Free Rad. Res. Comms., Vol. 14, Nos. 5-6, pp. 343-361
Reprints available directly from the publisher
Photocopying permitted by license only

PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE IS A SELENO-ENZYME DISTINCT FROM THE CLASSICAL GLUTATHIONE PEROXIDASE AS EVIDENT FROM cDNA AND AMINO ACID SEQUENCING

R. SCHUCKELT*, R. BRIGELIUS-FLOHÉ*, M. MAIORINO**, A. ROVERI**, J. REUMKENS*, W. STRAßBURGER*, F. URSINI****, B. WOLF* and L. FLOHÉ***

**Grünenthal GmbH, Center of Research, W-5100 Aachen, FRG*
***Institute of Biological Chemistry, University of Padova, 35100 Padova, Italy*
****Gesellschaft für Biotechnologische Forschung, W-3000 Braunschweig-Stöckheim, FRG*
*****Institute of Chemistry, University of Udine, Italy*

(Received November 28, 1990; in final form January 9, 1991)

The primary structure of phospholipid hydroperoxide glutathione peroxidase (PHGPx) was partially elucidated by sequencing peptides obtained by cyanogen bromide cleavage and tryptic digestion and by isolating and sequencing corresponding cDNA fragments covering about 75% of the total sequence. Based on these data PHGPx can be rated as a selenoprotein homologous, but poorly related to classical glutathione peroxidase (GPx). Peptide loops constituting the active site in GPx are, however, strongly conserved in PHGPx. This suggests that the mechanism of action involving an oxidation/reduction cycle of a selenocysteine residue is essentially identical in PHGPx and GPx.

KEY WORDS: Selenocysteine, phospholipid hydroperoxide glutathione peroxidase, glutathione peroxidase, primary structure.

ABBREVIATIONS: GPx: glutathione peroxidase; GSH: glutathione; PGHPx: phospholipid hydroperoxide glutathione peroxidase; CB: cyanogen bromide; TFA: trifluoroacetic acid; HPLC: high-performance liquid chromatography; TPCK: N-tosyl-L-phenylalanine chloromethyl ketone; I: inosine; PCR: polymerase chain reaction; EDTA: ethylene diamine tetracetic acid; IPTG: isopropyl β-D-thiogalactoside; bp: base pairs; X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

INTRODUCTION

Enzymatic reduction of hydroperoxides by glutathione peroxidase (GPx) was first described by Mills in 1957[1] and has since been discussed as a major defense mechanism against oxidative stress, in particular against oxidative biomembrane destruction.[2]

The "classical" glutathione peroxidase of bovine red blood cells discovered by Mills

Address for correspondence: PD Dr. Regina Brigelius-Flohé, Grünenthal GmbH, Center of Research, Zieglerstr. 6, W-5100 Aachen.

‡This publication contains data taken from the Ph.D. thesis of Rainer Schuckelt.

has meanwhile been fully characterized. It proved to be a tetrameric selenoprotein[3] with a selenocysteine in each of the four active sites.[4,5] Its amino acid sequence was elucidated[6] and confirmed by sequencing the corresponding cDNA.[7] Also, the three-dimensional organisation of the peptide backbone was analysed by X-ray crystallography.[8] This structural knowledge complemented by functional studies provides an almost precise idea of the catalytic mechanism which, in brief, consists of a fast oxidation of the selenolate form of the enzyme by a hydroperoxide, reaction of the selenenic acid derivative thus formed with GSH resulting in a selenadisulfide, and regeneration of the reduced enzyme form by a second GSH molecule.[2,9]

More recently, the GPx activity of plasma could be attributed to an immunologically distinct protein.[10,11] Its sequence proved to be distinct from, but homologous to that of the cytoplasmatic enzyme.[12]

In respect to its presumed role in biomembrane protection GPx proved disappointing. It could be convincingly demonstrated that the enzyme was unable to reduce hydroperoxo groups of biomembrane lipids without previous cleavage of the peroxidized fatty acids by a phospholipase.[13,14] In contrast, an other GSH peroxidase first isolated from pig liver by Ursini *et al.*[15] directly acted on peroxidized phospholipids integrated into biomembranes[15,16] and was therefore called phospholipid hydroperoxide glutathione peroxidase (PHGPx;).[16] Apart from this important functional difference and the apparently monomeric nature of PHGPx, the two enzymes appeared remarkably similar. The molecular mass of PHGPx was close to the subunit molecular mass of GPx, both enzymes contained selenium in almost identical stoichiometry, their amino acid compositions were rated to be similar, and the kinetic mechanisms were found to be identical.[16]

Due to these similarities it remained ambiguous whether PHGPx had to be considered as an enzyme in its own right or rather as a variant or derivative of GPx.

Our first attempts to solve this problem by protein chemical analysis, however, unmasked some additional differences between PHGPx and GPx. PHGPx could not be easily sequenced, since its N-terminus, in contrast to that of GPx, was blocked and the protein proved to be poorly soluble under conditions of proteolytic cleavage conventionally used for protein sequencing. These complications rendered a structural comparison of PHGPx and GPx based on protein sequencing data alone a frustrating approach. Attempts to isolate a cDNA encoding PHGPx by conventional colony hybridization from pig cDNA libraries were also unsuccessfull, since the message was obviously rarely represented in the libraries and the probes derived from the scarce protein chemical data proved not to be particularly specific. After amplification of the specific cDNA by PCR technique, however, we could finally identify and sequence three overlapping cDNA fragments corresponding to more than 75% of the PHGPx sequence. Based on these data we can now state that PHGPx is a selenoenzyme with a unique primary structure which reveals an only remote phylogenetic relationship to both intracellular and extracellular GPx.

MATERIALS AND METHODS

Source of the Enzyme

Phospholipid hydroperoxide glutathione peroxidase was purified from pig heart as previously described.[16]

Electrophoresis

Homogeneity and molecular mass of the reduced and carboymethylated protein was analysed according to Laemmli[17] in 12.5% acrylamide gel.

Amino Acid Analysis

The amino acid composition of the reduced and carboxymethylated enzyme[18] and of fragments was determined, after acid hydrolysis in 6 N HCl, 0.1% thioglycolic acid at 110°C for 20 h, on an amino acid analyser (LC 6001 Biotronic, Maintal/FRG) using postcolumn derivatization with ninhydrine.

Protein Fragmentation

The pure enzyme was reduced with β-mercaptoethanol and carboxymethylated with iodoacetic acid according to Crestfield *et al.*[18] The carboxymethylated protein was digested with TPCK-trypsin (Merck, Darmstadt/FRG) in 1.0% ammoniumbicarbonate, 0.1% Triton X100 pH 8.3 for 15 h at 37°C. The chemical cleavage of the protein was performed in 70% formic acid with cyanogen bromide (CB) according to Gross and Witkop.[19] Peptides were separated by HPLC on a reversed phase C 18 column using either a gradient of 0.1% aqueous TFA in acetonitrile at 50°C (tryptic peptides) or a gradient of 0.05% aqueous TFA in 0.025% TFA/acetonitrile at 25°C (CB-peptides).

