Elevation of Rat Liver mRNA for Selenium-dependent Glutathione Peroxidase by Selenium Deficiency*

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Selenium-dependent glutathione peroxidase (Se-GSH-Px, GSH-H$_2$O$_2$ oxidoreductase EC 1.11.1.9) is the best characterized selenoprotein in higher animals, but the mechanism whereby selenium becomes incorporated into the enzyme protein remains under investigation. To elucidate the mechanism of insertion of selenium into Se-GSH-Px further, we have systematically analyzed and compared the results of Western blot, in vitro translation immunoprecipitation, and Northern blot experiments conducted with liver proteins and RNAs obtained from rats fed on selenium-deficient and selenium-supplemented diets. The antisera employed in this study was raised against an electrophoretically pure Se-GSH-Px preparation obtained from rat livers by a simplified purification procedure involving separation by high performance liquid chromatography on a hydrophobic interaction column. Different forms of Se-GSH-Px, including apoprotein, cross-reacted with this antisem and Western blot analysis found no Se-GSH-Px protein present in livers from rats fed on selenium-deficient diets. By contrast, a distinct protein band corresponding to purified Se-GSH-Px was detected in livers from selenium-supplemented animals, a result consistent with the finding that the Se-GSH-Px activity was reduced to undetectable levels in livers of selenium-deficient rats. The in vitro translation experiments, however, indicated not only that mRNA for Se-GSH-Px was present during selenium deficiency but also that its translation products contained 2-3-fold as much immunoprecipitable protein as the products of poly(A) RNA from livers of selenium-supplemented rats. This result suggests that the Se-GSH-Px mRNA may be increased in the selenium-deficient state. Elevated levels of Se-GSH-Px mRNA were directly demonstrated in Northern blot experiments employing cDNA clone pGPX1211 as a probe. A similar increase in Se-GSH-Px mRNA was observed in such other tissues as kidney, testis, brain, and lung tissue, in selenium-deficient states. The present data support the co-translational mechanism for the incorporation of selenium into Se-GSH-Px in rat liver.

Our understanding of the molecular mechanisms underlying the biological activity of selenium is still somewhat limited, but a notable advance occurred in 1973 when selenium was found to be an essential component of mammalian glutathione peroxidase (GSH-Px, GSH:H$_2$O$_2$ oxidoreductase, EC 1.11.1.9) (1, 2). Since GSH-Px is known to be involved in cellular antioxidant defense, research has now established a broader role of selenium, complementary to that of vitamin E. One of the first enzymes shown to contain selenium, GSH-Px is the best characterized selenoprotein in higher animals. This enzyme, which catalyzes the reaction shown in equation I (where $R = H$ or alkyl; GSH = glutathione), plays an important role in eliminating potentially harmful peroxides formed by various nongaseous autoreduction mechanisms in cells. Additionally, Se-GSH-Px may also be involved in the metabolism of fatty acid hydroperoxides and cyclic endoperoxides formed, respectively, via the lipoxygenase and cyclooxygenase pathways (3-8). Capable of catalyzing the reduction of H$_2$O$_2$ as well as alkyl hydroperoxides, it differs from a group of selenium-independent GSH-Px, the glutathione S-transferases, that can use the latter only as peroxide substrates. Selenium-dependent GSH-Px isolated from various sources has a molecular weight of from 76,000 to 92,000 and is composed of four apparently identical subunits of molecular weight of from 19,000 to 23,000, each subunit containing a gram atom of selenium (8, 9).

During the past decade, selenium has also been found in several bacterial enzymes, in several amino proteins of unknown function, and in some tRNAs (9-11). Thus, although its exact function in most cases remains obscure, information is accumulating on its occurrence and chemical state in biological systems. In mammalian Se-GSH-Px as well as in the bacterial enzymes (formate dehydrogenase and glycine reductase) selenium is present as selenocysteine (9). All of these enzymes known to contain selenocysteine catalyze oxidation-reduction reactions, which suggests that oxidized forms of selenium are most likely involved in their catalytic function. But a specification of the mechanism by which selenium is incorporated into these proteins remains elusive. It is not entirely clear whether this occurs by post-translational (12) or co-translational (13) processes; however, the recent discovery of an (in-frame) UGA opal nonsense codon at position 47,

\[ \text{ROOH} + \text{GSH} \rightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O} \] (I)

