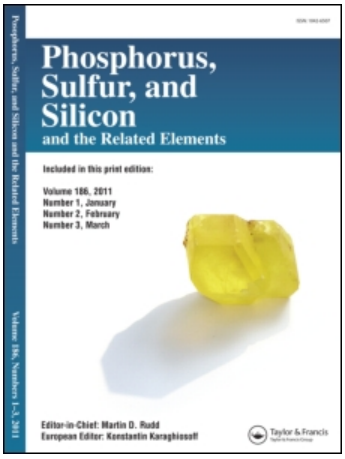

Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE

M. Maiorino[a]; A. Roveri[a]; F. Ursini[a]
[a] Institute of Biological Chemistry, University of Padova, Padova, Italy

To cite this Article Maiorino, M. , Roveri, A. and Ursini, F.(1988) 'PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE', Phosphorus, Sulfur, and Silicon and the Related Elements, 38: 1, 41 — 48
To link to this Article: DOI: 10.1080/03086648808079699
URL: http://dx.doi.org/10.1080/03086648808079699

Phosphorus and Sulfur, 1988, Vol. 38, pp. 41–48
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PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE

M. MAIORINO, A. ROVERI and F. URSINI
Institute of Biological Chemistry, University of Padova,
via F. Marzolo 3 35121 Padova Italy

Abstract "Phospholipid Hydroperoxide Glutathione Peroxidase" (PHGPX) is a new selenoenzyme recently identified and purified from mammalian tissues that reduces membrane hydroperoxides. This monomeric enzyme (M.W. 21,000), different from all previously known glutathione peroxidases and glutathione transferases, has been purified from different mammalian tissues and accounts for the cytosolic inhibition of microsomal lipid peroxidation induced by NADPH or ascorbate and iron. A selenocysteine is present at the active site, as suggested by the kinetics of the inhibition in the presence of iodoacetate. The kinetics, as in the case of GPX, is compatible with a ter-uni ping pong mechanism. The kinetic comparison between PHGPX and GPX in the presence of different substrates and detergents, indicates that, while GPX is more active on soluble hydrophilic substrates, PHGPX is more active on the hydroperoxides in the membranes.

INTRODUCTION

A great insight in the understanding of the physiological and nutritional roles of selenium in mammalian tissues was achieved by the discovery that Glutathione Peroxidase (GPX) contains selenium[1] and that this selenium is involved in the catalytic cycle of the enzyme[2,3]. The antioxidant role in vivo of this element, suggested by the study of the deficiency syndromes[4], was interpreted on the basis of the peroxidase reaction catalyzed by this enzyme that contains a selenocysteine at the active site[3] The protection of the cellular components against an oxidative challenge, was attributed to the reduction of hydroperoxides,

from which an oxygen centered radical can be generated by reductive cleavage of the O-O bond[5]. However, in spite of the wide substrate specificity toward hydroperoxides[3], GPX is inactive on phospholipid hydroperoxides[6] and therefore cannot prevent the free radical generation in the membranes, induced by decomposition of lipid hydroperoxides. In fact the NADPH or ascorbate - iron dependent microsomal lipid peroxidation[7], that, following the formation of the first lipid peroxides, proceeds through their decomposition that generates free radicals and then more lipid hydroperoxides[8], is not significantly inhibited by glutathione peroxidase (unpublished observation). The discovery of another selenoenzyme, present in mammalian tissues, the "Phospholipid Hydroperoxide Glutathione Peroxidase" (PHGPX)[9], that reduces the lipid hydroperoxides in the membranes and inhibits lipid peroxidation[10], seems to provide some insights into the relationship between selenium and protection against lipid peroxidation in living tissues.

PURIFICATION OF PHGPX

The identification and the purification of this enzyme were carried out following the glutathione dependent, peroxidation inhibiting activity of liver cytosol[10]. To follow the activity during the purification steps we took advantage of a simple peroxidation test. Soybean phosphatidyl choline dispersions or liposomes were peroxidized by the ferric iron triethylenetetramine complex, and the oxygen consumption was followed polarographically, using a Clark electrode. The preincubation of lipids with glutathione and fractions containing the activity, prevented the peroxidation. The original purification procedure has been recently simplified as follows. After the affinity chromatography on the bromosulphophthalein column the active fractions are concentrated by ultrafiltration and exhaustively dialyzed against a low ionic strength buffer (10 mM phosphate, pH 6.5). Following

this dialysis the bulk of contaminating proteins precipitates and are eliminated by centrifugation. Homogeneous PHGPX is then recovered by HPLC using a TSK-CM column and a gradient of KCl from 0 to 0.3 M in 10 mM phosphate buffer pH 6.5 lasting 30 min. The enzyme is eluted as a single peak at the end of the gradient, suggesting a cationic character which is confirmed by isoelectrofocusing (unpublished observation). Following this simplified procedure the resulting enzyme is homogeneous on SDS-PAGE, and the yield is substantially increased. The purified enzyme appears monomeric and the molecular weight is approximately 21,000 on SDS-PAGE.