Sequencing of Peptides

Tryptic peptides were covalently linked to aminopropylglass[20] by C-terminal coupling and then sequenced on a solid phase sequencer (LKB 4010), Freiburg/FRG) according to Laursen.[21] CB peptides were either sequenced on liquid phase sequencer (Beckman 890 M, München/FRG) or on a pulsed liquid phase sequencer (473A Protein Sequencer, Applied Biosystem, Foster City/USA). Phenylthiohydantoin derivatives of amino acids were identified by isocratic HPLC on a reversed phase C_8 column according to Lottspeich.[22]

Polymerase Chain Reaction (PCR)

The PCR was performed according to Saiki *et al.* (23) in 100 μl samples containing 1 μg cDNA (λgt 10 c-DNA library prepared from pig heart, lot no. 2511, twice amplified; Clontech Laboratories, Palo Alto CA, USA) in 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 20 pmoles of each primer, 100 μg/ml gelatine and 2.5 U Taq polymerase (Pharmacia, Freiburg, FRG). The samples were overlaid with 100 μl paraffin oil. Prior to the addition of Taq polymerase the samples were boiled for 5 min and then cooled on ice and transferred to a LEP-PremIII thermocycler (Biozym, Hameln, FRG).
Reaction times and temperatures were adjusted to the different conditions as specified below:

1) With probes containing degenerate codons the following conditions were applied: 1 min 94°C, 1 min 35°C, increase from 35°C to 50°C within 1 min, 1.5 min 74°C. After

40 cycles temperature was kept constant at 74°C for 7 min. The samples then were cooled until use.

2) Conditions for probes with unequivocal codons were 1 min 94°C, 1 min 55°C, 1.5 min 74°C. After 40 cycles temperature was kept constant at 74°C for 7 min. The samples then were cooled until use.

3) In the asymmetric PCR the primer with unequivocal codons complementary to a sequenced part of the PHGPx was applied in a 100-fold excess (20 pmoles) over the second primer (0.2 pmoles). The program used was: 1 min 94°C, 1 min 55°C, 30 sec 74°C, 25 cycles, then cooling. In this way the template corresponding to the PHGPx insert is specifically amplified. The asymmetric amplified sample then was used for the PCR procedure described under 2).

Cloning of PCR Fragments

For detailed procedures of molecular cloning see Maniatis *et al.*[24] or Ausubel *et al.*[25] After finishing the PCR procedure the toplayer of paraffin oil was removed from the samples. Residues of oil were extracted with ether. DNA fragments were precipitated with ethanol and redissolved. The DNA was then treated with Klenow polymerase in the presence of all four deoxynucleotides to fill-in incompletely synthesized ends and to generate homogenous blunt ends. The 5′-ends then were phosphorylated with polynucleotide kinase. Samples were applied to a 1.5% NuSieve agarose gel (Biozym, Hameln, FRG) and subjected to electrophoresis for 5 hours at 45 V in a TBE (0.1 M Tris borate; 84 mM boric acid; 2 mM EDTA) buffer. The gels were stained with ethidium bromide and promising bands selected for cloning and sequencing. The bands were cut out of the gel and directly used[26] for ligation with a HincII digested pUC19 vector, which had been extensively dephosphorylated before. Ligation was performed at 16°C. *E. coli* JM103 cells were then transformed with the ligated DNA. The clones containing the insert were selected on ampicillin xGal plates after induction with IPTG. The plasmid was isolated from positive (white) clones for sequencing by means of a plasmid miniprep kit from Diagen, Düsseldorf, FRG.

DNA-Sequencing

The PCR generated inserts were sequenced in both directions directly from the pUC-plasmids with the dideoxy method described by Sanger *et al.*[27] The sequencing kit (T 7 polymerase) from Pharmacia and the protocol provided by the manufacturer was applied. A 17-mer sequencing primer (−40) and a 17-mer reverse sequencing primer from Biolabs, Beverly MA, USA, were used as pUC-specific oligonucleotides.

Synthesis of Oligonucleotides

Oligonucleotides were obtained by the solid-phase oligonucleotide synthesis method of Adams *et al.*[28] at a 1 μMol scale. Using a Biosearch 8600 synthesizer of New Brunswick Scientific Co., Heusenstamm, FRG, commercially available beta-cyanoethyl-protected diisopropylamino phosphoamidites of the respective desoxyribonucleotides were used as the monomer-building blocks. After cleavage from the solid support the crude and still trityl-bearing strands were desalted and re-purified under sterile conditions by gel filtration and then submitted twice to reversed phase

HPLC. The main products were detritylated and then purified twice again by reversed phase HPLC.

RESULTS

Proteinchemical Analyses

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) was isolated from pig heart and purified to homogeneity by chromatographic steps including ion exchange-, affinity chromatography on a bromosulphophthalein column, and gel permeation chromatography.[16] The reduced and carboxymethylated (RCM) protein proved to be homogeneous in SDS polyacrylamide electrophoresis and showed an apparent molecular weight of about 20.0 kD (Figure 1), which corresponds to the value found for the native enzyme.[16]

The amino acid composition of RCM-PHGPx is listed in Table 1. The presence of selenocysteine in PHGPx was strongly supported by a peak corresponding to the carboxymethyl derivative of selenocysteine (Figure 2), but due to the instability and the incomplete derivatization with iodoacetic acid, it could not be quantified. The

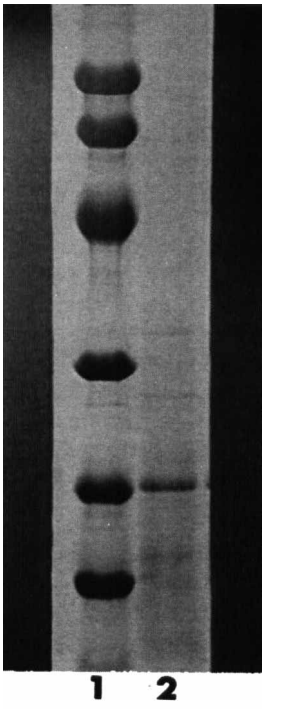

FIGURE 1 Dodecylsulfate/polyacrylamide gel electrophoresis of pig heart phospholipid hydroperoxide glutathione peroxidase (PHGPx). The sample was reduced with β-mercaptoethanol and carboxymethylated with iodoacetic acid. The gel was stained with Coomassie Blue R250. Lane 1: standard proteins, phosphorylase b (MW = 94,000), bovine serum albumin (MW = 67,000), ovalbumin (MW = 43,000), carbonic anhydrase (MW = 30,000), trypsin inhibitor (MW = 20,100) and lactalbumin (MW = 14,400); lane 2: 5 μg PHGPx

TABLE I
Amino acid composition of pig heart PHGPx. The composition is calculated on the basis of 190 amino acids. Values are given in residues per molecule. Selenocysteine and cysteine are determinated as carboxymethyl-derivatives.

Amino acid	residues/molecule
CM-Cys	6.2
Asx	23.2
Thr	6.0
Ser	8.2
Glx	20.6
Gly	17.6
Ala	13.6
Val	11.7
Met	8.5
Ile	8.3
Leu	11.3
Tyr	7.5
Phe	9.1
His	5.0
Lys	15.0
Arg	9.8
Pro	8.4
Total	190.0

N-terminus of CM-PHGPx was not susceptible to Edman degradation and is therefore assumed to be blocked.