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1 The abbreviations used are: P, peroxidase; Se-GSH-P, selenium-dependent glutathione peroxidase; GST, glutathione S-transferase; non-Se-GSH-P, selenium-independent glutathione peroxidase; HPLC, high performance liquid chromatography; HIC, hydrophobic interaction chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
which appears to be responsible for the incorporation of selenocysteine into bacterial formate dehydrogenase (14) and mammalian Se-GSH-Px (15-17) may help to resolve this issue. Evidence for the co-translational incorporation of selenium into bacterial formate dehydrogenase has been recently reported (18, 19), but such details remain unavailable for the mammalian Se-GSH-Px. In the present investigation we report evidence for the co-translational insertion of selenium into rat liver Se-GSH-Px, and also for the elevation of mRNA for Se-GSH-Px in a selenium-deficient state. (A preliminary account of this investigation appeared earlier (40).)

EXPERIMENTAL PROCEDURES

RESULTS

Effect of Selenium Deficiency on the Expression of Se-GSH-Px in Rat Liver—The interrelatedness of the metabolic functions of selenium and vitamin E having been established (20, 21), we investigated the effects of altered selenium and vitamin E nutrition on the expression of Se-GSH-Px in rat livers. Evidence for the absence of Se-GSH-Px activity in selenium-deficient rat liver is presented in Table I. Whole blood was analyzed for selenium and plasma for vitamin E, and as expected, blood selenium levels were significantly reduced in selenium-deficient animals (p < 0.05). Also, vitamin E concentration, measured as total tocopherol, was decreased to below detectable limits in rats fed vitamin E-deficient diets. The Se-GSH-Px activity of liver cytosol was decreased by almost 70-fold in selenium deficiency. There was approximately a 25% increase in the liver cytosolic non-Se-GSH-Px activity of selenium-deficient animals, which is consistent with our previous observations (24). Dietary vitamin E status had no effect on either selenium or non-Se-GSH-Px activities.

Expression of Se-GSH-Px Protein during Selenium Deficiency—The results of a protein immunoblot analysis for the detection of immunoreactive Se-GSH-Px in liver cytosol obtained from rats fed selenium-deficient and selenium-supplemented diets are illustrated in Fig. 1. The immunoreactive Se-GSH-Px protein was observed in the liver cytosols of only the selenium-supplemented rats (Fig. 1, lanes A and B), not in selenium-deficient rats (Fig. 1, lanes C and D). Dietary vitamin E status had no effect on the expression of Se-GSH-Px protein. These observations suggested that Se-

Fig. 1. Autoradiograph of 125I-protein A-labeled Western blot. Equal amounts of rat liver cytosolic protein from selenium-supplemented and selenium-deficient animals were run on SDS-PAGE, blotted, and exposed to antiserum against the purified rat liver Se-GSH-Px. The lanes are: A, +vitamin E, +selenium group; B, −vitamin E, +selenium group; C, +vitamin E, −selenium group; D, −vitamin E, −selenium; lanes E–H are the same as A–D, respectively, except that these samples were incubated with 10 μg/ml of sodium selenite for 15 min prior to electrophoresis; lane I, high performance liquid chromatography purified rat liver Se-GSH-Px (1 μg). GSH-Px protein was either not synthesized in the absence of selenium or significantly modified by the selenium deficiency so that it did not cross-react with the antibodies raised against the holoenzyme.

To distinguish between these two possibilities, we attempted to reconstitute both the Se-GSH-Px activity and immunocross-reactive protein in freshly prepared liver cytosolic fractions from selenium-deficient rats. When these fractions were incubated with various concentrations of sodium selenite for different intervals of time, no increase in Se-GSH-Px activity above the background was observed (data not shown) nor could any immunocross-reactive protein be detected in the selenium-deficient cytosolic fractions incubated with sodium selenite (Fig. 1, lanes G and H). To investigate further whether Se-GSH-Px apoprotein could cross-react with the antibodies raised against the holoenzyme, we removed the selenium moiety from the purified enzyme by treating the fully oxidized enzyme with cyanide and tested immunocross-reactivity by Western blot analysis. The treatment of Se-GSH peroxidase with H2O2 followed by cyanide has been shown to remove >95% of selenium from the protein (25). As shown in Fig. 2 (lane 6), antiserum raised against holoenzyme cross-reacted with the cyanide treated enzyme.

