Glutathione peroxidase catalyzes the reduction of hydrogen peroxide and organic hydroperoxide by glutathione and functions in the protection of cells against oxidative damage. Glutathione peroxidase exists in several forms that differ in their primary structure and localization. We have also shown that selenoprotein P exhibits a glutathione peroxidase-like activity (Saito, Y., Hayashi, T., Tanaka, A., Watanabe, Y., Suzuki, M., Saito, E., and Takahashi, K. (1999) J. Biol. Chem. 274, 2866–2871). To understand the physiological significance of the diversity among these enzymes, a comparative study on the peroxide substrate specificity of three types of ubiquitous glutathione peroxidase (cellular glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, and extracellular glutathione peroxidase) and of selenoprotein P purified from human origins was done. The specific activities and kinetic parameters against two hydroperoxides (hydrogen peroxide and phosphatidylcholine hydroperoxide) were determined. We next examined the thiol specificity and found that thioredoxin is the preferred electron donor for selenoprotein P. These four enzymes exhibit different peroxide and thiol specificities and collaborate to protect biological molecules from oxidative stress both inside and outside the cells.

Glutathione peroxidases (GPx) containing selenocysteine at the catalytic center play an important role in the detoxification of various hydroperoxides (1, 2). Four types of GPx (cellular GPx (cGPx), gastrointestinal GPx (GIGPx), extracellular GPx (eGPx), and phospholipid hydroperoxide GPx (PHGPx)) have been identified, and each enzyme is antigenetically, structurally, and enzymatically different. cGPx is a ubiquitously distributed enzyme that was the first of the GPx family to be discovered (3). GIGPx is also an intracellular enzyme but is expressed only at the epithelium of the gastrointestinal tract as a first line of defense against ingested lipid hydroperoxides (4). eGPx is mainly expressed by the kidneys from where it is released into the blood circulation (5, 6). Of the GPx family, eGPx is the only enzyme distributed in the extracellular fluids such as plasma (7). PHGPx can reduce a variety of hydroperoxides including hydroperoxides integrated in membranes, hydroperoxy lipids in low density lipoprotein (8), or thymine (9). Although cGPx, GIGPx, and eGPx are homotetramers, PHGPx is a monomer with a molecular size smaller than the subunits of the other GPxs (10). PHGPx has recently been shown to act as a structural component of the mitochondrial capsule and thus is needed in the formation of the flagellum (11), whereas in the nuclei, specific sperm nuclei GPx with properties similar to PHGPx and its processed products are involved in chromatin condensation and protection of the germline against oxidative damage (12).

We recently reported that selenoprotein P (SeP), presumed to contain ten selenocysteine residues, reduces phospholipid hydroperoxide in a GPx-like manner (13). SeP is an extracellular protein and is the major selenoprotein in rat and human plasma (14, 15). The physiological function of SeP is currently unknown, but several reports have led to the hypothesis that it functions as an antioxidant (13, 16). It is generally known that all members of the GPx family reduce hydrogen peroxide at the expense of glutathione (GSH) and that PHGPx and eGPx reduce hydroperoxides of more complex lipids such as phosphatidylcholine hydroperoxide (17, 18). However, the literature shows that the specific activity of each GPx was determined using a different kind of peroxide substrate at a different pH, temperature, and GSH concentration. Furthermore, as the enzyme was obtained from different origins (human, rat, and pig), a successful comparison of the enzyme activities of GPx family would appear impossible. We consider that to understand the physiological significance of GPx diversity, it is important to compare the specific activities and kinetic parameters of enzymes purified from the same origin determined under the same conditions.

In this study, we first purified three ubiquitous forms of GPx together with human SeP and determined the specific activities and kinetic parameters of these enzymes using three kinds of synthetic peroxide substrates. We next compared the thiol (especially thioredoxin) specificity and determined the kinetic parameters of SeP against thioredoxin. Finally, we considered the physiological significance of GPx diversity.
EXPERIMENTAL PROCEDURES

**Chemicals**—Tertiary butyl hydroperoxide (t-Bu-OOH), cumene hydroperoxide, and hydrogen peroxide were obtained from Nakalai Tesque, Kyoto. 1-Palmitoyl-2-oleoyl-3-phosphatidylcholine (PC), cholesterol, linoleate, GSH, and GSH reductase were from Sigma. Soybean lipoygenase was from Biozyme Laboratories Ltd. (Blaenavon, United Kingdom). Recombinant human thioredoxin (TRX) was prepared as described previously (19) and kindly provided by Ajinomoto, Co. Inc. (Kawasaki, Japan). Human out-dated red blood cells and frozen plasma were kindly donated by the Hokkaido Red Cross Blood Center. Methanol and 2-propanol were distilled before use. PC-OOH was prepared from PC by oxidation with soybean lipoygenase as described previously (13). Other chemicals were of the highest quality commercially available.