Fragments of RCM-PHGPx were obtained by cyanogen bromide cleavage. The peptides were separated by HPLC (Figure 3) and characterized by N-terminal sequencing. The peptide sequences are listed in Table II. Due to low yield, peptide CB3 could only be identified by pulsed liquid phase sequencing. Like intact PHGPx the peptide CB2 was not degraded by Edman chemistry. A modest homology of the peptide CB5 with the primary structures of glutathione peroxidases was observed, whereas the other peptide sequences could not be aligned with any known structure.

Some peptides overlapping cyanogen bromide fragments were obtained by tryptic digestion of RCM-PHGPx. The elution profile of the tryptic peptides on HPLC is shown in Figure 4. A total of ten fractions were analyzed by N-terminal sequencing. The derived sequences are summarized in Table III. The peptide fractions T1 and T6 were blocked. The amino acid sequence of the peptide T10 exhibited an homology with bovine GPx of more than 70%. Tyrosin in peptide T4a could not be identified by N-terminal sequencing, but was definitely demonstrated by amino acid analysis of the peptide hydrolysate. T4b is a minor contaminating sequence of T4a which supported an overlapping sequence to CB3.

The sequencing strategy and the alignment of the peptides with the cDNA sequence are summarized in Figure 5.

Isolation and Sequencing of cDNA

A commercial pig heart cDNA library in λ gt10 was used to amplify DNA fragments encoding PHGPx by means of the PCR technique. Primers according to the peptides T10 and CB5 were selected for the first PCR run. The selection of these peptides was

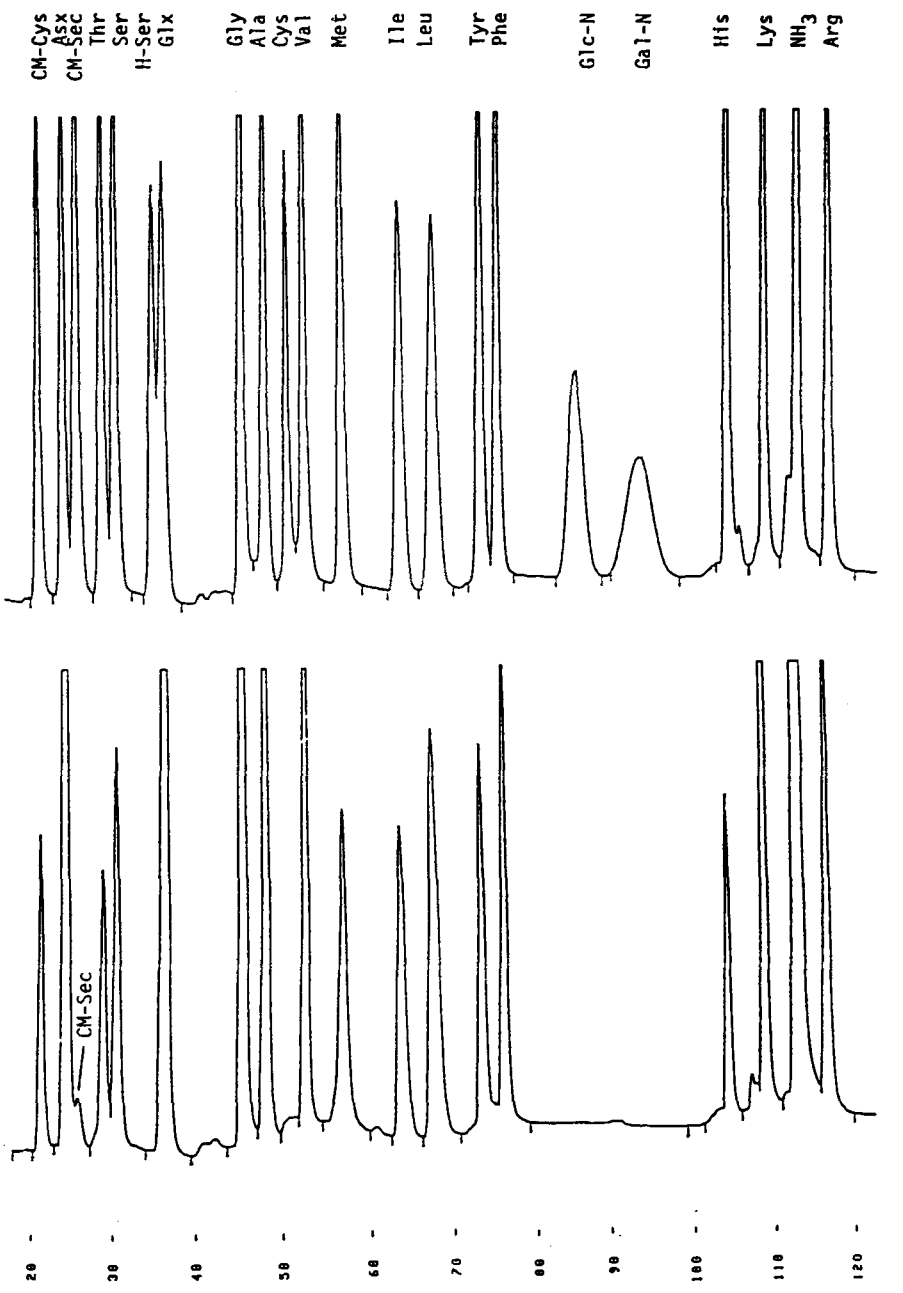

FIGURE 2 Amino acid analysis of phospholipid hydroperoxide glutathione peroxidase PHGPx from pig heart. Selenocysteine (CM-Sec) is identified as carboxymethylderivative. Upper panel: amino acid standard mixture. Lower panel: hydrolysate of PHGPx.