TABLE I

<table>
<thead>
<tr>
<th>Parameter measured</th>
<th>Dietary treatment</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>+ Vitamin E, + selenium</td>
<td>− Vitamin E, + selenium</td>
<td>+ Vitamin E, − selenium</td>
<td>− Vitamin E, − selenium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma vitamin E (μg/100 ml)</td>
<td>947 ± 108b</td>
<td>0b</td>
<td>975 ± 217b</td>
<td>0b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole blood selenium (μg/100 ml)</td>
<td>0.60 ± 0.04b</td>
<td>0.58 ± 0.04b</td>
<td>0.03 ± 0.002b</td>
<td>0.03 ± 0.003b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver Se-GSH-Px (nanomoles/min/mg protein)</td>
<td>194 ± 23b</td>
<td>188 ± 39b</td>
<td>3 ± 1c</td>
<td>3 ± 1c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver non-Se-GSH-Px (nanomoles/min/mg protein)</td>
<td>88 ± 23b</td>
<td>97 ± 24b</td>
<td>116 ± 6b</td>
<td>116 ± 19b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Vitamin E, as total tocopherol, was determined by the method of Taylor et al. (22).
b Vitamin E concen-
tration, measured as total tocopherol, was decreased to below detectable limits in rats fed vitamin E-deficient diets.

c Selenium levels were determined spectrofluorometrically as described by Whetter and Ullrey (23).

2 Portions of this paper (including "Experimental Procedures," Figs. 5 and 6, and Tables II and III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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samples (2 pg each) subjected to different treatments. Preparation of apoenzyme by cyanide treatment was done according to the procedure of Kraus et al. (23) with some modifications. The lanes are: 1, control Se-GSH-Px sample; 2, enzyme incubated with 0.2 mM GSH for 10 min; 3, enzyme incubated with 0.2 mM GSH for 10 min followed by 18-h incubation with 10 mM KCN; 4, same as the sample in lane 3 except that KC1 was used in place of KCN; 5, enzyme treated with 0.1 mM H2O2 for 10 min; 6, enzyme incubated with 0.1 mM H2O2 for 10 min followed by 10 mM KCN for 18 h; 7, same as lane 6 except that KC1 was substituted for KCN; 8, same as lane 6 except that it was electrophoresed immediately after the addition of KCN. The intensity of band in each lane was quantitated by densitometry.

The data shown in Fig. 2 also suggest that the antibody for the holoenzyme cross-reacts very well with different forms of the enzyme, that is to say, both the oxidized and reduced forms. This observation was substantiated by quantitation of the bands by densitometry.

**Presence of Immunoprecipitable Se-GSH-Px Protein in Rat Liver mRNA in Vitro Translation Products**—We used rat liver poly(A) RNAs purified twice through an oligo(dT)-cellulose affinity column to program an in vitro translation in the rabbit reticulocyte lysate system. The translation products of poly(A) RNA from selenium-deficient and selenium-supplemented rat livers, both deficient and supplemented with vitamin E, were immunoprecipitated with the antiserum raised against rat liver Se-GSH-Px, which yielded predominantly a single band corresponding to rat liver Se-GSH-Px on SDS-polyacrylamide gel electrophoresis in all four dietary groups (Fig. 3). Upon quantitating of the bands by densitometry and determining the total radioactivity in the immunoprecipitable in vitro translation products, we found that the amount of immunoprecipitated products was more than two times greater with mRNA from selenium-deficient livers than with selenium-supplemented livers (Fig. 3, lanes 3 and 4). A similar increase in immunoreactive product per µg of RNA was also observed with poly(A) RNAs prepared from testis, brain kidney, and lung tissues of selenium-deficient rats (data not shown). These results clearly demonstrate that Se-GSH-Px mRNAs are synthesized and accumulate to elevated levels in tissues of rats fed selenium-deficient diets. An additional band at around 30,000 daltons was observed consistently in an in vitro translation experiment. At present this band is not fully characterized.

In our in vitro translation experiments, we conducted the synthesis of Se-GSH-Px protein with the rabbit reticulocyte lysate system. This system probably contained the specific biological form of selenium as well as the other components needed for the ultimate transfer of selenium into protein, since erythrocytes actively synthesize Se-GSH-Px and we made no effort to exclude them from our in vitro translation system. We have also observed in a wheat germ in vitro translation system a failure to synthesize immunoprecipitable Se-GSH-Px protein; however, when we included 10 µg of sodium selenite in the system, we detected a very faint band corresponding to rat liver Se-GSH-Px on SDS-polyacrylamide gel electrophoresis (data not shown). These latter observations suggest that the wheat germ system may not have the elements necessary for the transfer of selenium into protein during the translational process.

