Phospholipid Hydroperoxides Are Substrates for Non-selenium Glutathione Peroxidase*

(Received for publication, September 9, 1998, and in revised form, February 1, 1999)

Aron B. Fisher‡, Chandra Dodia, Yefim Manevich, Jin-Wen Chen, and Sheldon I. Feinstein

From the Institute for Environmental Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104-6068

This study investigated phospholipid hydroperoxides as substrates for non-selenium GSH peroxidase (NS-GPx), an enzyme also called 1-Cys peroxiredoxin. Recombinant human NSGPx expressed in Escherichia coli from a human cDNA clone (HA0683) showed GSH peroxidase activity with sn-2-linolenoyl- or sn-2-arachidonoyl-phosphatidylcholine hydroperoxides as substrate; NADPH or thioredoxin could not substitute for GSH. Activity did not saturate with GSH, and kinetics were compatible with a ping-pong mechanism; kinetic constants (mM⁻¹ min⁻¹) were \( k_1 = 1 - 3 \times 10^9 \) and \( k_2 = 4 - 11 \times 10^8 \). In the presence of 0.36 mM GSH, apparent \( k_m \) was 120–130 \( \mu \)M and apparent \( V_{max} \) was 1.5–1.6 \( \mu \)mol/min/mg of protein. Assays with \( \text{H}_2\text{O}_2 \) and organic hydroperoxides as substrate indicated activity similar to that with phospholipid hydroperoxides. Maximal enzymatic activity was at pH 7–8. Activity with phospholipid hydroperoxide substrate was inhibited noncompetitively by mercaptoethanol with \( K_i \) of 4 \( \mu \)M. The enzyme had no GSH S-transferase activity. Bovine cDNA encoding NSGPx, isolated from a lung expression library using a polymorphic 5'-transferase activity, showed >95% similarity to previously published human, rat, and mouse sequences and does not contain the TGA stop codon, which is translated as selenocysteine in selenium-containing peroxidases. The molecular mass of bovine NSGPx deduced from the cDNA is 25,047 Da. These results identify a new GSH peroxidase that is not a selenoenzyme and can reduce phospholipid hydroperoxides. Thus, this enzyme may be an important component of cellular antioxidant defense systems.

The integrity of biomembranes and other phospholipid-enriched cellular components requires a mechanism to repair oxidized phospholipids generated by spontaneous or pathologic oxidation. Intracellular glutathione peroxidase (GSH Px) plays an important role in reducing fatty acid hydroperoxides and \( \text{H}_2\text{O}_2 \), although this enzyme has no activity toward phospholipid hydroperoxides (1). Detoxification of phospholipid hydroperoxides can be accomplished through the combined enzymatic activity of phospholipase \( \text{A}_2 \) (PLA₂) and reduction of the resultant fatty acid hydroperoxides with GSH Px (2). Recently, a phospholipid hydroperoxide GSH Px (PHGPx) has been described as a 19-kDa selenoenzyme that uses GSH to reduce peroxidized phospholipids to the nontoxic hydroxy derivative (3–5). Reduction of phospholipid hydroperoxides also has been described as a minor activity for the selenoenzyme plasma GSH Px and for some GSH S-transferase enzymes (6, 7).

In this report, we investigated phospholipid hydroperoxides as substrates for a novel 25-kDa GSH Px. The protein was first isolated from the bovine ciliary body and was shown to catalyze the reduction of \( \text{H}_2\text{O}_2 \) and organic hydroperoxides using GSH as electron donor (8). Activity toward phospholipid hydroperoxides was not tested. The absence of selenium in the protein was demonstrated by assay with 2,3-diaminonaphthalene (8). The enzyme, which was called a non-selenium GSH Px (NS-GPx), had no GSH S-transferase activity when assayed with a spectrum of potential SH acceptors (8).

A full-length cDNA sequence (HA0683) ultimately shown to encode NSGPx was first isolated from a human myeloblast cell line (KG-1) (9–11) and described as a Ca²⁺-independent PLA₂ (12). Subsequently, an identical cDNA from a human lymphoma cell line (U937) was described; protein encoded by this cDNA was expressed in \( E. \text{coli} \) and NIH 3T3 cells and shown to be a peroxidase with minimal PLA₂ activity (13). In contrast to the prior publication using the native protein (8), an \( \text{in vitro} \) assay with the protein product of this clone indicated that GSH was not an effective reductant, and the report concluded that the physiologic reductant for the peroxidase was unknown (13). Based on homology to the peroxiredoxin family, the enzyme was called 1-Cys peroxiredoxin, since only one Cys residue was conserved (13). More recently, protein expressed \( \text{in vitro} \) with a bovine cDNA clone was found to utilize GSH as a reductant for peroxidase activity, although the discrepancy with the previous study was not explained (14). One possibility, although unlikely, is that the human and bovine enzymes have different cofactor requirements.

The present study investigated substrate utilization of recombinant NSGPx using the human clone (HA0683) that has been previously described (12) as well as a newly isolated cDNA clone from a bovine lung cDNA library. Our results with both phospholipase \( \text{A}_2 \); DTT, dithiothreitol; PLPC, 1-palmitoyl-2-linolenoyl-sn-glycerol-3-phosphocholine; PAPC, 1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphocholine; DNP, diethyl \( p \)-nitrophenyl phosphate; MJ33, 1-hexadecyl-3-trifluoromethylglycerol-3-2-phosphonethanol; pPPB, \( p \)-bromophenacyl bromide; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; MES, 4-morpholinopropanesulfonic acid.