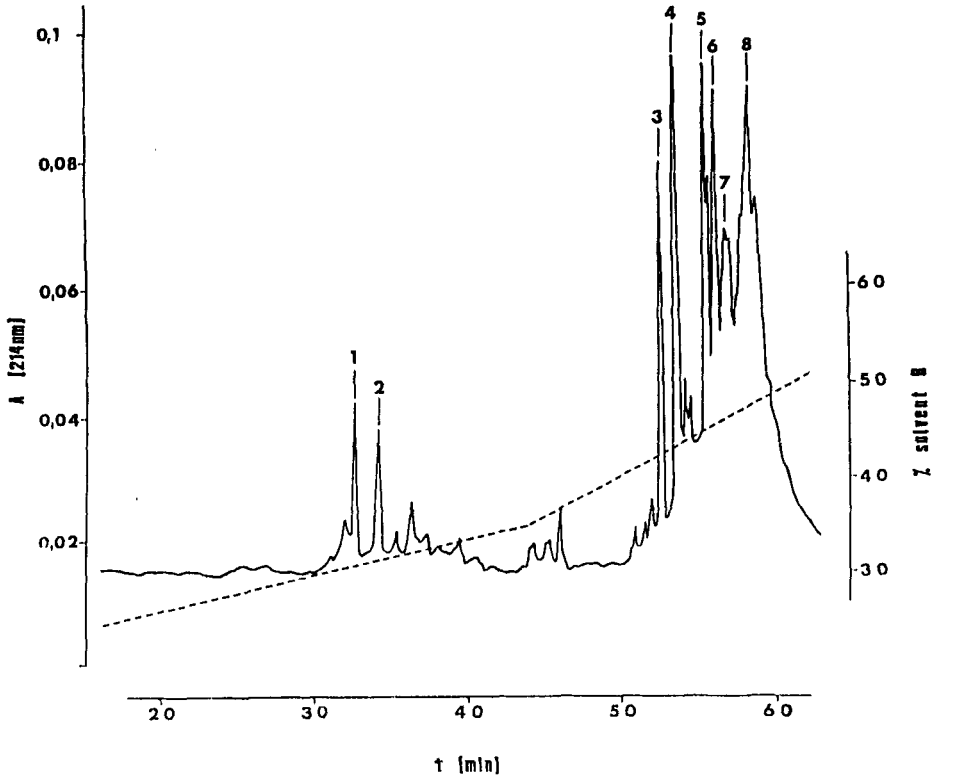

FIGURE 3 Elution profile of peptides obtained by cyanogen bromide cleavage of reduced and carboxymethylated phospholipid hydroperoxide glutathione peroxidase. The peptides were separated by reversed phase HPLC on a Nucleosil C 18 column (250 × 8 mm) with a gradient of 0.05% aqueous TFA (solvent A) in 0.025% TFA/acetonitrile (solvent B). Peptides are designated according to the order of elution.

based on their possible homology to the sequence of the bovine GPx.[6] It was assumed that the peptide T10 might represent a part of the N-terminus of PHGPx and CB5 a part of the C-terminus. The corresponding DNA probes were therefore prepared in the sense and antisense direction, respectively. Peptide and primer sequences are shown in Table IV. The primers were synthetisized according to the selected peptide sequences in all possible codon combinations with some wobble bases expressed as I (inosine). A mixture of such primers was applied in the PCR.

With this first PCR (see methods for degenerate codons) a DNA fragment of about 200 bp was obtained. This fragment was cloned as described in methods and sequenced. The sequence of the first PCR-product corresponds to bases 169 to 371 in Figure 5. From Figure 5 it is also obvious that the amino acid sequence deduced from the nucleotide sequence contains the oligopeptides T10 and CB5, which were used for the construction of the primers, and also a sequence encoding the peptide CB4, T7 and T8 (bases 253 to 300; Figure 5).

This first fragment now allowed to synthesize an unequivocal primer ($PCB4_{inv}$). It comprises the bases 306–277 of Figure 5 in the inverse direction. As second primer an

TABLE II

Amino acid sequence of peptides obtained by cyanogen bromide cleavage of carboxymethylated PHGPx. Peptides were separated by reversed phase HPLC on a C 18 column and then N-terminal sequenced. They are designated according to their consecutive order of elution (see Figure 3). X symbolizes unidentified amino acids, o indicates peptides overlapping with tryptic peptides.

CB 1:o	(M)	-	H	-	E	-	F	-	S	-	A	-	K	-	D	-	I	-	D	-	G									
CB 2:	blocked ?																													
CB 3:o	(M)	-	E	-	E	-	P	-	Q	-	V	-	I	-	E	-	K	-	D	-	L	-	P	-	X	-	Y	-	L	
CB 4:o	(M)	-	F	-	S	-	K	-	I	-	C	-	V	-	N	-	G	-	D	-	D	-	A	-	H					
CB 5:	(M)	-	L	-	G	-	N	-	A	-	I	-	K	-	W	-	N	-	F	-	T	-	K	-	F	-	L	-	I	-	D
CB 6:	(M)	-	L	-	G	-	N	-	A																					
CB 7:	(M)	-	L	-	G	-	N	-	A																					
CB 8:o	(M)	-	V	-	N	-	L	-	D	-	K	-	Y	-	X	-	G	-	Y	-	V	-	C	-	I	-	V			

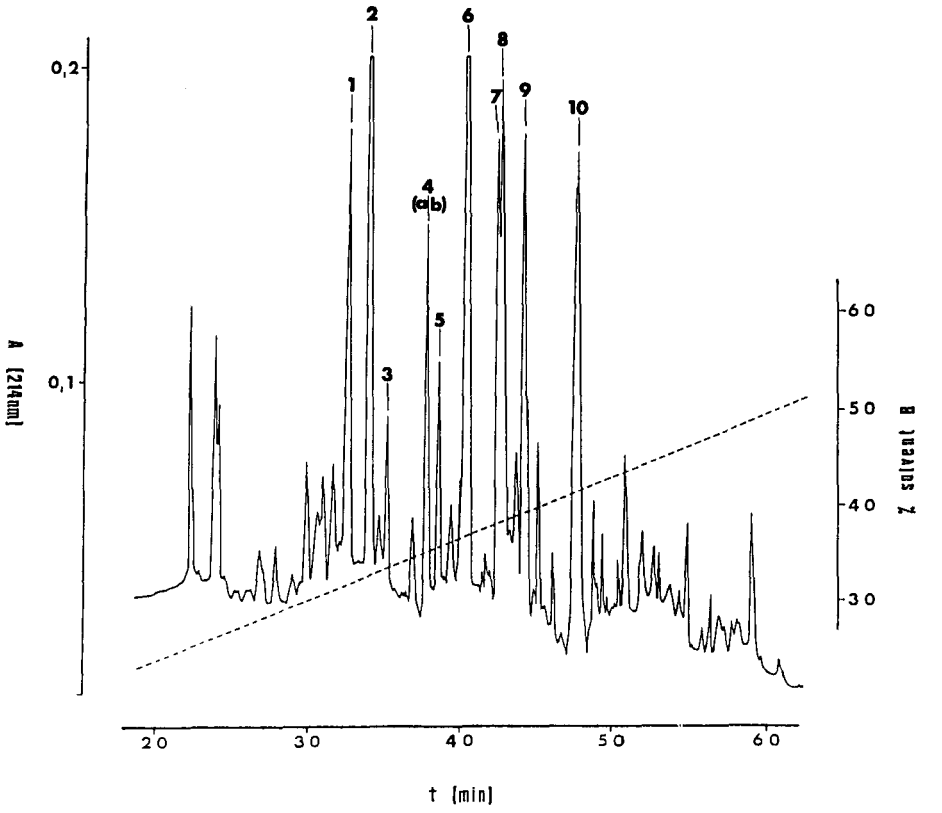

FIGURE 4 Trytic fingerprint of phospholipid hydroperoxide glutathione peroxidase (PHGPx) from pig heart. Peptides were obtained by digestion of reduced and carboxymethylated PHGPx with TPCK-trypsin. The tryptic fragments were separated by reversed phase HPLC on a Nucleosil C 18 column (250 × 8 mm) with a gradient of 0.1% aqueous TFA (solvent A) in acetonitrile (solvent B). Peptides are designated according to the order of elution.

oligonucleotide complementary to the λgt10-sequence on the right side of the λ-EcoRI cloning site was chosen (see Table 4, Pλr).

In order to increase the PHGPx-specific DNA the second PCR was run asymmetrically; the $PCB4_{inv}$ primer was added in a 100-fold excess over the Pλr primer. Thereby a specific amplification of the desired fragment in the single strand form was obviously achieved, which then was preferentially used as template in the following normal PCR run.