**Induction of Rat Liver Se-GSH-Px Poly(A) RNA Under Selenium Deficiency Determined by RNA Blot Analysis**—Using our cDNA clone pGPX1211 (17) as a probe for rat liver Se-GSH-Px mRNA, Northern blot analysis confirmed the elevation of rat liver mRNA coding for Se-GSH-Px as a consequence of selenium deficiency. After electrophoresis and blotting to the nitrocellulose of poly(A) RNA obtained from livers of rats fed selenium-deficient and selenium-adequate diets, mRNA for Se-GSH-Px was detected by hybridization with a nick translation labeled cDNA probe, pGPX1211. The results, shown in Fig. 4A, indicate the presence of Se-GSH-Px mRNA regardless of the selenium status of the rats. As Fig. 4A clearly shows (compare lanes 3 and 4 with lanes 1 and 2), there is more Se-GSH-Px mRNA in selenium-deficient states than in selenium-supplemented states. Upon quantitation by scanning densitometry, it was determined that there was approximately a 3-fold higher concentration of Se-GSH-Px mRNA in poly(A) RNAs of livers from rats fed selenium-deficient diets than in selenium-supplemented rat livers. Since different batches of poly(A) RNAs may contain variable amounts of rRNAs, we used the amount of poly(A) RNA that was approximately a 3-fold higher concentration of Se-GSH-Px mRNA in selenium-deficient states than in selenium-supplemented states. Upon quantitation by scanning densitometry, it was determined that there was approximately a 3-fold higher concentration of Se-GSH-Px mRNA in poly(A) RNAs of livers from rats fed selenium-deficient diets than in selenium-supplemented rat livers.
hybridized with a human $\beta$-actin cDNA probe as an internal standard to the results of A. Different lanes had rat liver poly(A) RNAs from the same source, respectively, as described under A except that they were hybridized with 32P-labeled human $\beta$-actin cDNA insert. The 28 and 18S indicate the mobilities of RNA markers.

DISCUSSION

A Western blot analysis of liver cytosols obtained from rats fed selenium-deficient diets, detected no immunoreactive Se-GSH-Px protein which is consistent with previous reports (17, 26, 27). Additionally we demonstrated that different forms of Se-GSH-Px, that is to say, fully reduced, fully oxidized and apoenzyme (selenium removed), can readily react with the antiserum raised against Se-GSH-Px from rat liver or from bovine erythrocyte enzyme. Our attempts to reconstitute either the Se-GSH-Px activity or the immunoprecipitable protein in the presence of sodium selenite in freshly prepared liver cytosol from rats fed selenium-deficient diets were unsuccessful. Therefore, the data suggest that a post-translational modification of existing protein is an unlikely mechanism for the insertion of selenium into Se-GSH-Px and the present results appear to be consistent with the co-translational mechanism (18, 19).

When one considers a new codon UGA for selenocysteine (18, 19) and a specific tRNA, selenocysteine tRNA (13) one can envision a mechanism for the co-translational incorporation of selenium into Se-GSH-Px. We have recently reported the presence of an (in-frame) UGA opal nonsense codon at position 47 in a cDNA clone pGPX1211 for rat liver Se-GSH-Px, which appears to be responsible for directing the incorporation of selenocysteine into Se-GSH-Px protein (17). The hybrid formation between the cDNA probe, pGPX1211, and mRNA from both selenium-deficient and selenium-adequate livers, suggests the existence of translatable mRNA for the synthesis of Se-GSH-Px protein, even in tissues from selenium-deficient rats. However, the absence of Se-GSH-Px activity and immunoprecipitable protein in these tissues indicates their inability to translate Se-GSH-Px mRNA into immunodetectable protein in the absence of selenium. Our in vitro translation studies indicate the formation of immunoprecipitable protein from the poly(A) RNA of selenium-deficient livers in a rabbit reticulocyte lysate system, probably attributable to the presence of the appropriate biological form of selenium and the proper conditions for its incorporation into Se-GSH-Px. Interestingly, when the translation involved a wheat germ system, which unlike the reticulocyte lysate system is not known to contain high levels of selenium or to produce selenoproteins, no immunoreactive product was formed with poly(A) RNA from livers of either selenium-supplemented or selenium-deficient rats. But when the wheat germ system was supplemented with sodium selenite, the poly(A) RNAs from both sources were translated into immunoprecipitable products. Taken altogether, our results and recent findings on the insertion of selenium into bacterial formate dehydrogenase (18) favor the co-translational mechanism for selenium incorporation into Se-GSH-Px protein.