**Enzyme Purification**—GPx was purified from human red blood cells as described previously (20) with a slight modification. eGPx and SeP were purified from human plasma as described previously (12, 13). PHGPx was purified from human placental cytosol as described previously (21) with a slight modification. TRX reductase was purified from human placenta as described previously (22) with some modifications (23).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (24) in slab gels (12.5% gel) under reducing conditions. After electrophoresis, proteins in the gel were stained with 0.1% Coomassie Brilliant Blue G-250 in 10% acetic acid and 30% methanol for 30 min, and then the gel was destained in 10% acetic acid and 30% methanol.

**Quantitative Amino Acid Analysis for Determination of Protein Molar Concentration**—The protein was dialyzed against distilled water and hydrolyzed in 6 M HCl for 24, 48, or 72 h or in 3 M mercaptoethanesulfonic acid for 24 h. After the removal of the solvent in vacuo, quantitative amino acid analysis was performed on a PICO-TAG system (Waters, Millipore) (13).

**Enzyme Assay**—GPx activities were examined by following the oxidation of NADPH in the presence of GSH reductase, which catalyzes the reduction of oxidized GSH formed by GPx as described previously (25) with a slight modification. Both samples and reference cuvettes contained 0.1% Tris-HCl, pH 7.4, 0.2 mM NADPH, 0.5 mM EDTA, 2 mM GSH, and 1 unit of GSH reductase in a total volume of 1 ml. An aliquot of each enzyme was added to the sample cuvette only. The reaction mixture was preincubated at 37 °C for 5 min, after which the reaction was started by the addition of peroxide to both cuvettes. In the case of phospholipid hydroperoxide, Triton X-100 and deoxycholate were added to the reaction mixture at an appropriate concentration. The oxidation of NADPH was followed at 340 nm at 37 °C, and activity was expressed as micromoles of NADPH oxidized per minute. TRX peroxidase activity was determined analogously by replacing GSH with TRX (1–100 nM) and GSH reductase with TRX reductase (5 nM). To confirm the reduction of PC-OOH and examine the thiol specificity, a HPLC assay was also conducted (13). After incubating the reaction mixture, a 9-fold volume of ice-cold 2-propanol was added to the reaction mixture. An aliquot of the reaction mixture was injected directly into the HPLC system. The HPLC conditions were basically those of Bao et al. (26) with a slight modification (13). To examine the thiol specificity, the reduction of H₂O₂ by cGPx and eGPx was determined as described previously (13, 21).

**Kinetic Analyses**—Kinetic analysis was carried out by following the time progress curves of substrate consumption (NADPH oxidation) at the various fixed GSH concentrations. From these curves, the reciprocal concentrations of hydroperoxide were plotted against the reciprocal velocities at 12-s intervals, and different GSH concentrations led to parallel lines (Fig. 2A). The other three enzymes also showed similar kinetic patterns as is typical for bi-substrate reactions displaying a ping-pong mechanism without apparent saturation (28) as described in detail below. This allowed the distinct measurement of k₁ for the oxidative step of the enzyme and of k₂ for the sum of the two reductive steps of the regeneration of the oxidized enzymes by GSH. The values of k₁ and k₂ for each enzyme determined under the same conditions are shown in Table II. Essentially identical k₁ values for t-Bu-OOH and H₂O₂ were obtained from both cGPx and eGPx. The k₂ values for PC-OOH obtained from eGPx and SeP were 40-fold and 200-fold lower than that from PHGPx, respectively. This indicates that eGPx and SeP are less reactive to phospholipid