The reaction resulted in a fragment of about 300 bp. This fragment again was cloned and sequenced as described in methods. The sequence of this second PCR product corresponds to bases 1 to 306 in Figure 5. Bases 169–306 overlapping in the two fragments were thus sequenced twice and found to be identical. The sequence comprises the oligo-peptides and also contains a TGA codon presumably coding for selenocysteine. The adjacent cDNA fragments code for an amino acid sequence with an unusually high homology to GPx (see discussion). The identification of TGA within a coherent reading frame excludes the possibility that TGA here is a stop codon, but indeed codes for selenocysteine as in other selenoproteins.[2,7,29–34]

TABLE III

Amino acid sequence of peptides obtained by TPCK-trypsin-digested carboxymethylated PHGPx. Peptides were separated by reversed phase HPLC on a C 18 column and then N-terminal sequenced. They are designated according to their consecutive order of elution (see Figure 4). X symbolizes unidentified amino acids, O indicates peptides overlapping with fragments obtained by cyanogen bromide cleavage. The tyrosine residue framed in a box is supposed to be a site of phosphorylation.

T 1 :	blocked ?																													
T 2 :O	S	-	M	-	H	-	E	-	F	-	S	-	A	-	K															
T 3 :	Y	-	A	-	E	-	C	-	G	-	L	-	R																	
T 4a:	E	-	F	-	A	-	A	-	G	-	Y	-	N	-	V	-	K													
T 4b:O	Y	-	G	-	P	-	M	-	E	-	E	-	P	-	Q	-	V	-	I	-	E	-	K							
T 5 :O	D	-	I	-	D	-	G	-	H	-	M	-	V	-	N	-	L	-	D	-	K									
T 6 :	blocked ?																													
T 7 :O	F	-	D	-	M	-	F	-	S	-	K																			
T 8 :O	I	-	C	-	V	-	N	-	G	-	X	-	D	-	A	-	X	-	P											
T 9 :	T	-	E	-	V	-	N	-	Y	-	T	-	Q	-	L	-	V	-	D	-	L	-	H	-	A	-	R			
T 10 :	I	-	L	-	A	-	F	-	P	-	C	-	N	-	Q	-	F	-	G	-	R									

TABLE IV

Sequences of PHGPx peptides, the possible pertinent cDNA and the nucleotide sequences of the synthesized primers used for the polymerase chain reactions The unequivocal primers correspond to sequences unequivocally established from the first (PCB4$_{inv}$) or second PCR product (P 1) and to vector sequences (P λ r) and P λ 1).

T 10 amino acid sequences	:		Ala	Phe	Pro	Cys	Asn	Gln	Phe	Gly				
possible codons	:	5′	GC A C G T	TT C T	CC A C G T	TG C T	AA C T	CA A G	TT C T	GG A C G T	3′			
Probe PT 10	:	5′	GCI	TT C T	CCI	TG C T	AA C T	CA A G	TT C T	GG	3′			
CB5 amino acid sequence	:		Met	Leu	Gly	Asn	Ala	Ile	Lys	Trp	Asn	Phe	Thr	
possible codons	:	5′	ATG	A C C T T G T	GG A C G T	AA C T	GC A C G T	ATC A T	AA A G	TGG	AA C T	TT C T	AC A C G T	3′
Probe PCB 5	:	3′	TAC	AI A G	CCI	TT A G	CGI	TAI	TT C T	ACC	TT A G	AA A G	TG	5′
Probe Pλr		:	5′	AGAGGTGGCTTATGAGTATTTCTTCCAGGG					3′					
Probe PCB4$_{inv}$		:	5′	CCACAGAGGGTGGGCATCGTCCCCATTCAC					3′					
Probe P1		:	5′	TGCTGAGATCAAAGAATTTGCTGCTGGC					3′					
Probe Pλ1		:	5′	CGAGCTGCTCTATAGACTGCTGGGTAG					3′					

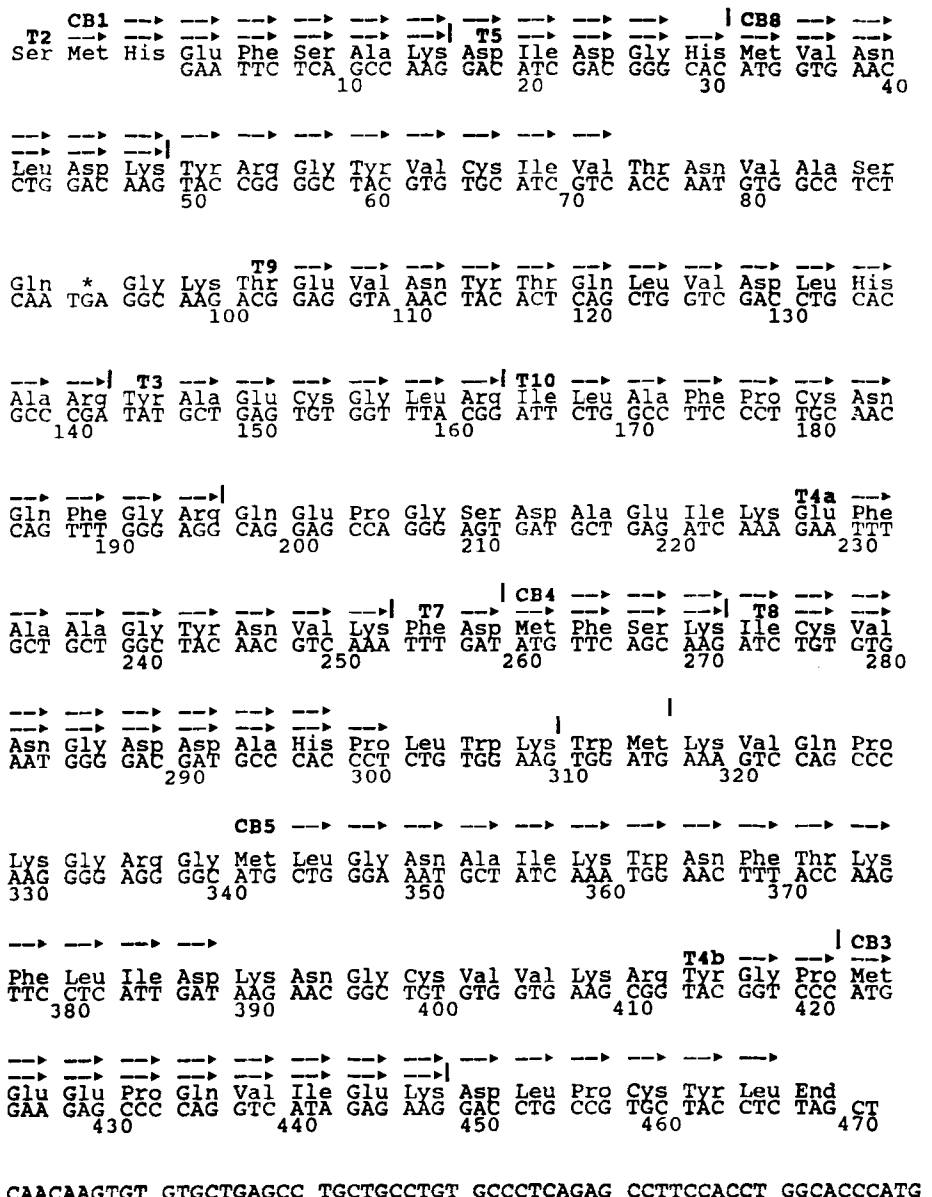

FIGURE 5 Summary of the identified primary structure of phospholipid hydroperoxide glutathione peroxidase (PHGPx). The protein sequencing strategy is indicated according to the cleavage methods used; cyanogen bromide cleavage (CB) and tryptic digestion (T). Amino acids identified by peptide sequencing are marked by → . * symbolizes selenocysteine.