* This work was supported by National Institutes of Health Grants HL19737 and HL60290. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was presented in part at the annual meeting of the Oxygen Society, Washington, D.C., November 19–23, 1998 (28), at EPAT '99, Goa, India, January 9–11, 1999, and at Experimental Biology '99, Washington, D.C., April 17–21, 1999 (29).

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM accession number(s) AF090194.

‡ To whom correspondence and reprint requests should be addressed. Institute for Environmental Medicine, University of Pennsylvania School of Medicine, 1 John Morgan Bldg., 3620 Hamilton Walk, Philadelphia, PA 19104-6068. Tel.: 215-888-9100; Fax: 215-888-0868; E-mail: abf@mail.med.upenn.edu.

† The abbreviations used are: GSH Px, glutathione peroxidase; NS-GPx, non-selenium glutathione peroxidase; hNSGPx, human NSGPx; PHGPx, phospholipid hydroperoxide glutathione peroxidase; PLA₂, phospholipase \( \text{A}_2 \).

‡ GenBankTM accession number DI4662.

28 This paper is available online at http://www.jbc.org
human and bovine NSGPx show that GSH is effective for reduction of \( H_2O_2 \) and organic hydroperoxides including phospholipid hydroperoxides.

**EXPERIMENTAL PROCEDURES**

**Materials**—GSH, glutathione reductase, \( \beta \)-NADPH, isopropl \( \beta \)-thiogalactopyranoside, diethyl p-nitrophenyl phosphate (DEPNP), \( \beta \)-bro-mophenacyl bromide (pBBP), dithiothreitol (DTT), \( H_2O_2 \), cumene hydroperoxide, \( \beta \)-butyryl hydroperoxide, ilinonic acid, arachidonic acid, 1-chloro-2,4-dinitrobenzene, GSH peroxidase (from bovine erythrocytes), GSH S-transferase (from rat liver), and soybean lipoxidase (EC 1.13.11.12 type V) were purchased from Sigma. Phospholipids, 1-palmitoyl-2-arachidonoyl PC (PAPC) and 1-palmitoyl-2-linolenoyl PC (PLPC), were purchased from Avanti Polar Lipids (Alabaster, AL). 13-Hydroperoxycetadecanoic acid and 15-hydroperoxycisatetraenoic acid were purchased from Cayman Chemicals (Ann Arbor, MI). The C\(_5\)-Sep-Pack column was purchased from Millipore Corp. (Milford, MA). Radiochemicals were purchased from DuPont NEN. Molecular mass standards for SDSPAGE, transblot membrane, protein dye-binding assay kit, horseradish peroxidase-conjugated goat anti-rabbit IgG, and Triton X-100 (electrophoretically purified) were purchased from Bio-Rad. Enhanced chemiluminescence kit and x-ray film were purchased from Amersham Pharmacia Biotech. pET28C vector and the His bind kit containing pET28C (Novagen, Madison, WI). A bovine lung cDNA library was purchased from Stratagene (La Jolla, CA). Lennox LB broth was obtained from Fisher. 1-Hexadecyl-3-trifluoroethylylgycero-sn-2-phosphomethyl (MJ33), a \( \alpha \)A\(_2\) inhibitor, was a gift from Dr. Mahendra Jain (University of Delaware).

**Preparation and Purification of Recombinant Protein**—The human cDNA clone (HA0683) (12) was digested with the restriction enzyme HindIII, and the insert was recloned into the HindIII site of pET28C (Novagen, Madison, WI) in both the forward (sense) and reverse (antisense) directions. The inserted fragment contained about 1040 nucleotide pairs and included the entire coding sequence of the human enzyme along with 43 nucleotides from the 5'-untranslated region and 320 nucleotides from the 3'-untranslated region. Plasmid DNAs were prepared from these HindIII digestion products using QIAGEN columns (QIAGEN, Chatsworth, CA); they were transformed into \( \text{E. coli} \) BL21(DE3) cells, which produce T7 RNA polymerase and express pET28C inserts efficiently; and were incubated at 37 °C overnight to get colonies. A single colony was inoculated in 50 ml LB broth containing kanamycin (30 \( \mu \)g/ml) and incubated with shaking at 37 °C overnight to get colonies. A single colony was inoculated in 50 ml of LB broth containing kanamycin (30 \( \mu \)g/ml) and incubated with shaking at 37 °C until optical density at 600 nm reached 0.6. Recombinant protein expression was induced by adding isopropyl \( \beta \)-thiogalactopyranoside to a final concentration of 1 mM and incubating at 37 °C for 5 min. The cells were harvested by centrifugation at 5000 \( \times \)g for 5 min at 4 °C, resuspended in 12.5 ml of cold 50 mM Tris-HCl (pH 8.0), and sonicated. The cell pellet was resuspended in 4 ml of cold 50 mM Tris-HCl and sonicated three times. For purification, the cell pellet was resuspended and sonicated in 5 ml imidazole buffer.

