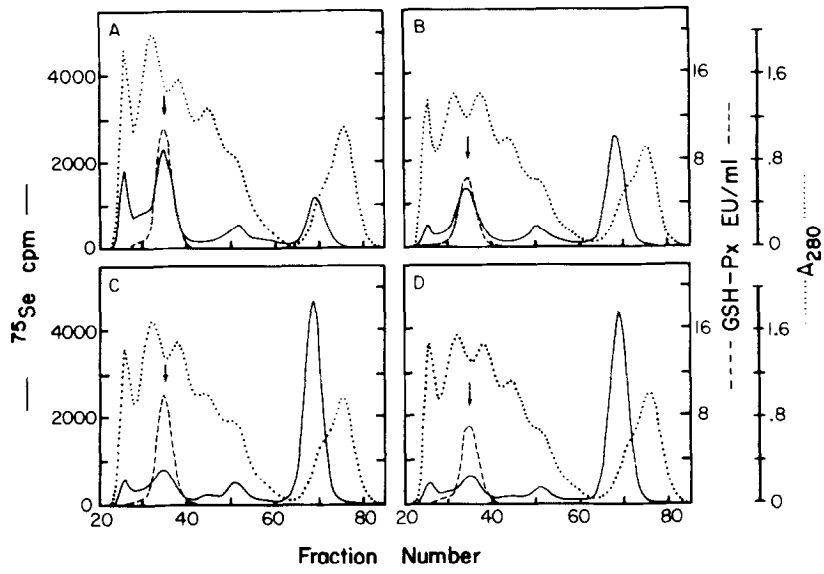

Fig. 2: Sephadex G-150 gel filtration chromatograms of liver supernatant. Livers were perfused for 4 hours with [^{75}Se]selenite alone (A), or with a 100-fold excess of Se as selenocystine (B), selenite (C) or selenide (D). Supernatant (8 ml) from individual livers was applied to a 2.2 x 97 cm column of Sephadex G-150 and eluted (20 ml/hr) with 0.05 M sodium phosphate buffer, pH 6.85, containing 0.5 mM GSH and 0.25 mM EDTA. Fractions contained 5 ml. The GSH-Px peak is indicated with an arrow in each chromatogram.

Figure 2 shows gel filtration chromatograms of liver supernatant when [^{75}Se]selenite alone (panel A) or when [^{75}Se]selenite plus a 100-fold excess of Se as Se-Cys$_2$, selenite, or selenide (panels B, C, or D, respectively) was included in the perfusate. ^{75}Se incorporation into GSH-Px was slightly reduced when a 100-fold excess of Se-Cys$_2$ was included with the [^{75}Se]selenite, whereas excess selenite or selenide very effectively reduced ^{75}Se incorporation. In a separate experiment (not shown), a 200-fold excess of unlabelled Se-Cys$_2$ also was relatively ineffective in reducing ^{75}Se incorporation from [^{75}Se]selenite.

Figure 3 shows gel filtration chromatograms when L-[^{75}Se]selenocystine was used. A 9-fold excess of selenite or selenide (panels C and D) resulted in barely discernible ^{75}Se peaks co-eluting with GSH-Px while added Se-Cys$_2$ (panel B) decreased ^{75}Se incorporation to a lesser extent. There was no

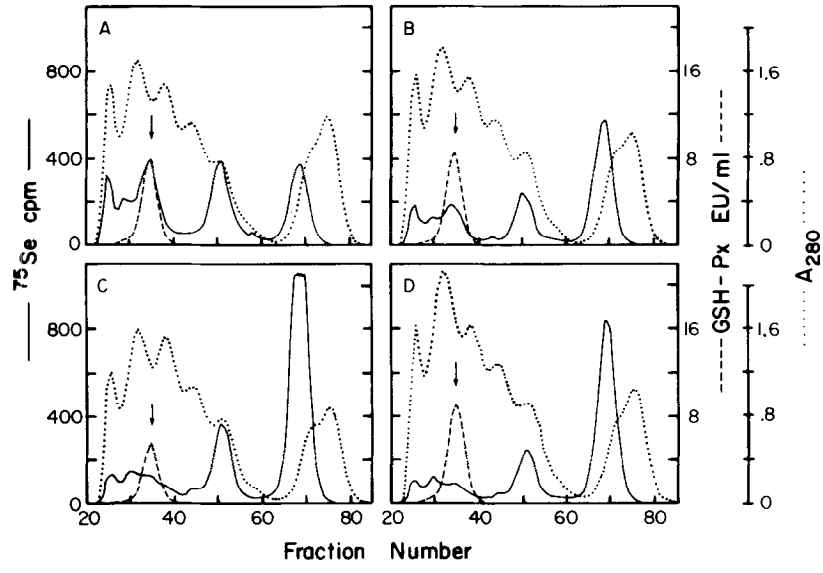

Fig. 3: Sephadex G-150 gel filtration chromatograms of liver supernatant. Livers were perfused for 4 hours with L-[^{75}Se]selenocystine alone (A), or with a 9-fold excess of Se as selenocystine (B), selenite (C) or selenide (D). Chromatograms were prepared as described in Fig. 2. The GSH-Px peak is indicated with an arrow in each chromatogram.

substantial difference between these chromatograms (Figures 2 and 3) and the chromatograms of replicate livers.