Since the sequence corresponding to the degenerate primer has to be considered ambiguous, a further asymmetric PCR procedure was attempted by means of an unequivocal primer P 1 (position 213–240 in Figure 5, see also Table 4) and a λgt10 primer (see Table 4, Pλ1). This yielded another fragment of about 480 bp covering the ambiguous part of the previously obtained fragment, the C-terminal sequence up to the stop codon TAG, about 200 untranslated bp and the poly-adenylation site.

As is obvious from the sequence shown in Figure 5 the fragment of PHGPx obtained so far ends up in an EcoRI site near the presumed N-terminus. Further screening of this library with the PHGPx primers appeared not very promising. The elucidation of the full-length cDNA would therefore require a library prepared with different restriction enzymes. Taking together the information from protein and cDNA sequencing, however, an appreciable part of the total PHGPx structure has become established and allows a comparison of PHGPx and GPx at a molecular level.

DISCUSSION

1. Validity of Sequence Data

The partial primary structure of PHGPx presented here is essentially based on the assumption that the cDNA fragments obtained from the library after specific DNA amplification by the polymerase chain reaction are indeed a transcript of the mRNA encoding PHGPx and are essentially correct. The validity of this assumption is proven by the finding that not only the primer sequences used were correctly recovered. Also the cDNA sequences coded correctly for the amino acids flanking those corresponding to the primers. Further, the amino acid transcript covered a total of 14 peptides, which have been isolated from PHGPx digests and sequenced. Assuming a molecular mass of PHGPx of 20 kD[16] the identified cDNA encodes at least 75% of the total protein, and from the deduced protein sequence again 66% are confirmed by direct peptide sequencing. In conclusion, therefore, it can be taken as granted that the cDNA encodes PHGPx and that the PHGPx sequence derived therefrom, although in parts based on sequencing a single cDNA fragment only, may be considered essentially correct.

2. The Selenium Moiety of PHGPx

It had been shown before by selenium deprivation experiments[35] and direct analysis[16] that PHGPx is a selenoprotein. The precise nature of the selenium moiety of PHGPx, however, remained unknown and might still be considered uncertain. In fact, the tiny peak indicating carboxymethyl-selenocysteine in Figure 2 by itself should hardly be taken as convincing evidence of chemical identity. The localization of this peak, however, coincides precisely with that of carboxymethyl-selenocysteine in the analysis of classical GPx and the respective peptide.[6] Unfortunately, peptide sequencing of CB8 to strengthen the chemical identity of the selenium moiety of PHGPx could not be further advanced due to scarcety of material. But since now a TGA codon, which encodes selenocysteine in intracellular[7,29–32] and extracellular GPx[12] and also in bacterial selenoproteins[33,34] was identified within an open reading frame of the cDNA encoding PHGPx, it can hardly be doubted that a selenocysteine residue is indeed also the selenium moiety of PHGPx.

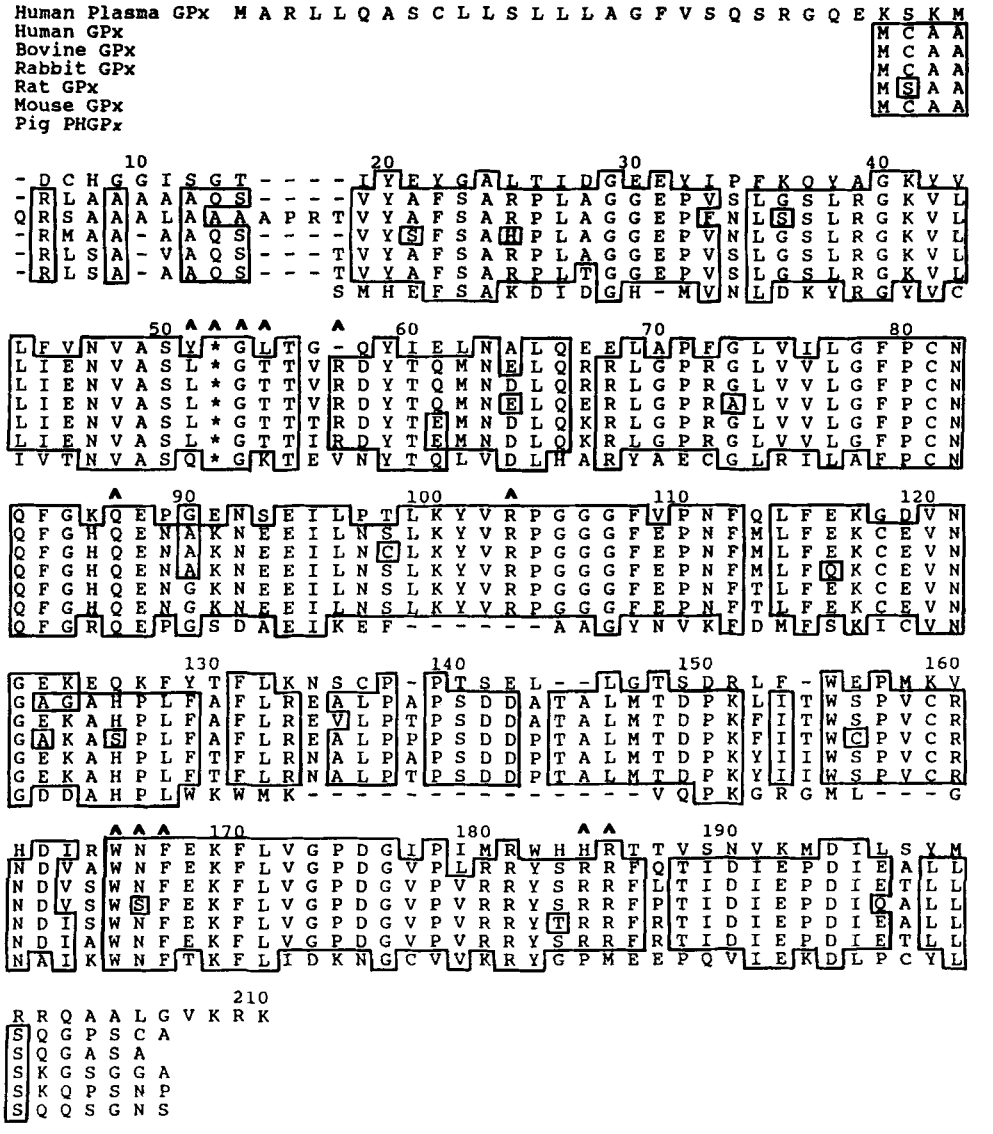

FIGURE 6 Alignment of the identified primary structure of phospholipid hydroperoxide glutathione peroxidase (PHGPx) with the sequences of intra-cellular glutathione peroxidases (GPx) of man,[29] cattle,[6,7] rabbit,[30] rat,[31] mouse[32] and extracellular human GPx[12] using bovine numbering. Amino acids supposed to be involved in substrate binding and catalysis of GPx are marked by ▲. * indicates selenocysteine (position 52). Amino acid homologies in four or more of the aligned sequences are boxed.