The HA0683 product was expressed as a fusion protein with a series of six histidine residues in pET28C (His tag) for purification. The His tag allows affinity purification of the fusion protein on a Ni\(^{2+}\) column using the His bind kit under native conditions by following the protocols recommended by the manufacturer. Briefly, after allowing the 5 ml imidazole buffer to drain to the top of the column bed (2.5 ml), the column was loaded with the prepared extract in the same buffer. The flow rate was 10 ml/h, the column was washed with 25 ml of 50 mM imidazole buffer and then washed with 15 ml 60 mM imidazole buffer, and the bound protein was eluted with 15 ml of 1 x imidazole buffer. Fractions of 1 ml were collected from the column and analyzed by SDS-PAGE (15). NSGPx as indicated by Western blot was concentrated in fractions 2–5, which were pooled. Western blot of the purified protein utilized a previously described polyclonal antibody prepared in rabbits to a 15-amino acid synthetic peptide representing a conserved internal sequence of NSGPx or a mouse monoclonal antibody (SH111) raised against the \( \text{E. coli} \) expressed HA0683 product (human NSGPx) (15).

HA0683 also was expressed with a wheat germ expression system in the presence of 0.5 mM \(^{35}\)S-methionine (63 Ci/mmol) as described previously; this procedure generated a single band of \(^{35}\)S-labeled protein by autoradiography of SDS-PAGE gels (12). Each translation included 1 ml of E. coli cell extract in which no absorbance was determined and no nonenzymatic oxidation of NADPH and used to calculate enzyme activity based on authentic NADPH standards. Protein was measured with Coomassie Blue (Bio-Rad Protein dye binding kit) using bovine \( \gamma \)-globulin as a standard.

**Enzyme Assays—**NSGPx activity was assayed by measuring consumption of NADPH in the presence of GSH and GSH reductase. Unless otherwise noted, all assays used the HA0683 product expressed in \( \text{E. coli} \) and affinity-purified. The standard reaction buffer (3 ml) was 50 mM Tris-HCl, 2 mM NaN\(_3\), 0.1 mM EDTA (pH 8.0), 0.3 mM NADPH, 0.36 mM GSH, and 0.23 units/ml GSH reductase. To assay PC hydroperoxides, 0.1% Triton X-100 was routinely added, since activity was approximately 40% less in its absence. For the standard assay, the mixture plus enzyme (2 \( \mu \)g of protein/ml) was preincubated for 5 min with continuous stirring. Fluorescence was continuously recorded at 460 nm (340-nm excitation) using a fluorescence spectrophotometer (Photon Technology Instruments, Bricktown, NJ). After a steady base line was achieved, the reaction was started by the addition of substrate generally at 250 \( \mu \)M, and the linear change in fluorescence was recorded for 5–10 min. The change in fluorescence was corrected for the relatively small base-line nonenzymatic oxidation of NADPH and used to calculate enzyme activity based on authentic NADPH standards. Protein was measured with Coomassie Blue (Bio-Rad Protein dye binding kit) using bovine \( \gamma \)-globulin as a standard. Enzymatic activity was expressed as nmol of NADPH oxidized/min/mg of protein. Data for multiple replications are presented as mean ± S.E. To confirm results with the fluorescence assay, the production of hydroxy-PLPC from PLPC hydroperoxide was determined by HPLC analysis,
assuming that the extinction coefficient at 235 nm is the same for both molecular species (18).

In some assays, NSGPx activity was determined by the disappearance of H₂O₂ by modification of the method of Kang et al. (13). The enzyme (6 µg/ml) was incubated for 2 or 10 min in 50 mM Tris, 0.1 mM EDTA buffer with 0.2 or 5 mM GSH and 250 mM H₂O₂. At the end of incubation, 2 mM Fe(II)₄(SO₄)₃ and 0.25 mM KSCN were added. The absorbance of the red ferrithiocyanate complex formed in the presence of peroxides was measured at 480 nm. This assay was used to test pH dependence; buffers were 50 mM glycine (pH 3), 50 mM sodium acetate (pH 4–5), 50 mM MES (pH 6), or 50 mM Tris-HCl (pH 7–10). This assay also was used to measure peroxidase activity in the presence of 2 mM DTT, substituting for GSH, as described previously (13).

GSH S-transferase activity was measured by continuous spectrophotometric assay with 1-chloro-2,4-dinitrobenzene substrate (8).

**Fig. 2.** Continuous assay of hNSGPx activity by fluorescence measurement at 460 nm (340-nm excitation) recorded as photon counts/s. Decreased fluorescence indicates NADPH oxidation coupled to reduction of hydroperoxide. The standard assay mixture contained purified recombinant human NSGPx (2 µg of protein/ml) from the *E. coli* expression system (HA0683 product), 0.36 mM GSH, 0.23 units/ml GSH reductase (GR), and 0.3 mM NADPH. Substrate (250 µM) was added after a stable base line. A, substrate t-butyl hydroperoxide (t-BUOOH); B, substrate PLPC hydroperoxide (PLPCOOH); 0.1% Triton X-100 was present; C, GSH omitted from the initial mixture and added after t-butyl hydroperoxide showing a requirement for GSH and showing that NADPH is not a co-substrate for NSGPx; D, thioredoxin (0.36 mM) and thioredoxin reductase (TR; 0.23 units/ml) substituted for GSH/GR with t-butyl hydroperoxide substrate showing that thioredoxin is not a co-substrate for NSGPx; the subsequent addition of GSH/GR restored activity.