**RESULTS**

**Purity of the Three Forms of GPx and SeP**—Specific activity (Table I) was determined using three kinds of peroxide substrate, t-Bu-OOH, H₂O₂, and PC-OOH, under the same conditions (2 mM GSH, 60 μM peroxide, pH 7.4, at 37 °C). cGPx and eGPx showed a superior reactivity with the watersoluble substrates, t-Bu-OOH and H₂O₂, whereas PHGPx or SeP showed only slight or almost no reactivity. The enzyme assay for PC-OOH reduction needs a detergent to solubilize PC-OOH in the buffer, and the optimum activity of each enzyme was acquired under the different detergent conditions. PHGPx activity against the PC-OOH substrate was the highest among four enzymes at 0.1% Triton X-100 and 0.24 mM sodium deoxycholate, whereas eGPx showed almost the same activity at 7 mM sodium deoxycholate. SeP reduced PC-OOH optimally at 0.025% Triton X-100 and 0.3 mM sodium deoxycholate. The specific activity of SeP to PC-OOH was one order lower than that of PHGPx or eGPx. Only in the case of eGPx did the presence of Triton X-100 lower the enzyme activity for PC-OOH but not for t-Bu-OOH (data not shown). On the other hand, eGPx had almost no reactivity with PC-OOH under any detergent conditions.

**Kinetic Analyses of the Three Forms of GPx and SeP**—The kinetic mechanism for the GSH-dependent reaction by SeP was studied following the time progress curves of substrate consumption (NADPH oxidation) at the various fixed GSH concentrations. From these curves, the reciprocal concentrations of hydroperoxide were plotted against the reciprocal velocities at 12-s intervals, and different GSH concentrations led to parallel lines (Fig. 2A). The other three enzymes also showed similar kinetic patterns as is typical for bi-substrate reactions displaying a ping-pong mechanism without apparent saturation (28) as described in detail below. This allowed the distinct measurement of k₁ for the oxidative step of the enzyme and of k₂ for the sum of the two reductive steps of the regeneration of the oxidized enzymes by GSH. The values of k₁ and k₂ for each enzyme determined under the same conditions are shown in Table II. Essentially identical k₁ values for t-Bu-OOH and H₂O₂ were obtained from both cGPx and eGPx. The k₂ values for PC-OOH obtained from eGPx and SeP were 40-fold and 200-fold lower than that from PHGPx, respectively. This indicates that eGPx and SeP are less reactive to phospholipid...
The activities were measured as initial rate and corrected for the spontaneous reaction. Enzyme concentration was determined by quantitative amino acid analysis. Each value is presented by μmol/min/mg protein.

|          | t-BuOOH | H2O2 | PC-OOH |
|----------|---------|------|--------|
| cGPx     | 552     | 793  | <0.02  |
| eGPx     | 199     | 373  | 54.1   |
| PHGPx    | 9.56    | 34.8 | 2.65   |
| SeP      | <0.02   | <0.02| 1.11   |

* Detergent conditions varied as follows: 1) 7 mM sodium deoxycholate, 2) 0.1% Triton X-100 and 0.24 mM sodium deoxycholate, and 3) 0.025% Triton X-100 and 0.3 mM sodium deoxycholate.

**Thiol Specificity of the GPx family and SeP**

As has been reported previously, cGPx used only glutathione as an electron donor. The other three enzymes could use dithiothreitol, mercaptoethanol, or cysteine at 2 mM. The rate constants $k_2$ for the oxidative reaction and $k_3$ for the sum of the two reductive steps can be calculated from the slope and intercept, respectively, of a Lineweaver-Burk plot described under “Experimental Procedures.” $k_2$ values for each GPx were averaged from all of the experiments irrespective of the peroxide substrate.

|          | t-BuOOH | H2O2 | PC-OOH |
|----------|---------|------|--------|
| cGPx     | 4.2 × 10^6 | 4.1 × 10^7 | n.d. |
| eGPx     | 2.3 × 10^6 | 4.0 × 10^7 | 3.4 × 10^7 |
| PHGPx    | n.d.     | n.d.  | 1.5 × 10^7 |
| SeP      | n.d.     | n.d.  | 8.8 × 10^4 |

n.d., not determined.