3. Homology Consideration

Not surprisingly, some parts of the PHGPx structure proved to be similar to that of GPx. As discussed above, the selenium moiety of PHGPx may also be considered a selenocysteine residue. This selenocysteine residue is located in a region with high

homology to the corresponding sequences of classical GSH peroxidases, whereas in other parts of the PHGPx sequence hardly any homology to the GPx sequence can be detected. Nevertheless, our partial sequence of PHGPx could be tentatively aligned to those of known GPx species (Figure 6). Whereas the homology between the established mammalian intracellular GPx species is about 85%, the homology between PHGPx and those GPx species is about 35%. At the cDNA level the homology between PHGPx and the known mammalian GPx species is about 45%. Also, the homology between PHGPx and the extracellular GPx is low (41% in terms of cDNA homology and 25% in terms of amino acid homology). This degree of homology is almost as low as that between homologous mammalian and bacterial enzymes as e.g. shown for the Cu/Zn superoxide dismutases.[36] On the other hand, the homology is by far too pronounced to be suggestive of a mere convergence. This would imply that nature must have invented the use of selenoproteins to reduce hydroperoxides at least a billion of years ago and, since, these selenoenzymes are diverging and specialising functionally. In view of the structural dissimilarity, the divergence of PHGPx and GPx must have occurred substantially earlier than the evolutionary divergence of mammalian species.

4. Special Structural Features

The identities of amino acid residues between GPx and PHGPx are not evenly distributed but centered in three clusters at the positions 47–55, 77–88 and 165–171

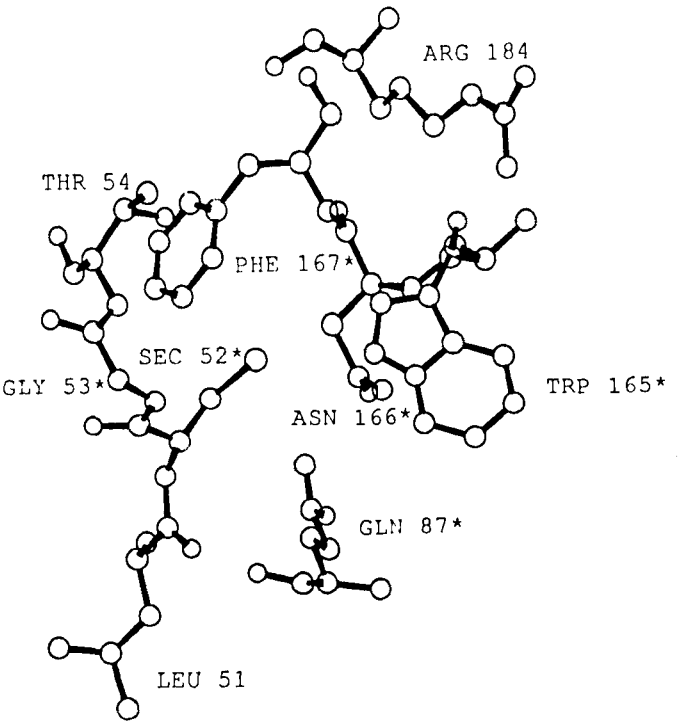

FIGURE 7 Active site of bovine glutathione peroxidase according to Epp *et al.*[37] The residues marked with an asterisk are conserved in phospholipid hydroperoxide glutathione peroxidase (PHGPx).

(Figure 6). Interestingly, these distant sequences are exactly those constituting the active site in GPx.

According to the X-ray analysis of the bovine GPx[8,9] the selenocysteine residue (residue 52) is located in the center of a flat hydrophobic impression flanked by the arginine residues 57, 103, 184 and 185 (position numbers according to Figure 6).[2,8,9] These were discussed to be involved in the electrostatic orientation of glutathione towards the active site selenium. In fact, these four arginine residues are conserved in the intracellular GPx of man, cattle, rabbit, rat and mouse, and also two of them in the extracellular human GPx, a finding supporting their functional relevance. All these arginine residues, however, are mutated or deleted, respectively, in PHGPx. Since the glutathione binding mechanism proposed for GPx[2,8,9] depends on the presence of at least two of the missing arginine residues, PHGPx must handle its substrate GSH in a different way. In fact, PHGPx has been reported to be less specific for the donor substrate than GPx.[16]

In contrast, the intimate environment of the selenocysteine residue of PHGPx could look almost identical to that of GPx (Figure 7). Tryptophane 165 and glutamine 87 supposed to sustain hydrogen bonds to the selenium are conserved at identical positions. Also, from the remaining five residues surrounding the selenocysteine (Leu51, Gly53, Thr54, Asn166 and Phe167) only two are changed (Leu51 → Gln, Thr54 → Lys). It is remarkable that also in the extracellular human GPx only these two residues are mutated. Altogether, from the 9 residues constituting the active site of bovine GPx, as depicted in Figure 7, six are conserved in PHGPx. This strongly supports the assumption that the catalytic mechanisms of GPx and PHPGx are identical in principle, as had already been deduced from kinetic measurements.[16]

A curiosity of PHGPx became obvious during sequencing of peptide T4. When sequenced by the solid-phase technique, a sequence with an unidentified amino acid was obtained. This ambiguous residue, however, could unequivocally be identified as tyrosine from the amino acid composition of T4a (data not shown) and later from cDNA sequencing (Figure 5, bases 241–243). This indicated that the tyrosine residue is obviuosly modified and can therefore not be identified. This observation led to the discovery that PHGPx can indeed be reversibly phosphorylated (unpublished). This surprises since the environment of the tyrosine residue does not represent a known phosphorylation site.[38] The biological implications of this finding are not yet understood but certainly it is suggestive of a further specialization of PHGPx and rather reminds of a modulation of membrane function than of defense against unspecific oxidant attack.

5. *Conclusions*

Based on partial sequence analysis, phospholipid hydroperoxide glutathione peroxidase is a selenoprotein poorly related to intracellular and extracellular glutathione peroxidase (GPx). The overall homology is below 40% for the intracellular GPx and about 25% for the extracellular enzyme of the aligned amino acid residues, but a higher conservation of amino acid residues in homologous positions is obvious in structural domains of presumed functional relevance. These results suggest that the divergence in the molecular evolution of these selenoproteins occurred earlier than the evolution of mammalian species. The emerging functional specialization of the selenoproteins certainly merits further attention.

Acknowledgements

We would like to thank Mr. H.-G. Döteberg, Mrs. A. Huppertz, Mrs. U. Leinz-Hanke, Mrs. D. Peters-Groth and Mrs. G. Wittlinger for their skilful assistance and Mrs. W. Berlin for preparing the manuscript.