**Isolation and Sequencing of Bovine cDNA—** Since the initial isolation of this enzyme was from bovine sources (8), we isolated a bovine cDNA to complement previous descriptions of human, rat, and mouse clones.
Non-selenium GSH Px

**TABLE I**

**NSGPx activity with various hydroperoxide (HP) substrates**

| Substrate         | NSGPx activity | $V_{\text{max(app)}}$ | $K_m$ | $k_1$ | $k_2$ |
|-------------------|----------------|------------------------|-------|-------|-------|
|                   | nmol/min/mg protein | nmol/min/mg protein | 
| $\text{H}_2\text{O}_2$ | 1.850 ± 32 | 1810 | 180 | $1.8 \times 10^4$ | $9.0 \times 10^4$ |
| Cumene HP         | 1.140 ± 30 | 1120 | 120 | $1.2 \times 10^4$ | $4.0 \times 10^4$ |
| t-Butyl HP        | 1.320 ± 39 | 1270 | 142 | $1.6 \times 10^4$ | $6.1 \times 10^4$ |
| Linolenoyl HP     | 1.400 ± 21 | 1390 | 141 | $1.7 \times 10^4$ | $6.8 \times 10^4$ |
| Arachidonoyl HP   | 1.380 ± 30 | 1350 | 135 | $1.8 \times 10^4$ | $7.2 \times 10^4$ |
| PLPC HP           | 1.470 ± 37 | 1500 | 120 | $3.2 \times 10^4$ | $11 \times 10^4$ |
| PAPC HP           | 1.670 ± 29 | 1640 | 129 | $2.3 \times 10^4$ | $7.9 \times 10^4$ |

![Image 323x402 to 539x550](image_url)

**Fig. 4.** Profile of NSGPx activity with $\text{H}_2\text{O}_2$ substrate versus pH. hNSGPx (6 μg of protein/ml) was incubated in buffer of varying pH with 0.1 mM EDTA, 0.2 mM GSH, and 250 μM $\text{H}_2\text{O}_2$ for 2 min, and then the remaining $\text{H}_2\text{O}_2$ was determined (see “Experimental Procedures”). The open and closed symbols represent separate experiments.

PAGE compared with the mass of 25,032 Da deduced from HA0683 (12) is due in part to the presence of the “His tag” plus additional amino acids from the vector and from the 5′ normally untranslated region; we estimate the mass of these additional amino acids as 5996 Da. Based on densitometric scanning of Coomassie Blue-stained gels, the expressed hNSGPx protein in the crude cell homogenate was 13% of total protein (not shown), and this increased to 85% after the Ni$^{2+}$ column (Fig. 1). This figure for purity represents a lower limit, since immunoreactivity at higher molecular mass on the Western blot suggests the presence of dimers or multimeric forms.

The expressed hNSGPx demonstrated GSH peroxidase activity as indicated by the linear decrease in NADPH fluorescence following the addition of organic hydroperoxide substrate to the assay buffer (Fig. 2). Fig. 2A shows t-butyl hydroperoxide as substrate; $\text{H}_2\text{O}_2$ and hydroperoxides of cumene, linolenic acid, and arachidonic acid were equally effective (Table I). There was zero activity (not shown) with the antisense preparation, indicating specificity of the translated gene fragment. Hydroperoxides of phosphatidylcholine likewise were reduced by hNSGPx (Fig. 2B). PLPC and PAPC hydroperoxides were equally effective substrates (Table I). Enzymatic activity required GSH, which in combination with glutathione reductase resulted in oxidation of NADPH (Fig. 2C). NADPH itself was not a cofactor for the enzyme, and substitution of thioredoxin/thioredoxin reductase for GSH/glutathione reductase also was ineffective (Fig. 2D). In contrast to hNSGPx, authentic GSH Px (selenoenzyme from erythrocytes) reduced $\text{H}_2\text{O}_2$ and t-butyl hydroperoxide in the presence of GSH but had no reactivity toward PLPC hydroperoxide (Fig. 3).
hNSGPx also reduced $H_2O_2$ with DTT as electron donor as determined with the end point assay, consistent with previous reports (13, 23). Peroxidase activity of the crude cell lysate from *E. coli* prior to hNSGPx affinity purification was 61 ± 2 nmol/min/mg of protein ($n = 4$) for the end point assay, and no activity was seen in the absence of DTT. The corresponding activity in nmol/min/mg of protein with this preparation using 5 mM GSH in place of DTT was 60 ± 1 ($n = 4$). Activity for affinity-purified hNSGPx assayed with 0.2 mM GSH was 1275 ± 16 nmol/min/mg of protein ($n = 4$).