The liver supernatant retained 11 to 12% of the ^{75}Se at the end of the perfusion when [^{75}Se]selenite was used and 5 to 8% when ^{75}Se-Cys$_2$ was used, whether or not excess unlabelled Se was included in the perfusate. Bile flow averaged 2.3 ml/4 hours and was not significantly different between Se treatments. The recovery of ^{75}Se from the perfusion apparatus averaged 99%.

DISCUSSION: These experiments demonstrated that a 9-fold excess of Se as selenite or selenide very effectively reduced ^{75}Se incorporation into GSH-Px from ^{75}Se-Cys$_2$. Furthermore, a 100- or 200-fold molar excess of Se-Cys$_2$ was relatively ineffective in diluting out ^{75}Se incorporation from [^{75}Se]selenite These results indicate that Se-Cys is not the immediate precursor for GSH-Px synthesis. If Se-Cys were this precursor, the large excess of unlabelled Se-Cys$_2$ should have effectively diluted out the ^{75}Se labelling from [^{75}Se]-selenite; incorporation of ^{75}Se into GSH-Px from ^{75}Se-Cys$_2$ should have been less susceptible to dilution by a small excess of selenite or selenide. In-

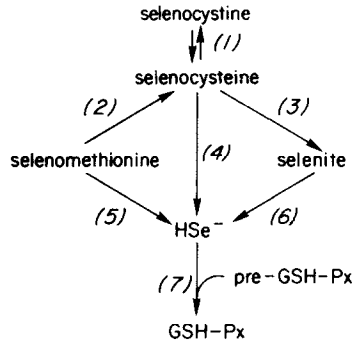

Fig. 4: Proposed pathways of selenium metabolism. Reactions 1-3: analogous to sulfur amino acid metabolism; reaction 4: selenocysteine may be degraded with loss of HSe^-, as Se metabolism is reductive (16); reaction 5: selenomethionine may be degraded with release of HSe^-, analogous to the methionine transamination pathway (20); reaction 6: reductive pathway of selenite to selenide (15); reaction 7: hypothetical GSH-Px selenocysteine synthase.

stead, these results suggest that selenite or selenide are more readily metabolized to the form of Se actually incorporated into GSH-Px. Apparently the Se from Se-Cys must first be released into the pool that readily equilibrates with Se from selenide and selenite before it is incorporated into GSH-Px.

These isotope dilution experiments did not distinguish between the ability of selenite or selenide to serve as Se precursors, most likely because selenite is readily reduced in the liver to selenide (15). At physiological pH, selenide would exist as HSe^-, which could serve as the immediate precursor for GSH-Px synthesis.

Figure 4 shows possible pathways of Se metabolism consistent with the results obtained in these experiments. Se-Cys degradation probably releases HSe^- directly rather than by way of selenite because Se compounds in animals have a tendency toward reductive metabolism (16). If this is the case, these experiments indicate that selenite reduction to selenide occurs more readily than release of Se from Se-Cys. In the proposed pathway, selenide is specifically incorporated posttranslationally into an amino acid residue (e.g., dehydroalanine, serine or cysteine) in the pre-GSH-Px, possibly by an enzyme similar to cysteine synthase (17). The model does not require a Se-Cys tRNA

and specific codon, and it does not require synthesis of free Se-Cys from selenite, a conversion which may occur but apparently only to a minimal extent (14,17).

Several alternative explanations of these results are possible but unlikely. The most improbable is that there are two immediate Se precursors for GSH-Px, one metabolically closer to Se-Cys but from which incorporation occurs at a rate much slower than from selenide. A second possibility is that Se-Cys is the immediate precursor, but the reduction of $Se\text{-}Cys_2$ to Se-Cys is much slower than the rate of Se-Cys synthesis from inorganic Se. This, too, is unlikely because a) GSH, maintained at a high concentration in liver, will non-enzymatically reduce $Se\text{-}Cys_2$, and b) the GSH:disulfide oxidoreductases present in rat liver will reduce L- or D-cystine readily (18,19), and thus most likely reduce $Se\text{-}Cys_2$.

These dilution experiments have indicated that free Se-Cys is an unlikely immediate precursor for Se incorporation into GSH-Px, but have suggested that a precursor metabolically close to selenide serves as this precursor. Identification of this compound, the mechanism of Se incorporation into GSH-Px, and the pathway of Se-Cys degradation are areas for further experimentation.

ACKNOWLEDGEMENT: The authors would like to thank Dr. H. E. Ganther for his helpful advice and review of the manuscript.

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