References

1. G.C. Mills, (1957) Hemoglobin catabolism. I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. *Journal of Biological Chemistry*, **229**, 189–197.
2. L. Flohé, (1989) The selenoprotein glutathione peroxidase. In: *Glutathion* (Dolphin, D., Poulson, R., Avramovic, O., eds.) p. 643–731, John Wiley & Sons, New York.
3. L. Flohé, W.A. Günzler and H.H. Schock, (1973) Glutathione peroxidase: a selenoenzyme. *FEBS Lettes*, **32**, 132–134.
4. J.W. Forstrom, J.J. Zakowski and A.L. Tappel, (1978) Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. *Biochemistry*, **17**, 2639–2644.
5. A. Wendel, B. Kerner and K. Graupe, (1978) The selenium moiety of glutathione peroxidase. *Hoppe-Seyler's Z. Physiol. Chem.*, **359**, 1035–1036.
6. W.A. Günzler, G.J. Steffens, A. Grossmann, S.-M.A. Kim, F. Ötting, A. Wendel and L. Flohé, (1984) The amino-acid sequence of bovine glutathione peroxidase. *Hoppe-Seyler's Z. Physiol. Chem.*, **365**, 195–212.
7. G.T. Mullenbach, A. Tabrizi, B.D. Irvine, G.I. Bell, J.A. Tainer and R.A. Hallewell, (1988) Selenocysteine's mechanism of incorporation and evolution revealed in cDNAs of three glutathione peroxidases. *Protein Engineering*, **2**, 239–246.
8. R. Ladenstein, O. Epp, K. Bartels, A. Jones, R. Huber and A. Wendel, (1979) Structure analysis and molecular model of the selenoenzyme glutathione peroxidase at 2.8 Å resolution. *Journal of Molecular Biology*, **134**, 199–218.
9. R. Ladenstein, O. Epp, W.A. Günzler and L. Flohé, (1986) Glutathione peroxidase on approval. *Life Chemistry Reports*, **4**, 37–55.
10. K. Takahashi, P.E. Newburger and H.J. Cohen, (1986) Glutathione peroxidase protein. Absence in selenium deficiency states and correlation with enzymatic activity. *Journal of Clinical Investigation*, **77**, 1402–1404.
11. K. Takahashi and H.J. Cohen, (1986) Selenium-dependent glutathione peroxidase protein and activity: immunological investigations on cellular and plasma enzymes. *Blood*, **68**, 640–645.
12. K. Takahashi, M. Akasaka, Y. Yamamoto, C. Kobayashi, J. Mizoguchi and J. Koyama, (1990) Primary structure of human plasma glutathione peroxidase deduced from cDNA sequences. *Journal of Biochemistry*, **108**, 145–148.
13. A. Grossman and A. Wendel, (1983) Non-reactivity of the selenoenzyme glutathione peroxidase with enzymatically hydroperoxidized phospholipids. *European Journal of Biochemistry*, **135**, 549–552.
14. A. Sevanian, S.F. Muakkassah-Kelly and S. Montestruque, (1983) The influence of phospholipase A2 and glutathione peroxidase on the elimination of membrane lipid peroxides. *Archies in Biochemistry and Biophysics*, **223**, 441–452.
15. F. Ursini, M. Maiorino, M. Valente, L. Ferri and C. Gregolin, (1982) Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. *Biochimica et Biophysica Acta*, **710**, 197–211.
16. F. Ursini, M. Maiorino and C. Gregolin, (1985) The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. *Biochimica et Biophysica Acta*, **839**, 62–70.
17. U.K. Laemli, (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
18. A.M. Crestfield, S. Moore and W.H. Stein, (1963) The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *Journal of Biological Chemistry*, **238**, 622–627.
19. E. Gross and B. Witkop, (1961) Selective cleavage of the methionyl peptide bonds in ribonuclease with cyanogen bromide. *Journal of American Chemistry*, **83**, 1510–1511.
20. W. Machleidt and E. Wachter, (1977) New supports in solid-phase sequencing. *Methods in Enzymology*, **47**, 263–377.
21. R.A. Laursen, (1979) Solid-phase Edman degradation. *European Journal of Biochemistry*, **20**, 89–102.
22. F. Lottspeich, (1980) Identification of the phenylthiohydantoin derivatives of amino acids by high pressure liquid chromatography. Using a ternary, isocratic solvent system. *Hoppe-Seyler's Z. Physiol. Chem.*, **361**, 1829–1834.
23. R.K. Saiki, D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis and H.A. Erlich,

(1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239**, 487–491.
24. T. Maniatis, E.F. Fritsch and J. Sambrook, (1982) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory.
25. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl, (1987) *Current Protocols In Molecular Biology* John Wiley & Sons, New York.
26. M.M. Dumais, S. Nochumson, (1987) In-gel ligation and transformation of double-stranded DNA (dsDNA) *Bio Techniques*, **5**, 62.
27. F. Sanger, S. Nicklen and A.R. Coulson, (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA*, **74**, 5463–5467.
28. S.P. Adams, K.S. Kavka, E.J. Wykes, S.B. Holder and G.R. Galluppi, (1983) Hindered dialkylamino nucleoside phosphite reagents in the synthesis of two DNA 51-mers. *Journal of the American Chemistry Society*, **105**, 661–663.
29. Y. Sukenaga, K. Ishida, T. Takeda and K. Takagi, (1987) cDNA sequence coding for human glutathione peroxidase. *Nucleic Acids Research*, **15**, 7178.
30. M. Akasaka, J. Mizoguchi, S. Yoshimura and K. Watanabe, (1989) Nucleotide sequence of cDNA for rabbit glutathione peroxidase. *Nucleic Acids Research*, **17**, 2136.
31. Y.-S. Ho, A.J. Howard and J.D. Crapo, (1988) Nucleotide sequence of a rat glutathione peroxidase cDNA. *Nucleic Acids Research*, **16**, 5207.
32. I. Chambers, J. Frampton, P. Goldfarb, N. Affara, W. McBain and P.R. Harrison, (1986) The structure of the mouse glutathione peroxidase gene: the selenocysteine in the active site is encoded by the "termination" codon, TGA. *EMBO Journal*, **5**, 1221–1227.
33. T.C. Stadtman, (1987) Specific occurrence of selenium in enzymes and amino acid tRNAs. *FASEB Journal*, **1**, 375–379.
34. W. Leinfelder, E. Zehelein, M.A. Mandrand-Berthelot and A. Böck, (1988) Gene for a novel tRNA species that accepts L-serine and cotranslationally inserts selenocysteine. *Nature*, **331**, 723–725.
35. F. Weitzel, F. Ursini and A. Wendel, (1990) Phospholipid hydroperoxide glutathione peroxidase in various mouse organs during selenium deficiency and repletion. *Biochimica et Biophysica Acta*, **1036**, 88–94.
36. G.J. Steffens, J.V. Bannister, W.H. Bannister, L. Flohé, W.A. Günzler, S.-M.A. Kim and F. Ötting, (1983) The primary structure of Cu-Zn superoxide dismutase from Photobacterium leiognathi: ecidence for a separate evolution of Cu-Zn superoxide dismutase in bacteria. *Hoppe-Seyler's Z. Physiol. Chem.*, **364**, 675–690.
37. O. Epp, R. Ladenstein and A. Wendel, (1983) The refined structure of the selenoenzyme glutathione peroxidase at 0.2-nm resolution. *European Journal of Biochemistry*, **133**, 51–69.
38. B.E. Kemp and R.B. Pearson, (1990) Protein kinase recognition sequence motifs. *TIBS*, **15**, 342–346.

Accepted by Prof. H. Sies