The effect of pH on the peroxidase reaction was evaluated with affinity-purified hNSGPx by the disappearance of $H_2O_2$ using the end point assay with 0.2 mM GSH. The pH optimum was in the range of 7–8; activity was reduced by 65% at pH 6, and virtually no activity was seen at pH 5 and below (Fig. 4). The pH dependence for PLPC hydroperoxide reduction was evaluated at pH 5 and 8 using HPLC to determine formation of the hydroxyphospholipid. HPLC of the purified original substrate showed a phospholipid hydroperoxide peak at 20.9 min but no hydroxyphospholipid peak. After a 1-h incubation of hNSGPx with substrate at pH 8, the peak height of the hydroperoxide signal was significantly decreased, and a hydroxyphospholipid peak appeared at 23.4 min (Fig. 5C); hNSGPx activity calculated from the integrated hydroxyphospholipid peak was 1400 nmol/min/mg of protein, similar to the value obtained with the fluorescence assay. No hydroxyphospholipid peak was detected at pH 5, indicating the absence of NSGPx activity (Fig. 5D).

Double-reciprocal plots of hNSGPx activity versus substrate concentration were linear for the hydroperoxide substrates as illustrated in Fig. 6 and were used to calculate apparent kinetic constants at 0.36 mM GSH used for the standard assay (Table I). The apparent $K_m$ for phosphatidylcholine hydroperoxides was 120–130 $\mu$M with apparent $V_{\text{max}}$ of 1.5–1.6 $\mu$mol/min/mg of protein. Values were similar for the fatty acid and short chain hydroperoxides, while both apparent $K_m$ and $V_{\text{max}}$ for $H_2O_2$ were slightly greater.

hNSGPx activity did not show saturation with GSH, and a double-reciprocal plot of activity versus substrate concentration at different [GSH] showed parallel lines (Fig. 6C). These results imply a ping-pong mechanism as described for other GSH peroxidases (3). These plots were used to calculate the kinetic constants $k_1$ and $k_2$ from the equation,

$$E/V = (1/k_1[S]) + (1/k_2[GSH])$$

(Eq. 1)

where $E$ is the NSGPx enzyme concentration, $V$ is the initial reaction rate, $S$ is the hydroperoxide substrate concentration, and GSH is the GSH concentration.

Calculation of $k_1$ and $k_2$ assumes constant [GSH] as provided by the presence of NADPH and glutathione reductase and also that the hydrophobic phospholipid hydroperoxide interface does not influence the apparent binding equilibrium to the active site. The apparent $K_m$ for a given GSH concentration can be calculated as $[\text{GSH}]k_2/k_1$.

hNSGPx activity with hydroperoxide substrates was inhibited by mercurial sulfinic acid (Fig. 7), an inhibitor of cysteine- and selenocysteine-mediated reactions. Inhibition was noncompetitive with PLPC hydroperoxide substrate (Fig. 6B) with calculated $K_i$ of 4 $\mu$M. The serine protease inhibitor, DENP, the histidine-active agent, pBPB, and a transition state phospholipid analogue inhibitor of PLA$_2$ activity, MJ33 (12, 15), had no effect on hNSGPx activity (Fig. 7). Fig. 7 shows the addition of inhibitors to the cuvette; preincubation of enzyme with each inhibitor for 30 min gave similar results with both PLPC hydroperoxide (Table II) and $t$-butyl hydroperoxide (not shown) as substrate.

The purified *E. coli*-expressed hNSGPx had no GSH S-transferase activity with 1-chloro-2,4-dinitrobenzene substrate (data not shown) in agreement with a previous report for the enzyme isolated from bovine ciliary body (8). Authentic GSH S-transferase from rat liver was used as a positive control for these assays and demonstrated the appropriate activity indicated by the supplier.

NSGPx activity was evaluated following expression of HA0683 with a wheat germ *in vitro* translation system. [$^{35}$S]Methionine incorporation was used to determine enzyme protein concentration for calculation of specific activity; note that possible contamination of the radiolabeled band with other 35S-labeled proteins would result in an overestimation of hNSGPx protein and an underestimation of its specific enzymatic activity. hNSGPx activity (PAPC hydroperoxide substrate) for protein from the wheat germ *in vitro* translation system was 3.7 $\mu$mol/min/mg of protein. This value is about 2.5-fold greater than that obtained with hNSGPx from the *E. coli* expression system. *In vitro* translation in wheat germ with the bovine lung NSGPx cDNA clone yielded protein with NSGPx activity similar to that for the human clone (HA0683) when expressed per total wheat germ protein (data not shown). However, specific
activity of bovine NSGPx could not be calculated, since [35S]methionine was not included in the translation medium. No NSGPx activity was detected from the wheat germ expression system in the absence of cRNA.

Analysis of clones isolated from the bovine lung cDNA library indicated that nucleotide sequences of seven of the clones corresponded closely to the previously published sequences for NSGPx cDNA from human, rat, and mouse sources (15). These seven clones were composed of five shorter clones, which appeared to utilize the first polyadenylation signal (ATTAAA), and two longer clones, which utilized the second (consensus) polyadenylation signal, AATAAA (Fig. 8). These proportions may reflect ease of cloning rather than the in vivo proportions.

The nucleotide sequence of the coding region (Fig. 8) was identical in the overlapping portion of all of the clones and indicated a predicted molecular mass of 25,047 Da. Out of 672 nucleotides, only 29 positions differed from the sequence of the other three species (Fig. 8). It is interesting that the termination codon sequence in the bovine DNA is TAG, while the other three species have TAA. The predicted amino acid sequence of the bovine NSGPx contains amino acids that differ from all of the other three species at only 10 positions out of 224 (Fig. 8).