**Thiol specificities of the GPx family and SeP**

Each enzyme activity was measured using dithiothreitol, mercaptoethanol, or cysteine at 2 mM. Each enzyme activity was converted to a percentage of that shown when using GSH.

|          | GSH | Dithiothreitol | Mercaptoethanol | Cysteine |
|----------|-----|----------------|-----------------|---------|
| cGPx     | 100 | <2             | <2              | <2 |
| eGPx     | 100 | 160            | 67.6            | 59.5 |
| PHGPx    | 100 | 510            | 65.6            | 15.4 |
| SeP      | 435 | 31.6           | 59.0            |        |

**Experimental Procedures.**

Trx-dependent reduction activities of SeP. A. Trx peroxidase activities were examined by following the oxidation of NADPH in the presence of TRX reductase (TR) (5 mM), TRX (5 μM), and SeP (0.2 μM) as described under “Experimental Procedures.” B. PC-OOH reduction and PC-OH formation were directly confirmed by HPLC separation of PC-OOH and PC-OH as described previously (13). 100 pmol of PC-OOH and 100 pmol of PC-OH were eluted at 10.6 and 11.4 min, respectively. C. 100 pmol of PC-OOH equivalent of the reaction mixture (60 μM PC-OOH incubated with 0.2 μM SeP and 5 μM TRX for 5 min) was applied to a HPLC column.

HPLC analysis of the reaction products confirmed the conversion of PC-OOH to PC-OH by SeP with TRX (the loss of PC-OOH and the formation of PC-OH) (Fig. 3B). These results indicate that SeP reduces the peroxide at the expense of TRX.
We next examined the effect of GSH or TRX dose on the activity of the four enzymes (Fig. 4). cGPx showed almost the same reactivity to GSH and TRX. On the other hand, eGPx reduced peroxide at lower concentrations of GSH than those of TRX. In the case of PHGPx and SeP, TRX was more effective than was GSH. When SeP was used, TRX was 500-fold more effective than GSH.

Comparison of Kinetic Analyses of SeP—The kinetic mechanisms for the GSH- and TRX-dependent reactions by SeP were compared as shown in Equation 3.

\[ [E]v' = \Phi_0, \Phi_d [ROOH] + \Phi_2 [\text{thiol}] \quad \text{(Eq. 3)} \]

In the Dalziel equation (28, 30) shown above, \( \Phi_0 \) is defined as the reciprocal of the maximum turnover number, \( \Phi_1 \) is defined as the reciprocal rate constant \( k_1 \) for the oxidative reaction, and \( \Phi_2 \) is defined as the reciprocal \( k_2 \) for the sum of the two reductive steps of the regeneration of the reduced enzyme by GSH. The PC-OOH reduction of SeP using GSH revealed a ping-pong mechanism as described above. Replotting the reciprocal GSH concentrations against the reciprocal apparent \( V_{\text{max}} \) for infinite concentrations of PC-OOH yielded a straight line cutting the ordinate origin, i.e. the coefficient \( \Phi_0 \) approximates zero (Fig. 2B). Accordingly, infinite \( V_{\text{max}} \) and \( K_m \) values were obtained (Table IV). The kinetics of SeP with PC-OOH and TRX were analyzed next. As observed in GSH-dependent reduction, SeP also showed a ping-pong pattern (Fig. 2C). However, replotting the reciprocal TRX concentrations against the reciprocal apparent \( V_{\text{max}} \) revealed a difference in that the slope did not cut the ordinate at zero (Fig. 2D). This indicates Michaelis-Menten-type saturation kinetics with defined \( \Phi_0, V_{\text{max}}, \) and \( K_m \) values. The \( k_1 \) and \( k_2 \) were determined similarly as in the case of GSH. The \( k_1 \) value obtained by TRX was essentially the same as that obtained by GSH, but the \( k_2 \) value for TRX was 400-times higher than that for GSH (Table IV).

**DISCUSSION**

To our knowledge, this was the first comparative study on the peroxide substrate specificity of the three ubiquitous GPx types cGPx, PHGPx, and eGPx and of SeP purified from human origins. Unfortunately, we cannot obtain a full-length recombinant mammalian selenocysteine-containing protein from an expression system such as *Escherichia coli*, because the translational mechanism of selenocysteine differs markedly between eukaryotes and prokaryotes (31). Therefore, we have isolated these four enzymes from human origins. As shown in Fig. 1, all of the enzymes were highly purified.