Most noteworthy in the study described here was the amount of immunoprecipitable Se-GSH-Px synthesized from poly(A) RNAs obtained from livers of rats fed selenium-deficient diets. In vitro translation of liver poly(A) RNA isolated from selenium-deficient rats revealed three times higher level of Se-GSH-Px protein than that from selenium-supplemented rats. This result is supported by a RNA blot analysis using the cDNA probe, pGPX1211, which showed that approximately three times more poly(A) RNA coding for Se-GSH-Px was present in livers of selenium-deficient rats than in livers of selenium-supplemented rats. These observations contradict recent reports which indicate that mRNA levels are reduced in livers of selenium-deficient rats (28, 29). This discrepancy would have been more clearly documented had they performed in vitro translation immunoprecipitation experiments with poly(A) RNAs from livers of both selenium-deficient and selenium-supplemented rats. We have also observed a similar increase in Se-GSH-Px mRNA in lung, brain, testis, and kidney tissues.

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REFERENCES
Elevation of mRNA for Se-GSH-Px in Selenium Deficiency


Table II
Summary of purification profile of rat liver Se-GSH-Px

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units protein)</th>
<th>Specific Activity (units/mg)</th>
<th>Purification Fold</th>
<th>Recovery (%)</th>
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<tr>
<td>Crude extract</td>
<td>755</td>
<td>25,182</td>
<td>92</td>
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<td>1</td>
<td>100</td>
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<tr>
<td>Ammonium sulfate</td>
<td>575</td>
<td>2,771</td>
<td>4800</td>
<td>5.2</td>
<td>8</td>
<td>95</td>
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<tr>
<td>CM chromatography</td>
<td>450</td>
<td>200</td>
<td>4900</td>
<td>29.2</td>
<td>73</td>
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<td>Sephadex G-100</td>
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<td>38</td>
<td>2550</td>
<td>65.4</td>
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<td>DE chromatography</td>
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<td>7.2</td>
<td>2390</td>
<td>332</td>
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<td>RNA column</td>
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<td>2.6</td>
<td>548</td>
<td>588</td>
<td>1405</td>
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Table III
NH_{2}-terminal sequence analysis of Se-GSH-Px

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>NH_{2}-terminal amino acid sequence</th>
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<tr>
<td>Rat liver</td>
<td>VA GAV phường GAV</td>
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<td>Rat liver (developed from CRD)</td>
<td>MS AAV SAV PAV PAV GAV GAV GAV GAV GAV GAV GAV</td>
</tr>
<tr>
<td>Rat liver (developed from CRD)</td>
<td>MS AAV SAV PAV PAV GAV GAV GAV GAV GAV GAV</td>
</tr>
<tr>
<td>TNBC erythrocyte</td>
<td>AAAL AAAA PAV PAH PAV PAV GAV GAV GAV GAV GAV</td>
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</table>

* Data published by Reider et al. (17). ** Data reported by Masseter et al., 1986.

Fig. 4. Hydrophilic interaction chromatography of Se-GSH-Px fraction obtained from a CM column of the rat liver. The elution profile on RPC-100 (A) shows the presence of two peaks. The elution profile on RPC-100 (B) shows the presence of two peaks. The elution profile on RPC-100 (C) shows the presence of two peaks.

Fig. 5. Electrophoresis of purified Se-GSH-Px. The purified Se-GSH-Px was subjected to SDS-PAGE. The purified Se-GSH-Px was subjected to SDS-PAGE. The purified Se-GSH-Px was subjected to SDS-PAGE.

Fig. 6. Analytical gel of purified Se-GSH-Px. The purified Se-GSH-Px was subjected to analytical gel electrophoresis. The purified Se-GSH-Px was subjected to analytical gel electrophoresis. The purified Se-GSH-Px was subjected to analytical gel electrophoresis.
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