While this manuscript was under review, the cDNA sequence of bovine NSGPx was reported (14). That clone was obtained by polymerase chain reaction of RNA isolated from bovine ciliary body, while the clone reported here was isolated from a bovine lung cDNA library. Comparing the previous and present sequences, within the coding region there is a single nucleotide difference (C for T at nucleotide number 6), which does not affect the amino acid sequence. The sequence reported here does contain much more of the 3' untranslated region, and the
sequences of both 5'- and 3'-untranslated regions differ substantially between the present clone and the previous report (14).

**DISCUSSION**

This study shows that an enzyme previously reported as a NSGPx without S-transferase activity (8) can also reduce phospholipid hydroperoxides in the presence of GSH. Activity was demonstrated by a fluorescence assay coupled to NADPH oxidation as well as by HPLC assay. The NSGPx enzyme for assay was generated by expression of the human cDNA clone HA0683 in *E. coli* and also by its expression in a wheat germ *in vitro* translation system.

The initial reported isolation of NSGPx protein was from bovine ciliary body (8). N-terminal amino acid sequencing of the protein identified 25 of the 29 N-terminal amino acids (after the initial methionine); two other amino acids were represented by n.d.

**Fig. 8.** Nucleotide sequence of bovine lung NSGPx cDNA (*top*) and the corresponding amino acid sequence (*bottom*). Numbers on the right indicate nucleotide positions relative to the translational start; those on the left indicate amino acid positions. The sequence shown is a composite derived from several clones. The sequence of the 5'-end of the cDNA is based upon four clones that had identical sequences beginning at the indicated 5'-end, 42 nucleotides upstream from the translational start, and ending shortly after the second polyadenylation signal. A fifth clone had the same 3'-end but did not extend as far on the 5'-end. For two other clones, their sequences terminated shortly after the second polyadenylation signal and contained a much longer 3'-untranslated region but did not extend to the start codon. The start and termination codons are underlined. **Boldface letters** indicate nucleotides or amino acids that differ from those in the corresponding positions in the coding regions of the mouse, rat, and human NSGPx cDNAs (15). The two putative polyadenylation signals are in **boldface** type and underlined.

**AMINO ACID #**

| Enzyme (NSGPx) Source | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|-----------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bovine ciliary body (8) | X | P | G | G | L | L | G | D | E | A | P | N | F | E | A | N | T | I | G | (5) | (5) | F | X | X | W | L | G |
| Bovine ciliary body* (14) | M | P | G | G | L | L | G | D | E | A | P | N | F | E | A | N | T | I | G | R | I | K | F | H | D | Y | L | G |
| Bovine lung (this report) | M | P | G | G | L | L | G | D | E | A | P | N | F | E | A | N | T | I | G | R | I | K | F | H | D | Y | L | G |
| Rat effector mouse (22,23) | X | P | G | G | L | L | G | D | E | A | P | N | F | E | A | N | T | I | G | R | I | K | F | H | D | Y | L | G |
| Human myeloblast (12)* | M | C | A | S | R | B | D | W | R | C | A | R | S | M | H | E | F | S | A | K | D | I | D | G | H | V | N | L | O | K |

*Sequence deduced from cDNA. *Activity was measured in the present report and with an identical cDNA from a human lymphoma cell line (13). **The selenoenzyme, phospholipid hydroperoxide glutathione peroxidase. X = not determined, ( ) = uncertain, bold = amino acid differs from bovine ciliary body.

**Fig. 9.** N-terminal amino acid sequences for isolated proteins or deduced from cDNAs for reported sequences with similarity to NSGPx isolated from bovine ciliary body (8). The sequences shown are for proteins that have been evaluated for peroxidase activity. The selenoenzyme PHGPx from pig liver (5) is shown for comparison. Residues that differ from the bovine ciliary body are shown in **boldface** type. The selenoenzyme PHGPx from pig liver (5) is shown for comparison. Residues that differ from the bovine ciliary body are shown in **boldface** type.
ported with less certainty, and two amino acids were unidentified as shown in Fig. 9 (8). Based on N-terminal sequence homology (Fig. 9), NSGPx protein was subsequently isolated from rat olfactory mucosa (22, 23). cDNA clones for NSGPx have been isolated from human myeloblast and lymphoma cell lines (12, 13), bovine ciliary body epithelium (14), and now bovine lung and expressed with in vitro translation by E. coli and in NIH 3T3 cells to demonstrate peroxidase activity (Refs. 13 and 14; present report). The nucleotide sequences for the coding region of these clones demonstrate >90% identity to each other and, for the deduced N-terminal amino acids, to NSGPx protein isolated from the ciliary body (Fig. 9). Based on sequence similarity, NSGPx protein also has been isolated from human (25) and mouse (26) liver and from human erythrocytes (27), while cDNAs have been isolated from rat lung type 2 epithelial cells (15), human keratinocytes (19), mouse skin (20), and mouse liver and kidney (26); however, these isolates have not been evaluated for peroxidase activity, and their sequences are not illustrated in Fig. 9. Related sequences from nematode, yeast, archaean, plants, and other sources have been described as members of the 1-Cys peroxiredoxin family (13).