As shown in Table I, it is apparent that eGPx reduces \(-\text{Bu-OOH} \) and \( \text{H}_2\text{O}_2 \) effectively but does not reduce PC-OOH. To determine the enzymatic activity of eGPx, PHGPx, and SeP for PC-OOH, the optimum detergent conditions were reexamined and were found to differ for each enzyme. These differences may be attributed to the physical state of the lipid interface, suggesting that these three enzymes exhibit different reactivities to naturally occurring substrates such as lipid peroxides on lipoproteins and cell membranes. Under each optimum condition, PHGPx and eGPx showed the almost same specific activity to PC-OOH. Only in the case of eGPx did the presence of Triton X-100 lower the enzyme activity for PC-OOH but not for \(-\text{Bu-OOH} \) (32). Triton X-100 may not inhibit eGPx activity through the interaction with selenocysteine at the active site but may influence the physical state of the lipid interface. The differences in the required detergent conditions for the maximum PC-OOH reducing activity of PHGPx, eGPx, and SeP indicate that these three enzymes might be reactive to different kinds of phospholipid hydroperoxides such as oxidized lipoproteins and oxidized lipids on the plasma membrane of the cells.

High \( k_1 \) values (beyond \( 10^2 \text{ M}^{-1} \text{ s}^{-1} \)) for \( \text{H}_2\text{O}_2 \) were obtained from both cGPx and eGPx as commonly observed with other forms of mammalian GPx (17, 28). A high \( k_2 \) (beyond \( 10^2 \text{ M}^{-1} \text{ s}^{-1} \)) for PC-OOH was also obtained from PHGPx as reported previously for porcine PHGPx (17). Approximately, 100-fold lower \( k_1 \) and \( k_2 \) values were observed in SeP compared with PHGPx, suggesting that SeP is less reactive to PC-OOH than PH-GPx. All of the GPx family contains an active triad consisting of selenocysteine, glutamine, and tryptophan residues (1). SeP lacks one element of the active triad, the tryptophan residue. Deletion of this amino acid residue may be the reason why SeP shows a lower specificity to peroxide substrates. cGPx showed the highest \( k_2 \) value followed by eGPx, PHGPx, and SeP. This indicates that eGPx is significantly more reactive to GSH than are the other enzymes. As expected, cGPx used only GSH as an electron donor. The other three
enzymes could use diithiothreitol, mercaptoethanol, or cysteine as a reducing substance. Therefore, eGPx is a true glutathione peroxidase, whereas, strictly speaking, the other three enzymes are not glutathione peroxidases but are thiol peroxidases. cGPx contains a GSH binding site consisting of one lysine and four arginine residues (33). eGPx lacks three of the arginine residues of the GSH binding site. PHGPx and SeP lack all five residues of the GSH binding site. Deletion of these amino acid residues may be the reason why the other three enzymes show a lower specificity to GSH.

Because plasma GSH concentrations below 10 μM are not able to maintain a sufficient reduction capacity for eGPx and SeP, it has been implied that a physiologic substrate besides GSH might exist. It has been reported that eGPx is able to use TRX instead of GSH as a thiol substrate. Surprisingly, TRX is 500-fold more effective as an electron donor for GSH were in inverse proportion to the specificity for TRX. k_m for the low K_m GSH and TRX at almost the same rate. This seems odd because the TRX protein and the amino-terminal half of GPx protein share the same tertiary structure. This finding suggests that the TRX and GSH systems may be evolutionarily related. Therefore, we tried to determine whether the other GPxs including SeP also operate together with TRX reductase, which is also a thiol peroxidase by kinetic analysis. Following this, SeP and eGPx may be defined as an extracellular TRX peroxidase.

TRX operates together with TRX reductase, which is also a selenocysteine-containing enzyme, to reduce the disulfide bond of general proteins using NADPH (35). TRX is reportedly secreted in extracellular fluids (36), and significant expression of eGPx and PHGPx are found in the cytosol. These enzymes exhibit different substrate and thiol specificities and collaborate to protect the biological molecules from oxidative stress inside and outside the cells, respectively. However, further studies are necessary to clarify the reactivities of the GPx family and SeP against various hydroperoxides (i.e. oxidized lipoproteins and oxidized lipids on the cell membranes).

Acknowledgments—We thank the Hokkaido Red Cross Blood Center and Tonan Hospital for providing human blood cells, plasma, and placenta.

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