H₂O₂ and short chain hydroperoxides have been shown previously to be effective substrates for NSGPx, but none of the previous reports has evaluated phospholipid hydroperoxides as substrates for this enzyme. The present study demonstrates that NSGPx has similar activity with phospholipid and short chain hydroperoxides. The cofactor reductant for peroxidase activity of NSGPx has been considered previously in several studies. NSGPx isolated from the bovine ciliary body exhibited GSH peroxidase activity (8). However, GSH was not an effective reductant with protein expressed in E. coli using the human NSGPx cDNA clone (13) or with NSGPx protein isolated from rat olfactory epithelium (23), although both preparations showed peroxidase activity in the presence of DTT. These latter reports concluded that the physiological cofactor for reduction of peroxides by NSGPx was unknown. Paradoxically, DTT had been reported to be an ineffective reductant with NSGPx protein isolated from the bovine ciliary body (8). More recently, NSGPx expressed using a cDNA clone from bovine ciliary epithelium was shown to utilize GSH as cofactor for its peroxidase activity (14). The present study with recombinant human and bovine NSGPx confirms that the enzyme can utilize GSH and that DTT is also effective as reductant. Thioredoxin and NADPH were ineffective as co-factors, consistent with a previous report (13).

Because of the previous uncertainty regarding its activity, the trivial name given to the enzyme has been inconsistent. It was originally called a non-selenium GSH Px (8). A subsequent study noted sequence homology to members of the peroxiredoxin (formerly thioredoxin peroxidase and before that thiol-specific antioxidant) family of enzymes (13). However, unlike most members of that family, this enzyme contains only one conserved cysteine and, therefore, was termed 1-Cys peroxiredoxin (13). Our laboratory has called the enzyme αiPLA₂ based on PLₐ₂ activity that is Ca²⁺-independent with acidic pH optimum (12, 15, 24), although this activity appears to be much lower than the peroxidase activity (13). The protein without regard to its function also has been called HUMORF06 as a descriptor of the full-length human myeloblast coding sequence (11, 26) and LT4 protein based on its chromosomal location in the mouse genome (26). Based on the results in this study, we confirm non-selenium GSH peroxidase (NSGPx) as an appropriate name for this member of the peroxiredoxin family.

The molecular mass of NSGPx (in kDa) predicted from the cDNA is 24.8 for rat and mouse (15, 20) and 25.0 for human (12, 19) and bovine (Ref. 14; this report) enzymes. The apparent molecular mass of native NSGPx on reduced SDS-PAGE was 24–26 kDa for human (25, 27), 26–28 kDa for rat and mouse (12, 22, 26), and 29 kDa for bovine (8, 24) protein. The reported pl was 6.2 for the human (25, 27) and 5.6–5.9 for the mouse (26) proteins. The reason for the range in mobility on PAGE is not known. Endoglycosidase F treatment of bovine lung NSGPx did not affect its mobility on gel electrophoresis (24), indicating the probable absence of significant post-translational glycosylation.

Phospholipid hydroperoxides can be generated in vivo in response to oxidative stress and may have major effects on membrane structure and function. The ability to reduce phospholipid hydroperoxides has been demonstrated for the selenoenzyme PHGPx (3) and as a minor activity for both the selenoenzyme plasma GSH Px (6) and the non-selenium GSH S-transferases (7). This study demonstrates that NSGPx also can reduce phospholipid hydroperoxides. Kinetic analysis of NSGPx activity indicated a tert-uni ping-pong mechanism similar to that described for other GSH peroxidases including PHGPx (3). Further, the kinetic constants for NSGPx described in this report are of the same order of magnitude as shown previously for PHGPx (3). Despite these similarities, the N terminus for the 25-kDa NSGPx has no amino acid sequence homology with the 19-kDa selenoprotein PHGPx from pig liver (Fig. 9) or with the N terminus for the longer transcript (not shown) from rat testis (21). Although the molecular mass of NSGPx is similar to that for plasma GSH Px, these two enzymes have no significant amino acid sequence homology (7). A previous detailed analysis of the entire coding region identified a short sequence motif (20 amino acids) in selenium PHGPx and another short motif (10 amino acids) in GSH S-transferase (P₁ type) that show 60–70% similarity to sequences in the present enzyme (19). The cDNA for NSGPx does not contain the stop codon TGA (Fig. 8), which in GSH Px and PHGPx is translated as selenocysteine (5). This corroborates the previous chemical analysis of NSGPx protein, which failed to detect selenium (8). Thus, unlike the other enzymes with PHGPx activity, NSGPx does not contain selenium and has no GSH S-transferase activity. It therefore represents a novel enzyme for the reduction of phospholipid hydroperoxides.

Acknowledgments—We thank Dr. Tae-Suk Kim for the E. coli-expressed protein used in the initial phase of this work; Dr. John Powers for alerting us to the initial reported isolation of this enzyme (8); Drs. Henry Forman, Abu Al-Mehdi, and Mahendra Jain for excellent suggestions; Drs. Colin Funk and Maeve McDonald for use of the high pressure liquid chromatograph; Dajmie Fisher for technical assistance; and Elaine Primero for typing the manuscript.

REFERENCES
1. Michiels, C., Raes, M., Toussaint, O., and Remacle, J. (1994) Free Radical Biol. Med. 17, 235–248
2. van Kuijk, F. J. G. M., Sevanian, A., Handelman, G. J., and Dratz, E. A. (1987) Trends Biochem. Sci. 12, 31–34
3. Ursini, F., Maiorino, M., and Gregolin, C. (1985) Biochim. Biophys. Acta 839, 626–70
4. Maiorino, M., Gregolin, C., and Ursini, F. (1990) Methods Enzymol. 186, 444–457
5. Brigiouin-Flohé, R., Aumann, K-D., Blöcker, H., Gross, G., Kieß, M., Köppel, K-D., Maiorino, M., Roveri, A., Schuckelt, R., Ursini, F., Wingender, E., and Flohé, L. (1994) J. Biol. Chem. 269, 7342–7348
6. Yamamoto, Y., and Takahashi, K. (1993) Arch. Biochem. Biophys. 305, 541–545
7. Hurst, R., Bao, Y., Jemth, P., Mannervik, B., and Williamson, G. (1998) Biochem. J. 332, 97–110
8. Shichi, H., and Demar, J. C. (1990) Exp. Eye Res. 50, 513–520
9. Nomura, N., Miyajima, N., Suzuki, T., Tanaka, A., Kawarabayasi, Y., Sato, S., Nagase, T., Seki, N., Ishikawa, K., and Tabata, S. (1994) DNA Res. 1, 27–35
10. Nagase, T., Miyajima, N., Tanaka, A., Suzuki, T., Seki, N., Sato, S., Tabata, S., Ishikawa, K., Kawarabayasi, Y., Kotani, H., and Nomura, N. (1995) DNA Res. 2, 37–43
11. Chee, H. Z., Robison, K., Pool, L. B., Church, G., Storz, G., and Rhee, S. G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 7017–7021
12. Kim, T-S., Sundaresan, C. S., Feinstein, I. S., Dodia, C., Skach, W. R., Jain, N., Nagase, T., Seki, N., Ishikawa, K., Nomura, N., and Fisher, A. B. (1997) J. Biol. Chem. 272, 2542–2550
21334
Non-selenium GSH Px

13. Kang, S-W., Baines, I. C., and Rhee, S-G. (1998) J. Biol. Chem. 273, 6303–6311
14. Singh, A. K., and Shiichi, H. (1998) J. Biol. Chem. 273, 26171–26178
15. Kim, T-S., Dodia, C., Chen, X., Hennigan, B. B., Jain, M., Feinstein, S. I., and Fisher, A. B. (1998) Am. J. Physiol. 274, L750–L761
16. Funk, C. D., Gunne, H., Steiner, H., Izumi, T., and Samuelsson, B. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2592–2596
17. Therod, P., Couturier, M., Demelier, T. F., and Lemonnier, F. (1993) Lipids 28, 245–249
18. Gardner, H. W. (1989) Biochim. Biophys. Acta 1001, 274–281
19. Frank, S., Munz, B., Hübner, G., Olsen, E., and Werner, S. (1997) Biochem. J. 326, 579–585
20. Pushpa-Rekha, T. R., Burdsall, A. L., Oleksa, L. M., Chisolm, G. M., and Driscoll, D. M. (1995) J. Biol. Chem. 270, 26993–26999
21. Peshenko, I. V., Novoselov, V. I., Evdokimov, V. A., Nikolaev, Yu, V., Shuvaeva, T. M., Lipkin, V. M., and Fesenko, E. E. (1996) FEBS Lett. 381, 12–14
22. Peshenko, I. V., Novoselov, V. I., Evdokimov, V. A., Nikolaev, Yu, V., Shuvaeva, T. M., Lipkin, V. M., and Fesenko, E. E. (1998) Free Radical Biol. Med. 25, 654–659
23. Peshenko, I. V., Novoselov, V. I., Evdokimov, V. A., Nikolaev, Yu, V., Kamzalov, S. S., Shuvaeva, T. M., Lipkin, V. M., and Fesenko, E. E. (1998) Free Radical Biol. Med. 25, 654–659
24. Akiba, S., Dodia, C., Chen, X., and Fisher, A. B. (1998) Comp. Biochem. Physiol. Part B, 120, 393–404
25. Golaz, O., Hughes, G. J., Frutiger, S., Paquet, N., Bairoch, A., Pasquali, C., Sanchez, J. C., Tissot, J. D., Appel, R. D., Walser, C., Balant, L., and Hochstrasser, D. F. (1993) Electrophoresis 14, 1223–1231
26. Iakonbova, O. A., Pacella, L. A., Her, H., and Beter, D. R. (1997) Genomics 42, 474–478
27. Hochstrasser, D. F., Frutiger, S., Paquet, N., Bairoch, A., Ravier, P., Pasquali, C., Sanchez, J. C., Tissot, J. D., Bejelqvist, B., Vargas, R., Appel, R. D., and Hughes, G. J. (1992) Electrophoresis 13, 992–1001
28. Fisher, A. B., Dodia, C., Manevich, Y., Chen, J.-W., and Feinstein, S. I. (1998) Free Radical Biol. Med. 25, S31
29. Chen, J. W., Dodia, C., Feinstein, S. I., and Fisher, A. B. (1999) FASEB J. 13, 174 (abstr.)