This enzyme was identified in rat liver, brain, heart, kidney and lung[11], in dog liver, brain and kidney and purified to homogeneity from pig liver[10] and heart[12]. The purification of PHGPX is carried out using cytosol as starting material. However, because the high ionic strength homogenization buffers increase the recovery in the soluble fraction, an ionic interaction with the membranes is suggested. By using a high ionic strength extraction, PHGPX was released from all the subcellular organelles. In the mitochondria the enzyme seems to be localized either in the external membrane or in the intermembrane space.[11]

Due to practical considerations PHGPX used for molecular and enzymatic characterization was prepared from pig heart.

ENZYMATIC CHARACTERIZATION OF PHGPX

The reduction of lipid hydroperoxides by PHGPX was demonstrated by TLC[10], HPLC[13] and mass spectroscopic[14] analysis of the substrates and products of the reaction. While different substrates, such as hydrogen peroxide, tert-butyl hydroperoxide, cumene hydroperoxide and linoleic acid hydroperoxide are reduced both by GPX and PHGPX, phospholipid hydroperoxides are reduced only by PHGPX[15]. The activity was tested on hydroperoxy derivatives of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl

serine, phosphatidic acid and cardiolipine[9]. Evidence that PHGPX reduces all the titrable hydroperoxy groups in peroxidized membranes[16] suggests a very wide specificity for the peroxidic substrate. The presence of Se in PHGPX was demonstrated by proton induced x ray fluorescence analysis of the homogeneous protein, and its involvement in the catalytic cycle was strongly suggested by the kinetics of the inhibition in the presence of iodoacetate[9]. PHGPX, as well as GPX[17], is inhibited by iodoacetate only in the presence of suitable thiols with a first order kinetics suggesting the presence in the active site of a strong nucleophile. The presence of a selenol maintained continuously reduced by thiols accounts for the inhibition kinetics. This analysis allows also the study of the specificity of selenium dependent peroxidases for thiol substrates. The inhibition occurs only if the thiol is able to reduce the selenium at the active site since the enzymes, as prepared, are in the oxidized forms that are not susceptible to reaction with iodoacetate. While GPX is inhibited by iodoacetate only in the presence of glutathione[17], PHGPX is inhibited also in the presence of mercaptoethanol and dithioerythritol[9], suggesting that in the latter case the selenium is more easily accessible to reducing substrates. The kinetics of the reaction were studied using a spectrophotometric test in which the oxidation of glutathione, catalyzed by PHGPX in the presence of a peroxidic substrate, was measured using glutathione reductase and NADPH[9,15]. The digitalized readouts at 340 nm were processed by a computer to obtain the reaction rate and substrate concentration data required to draw the Lineweaver and Burk plot. Calculation of the kinetic parameters from a single trace of NADPH oxidation was not hampered by the occurrence of product inhibition or reverse reaction. The single traces were also processed by the computer to fit the integrated rate equation for a ping-pong reaction without the formation of central ternary complexes[17,18]. These analyses demonstrated that, in spite of

the different substrate specificities, the kinetic mechanisms of PHGPXa nd GPX are identical. In the presence of both enzymes, using either plot, different glutathione concentrations led to parallel lines from which the Dalziel coefficients and the rate constants for the interaction of the enzymes with the peroxidic substrate could be calculated. Although more direct evidence is still lacking, in the catalytic cycle of PHGPX, as well as in the case of GPX, the selenol is oxidized to selenenic acid by the hydroperoxide, and the selenenic acid is in turn reduced back by two reductive reactions in which the thiol substrate is oxidized to disulfide. Linoleic acid hydroperoxide is reduced equally well by either enzyme, hydrogen peroxide is reduced faster by GPX and phosphatidyl choline hydroperoxides are reduced only by PGHPX[15]. A more detailed kinetic comparison between GPX and PHGPX was carried out using linoleic acid hydroperoxide as a substrate for both enzymes and by measuring the effect of detergents on the kinetic coefficients[19]. This study showed that Triton X-100 inhibited the interaction between GPX and the hydroperoxide at very high concentrations. This evidence is in agreement with the interfacial character of PHGPX, already suggested by the stimulation of the activity when the substrate was a hydroperoxy derivative of a phospholipid[9]. This detergent apparently homogenizes the dispersed phospholipid, thus increasing its reactivity. This effect is not required when linoleic acid hydroperoxide is used as substrate and the observed inhibition at high Triton concentration is due to the surface dilution of the substrate[20]. In the case of GPX, on the other hand, the shift of the substrate toward a mixed micellar form hampers the enzyme substrate interaction. This interpretation is supported by the observation that, when the substrate is hydrogen peroxide, Triton is ineffective with both enzymes. Moreover when the substrate is linoleic acid hydroperoxide, in the absence of Triton the Lineweaver and Burk plots from single traces appear biphasic with

a break point approximately at the critical micellar concentration of the substrate[15]. The deflection is upward in the case of GPX and downward in the case of PHGPX. This behavior, according to the simulation model[21], suggests that the reactivity of the enzyme toward a micellar substrate increases in the case of GPX and decreases in the case of GPX.

PHYSIOLOGICAL ROLE OF PHGPX

PHGPX was purified as a "peroxidation inhibiting protein"[10], and consequently the major physiological role seems to be the control of lipid peroxidation. PHGPX obviously prevents lipid peroxidation induced in liposomes by an iron complex that generates free radicals from preexisting lipid hydroperoxides. However this enzyme also inhibits almost completely lipid peroxidation induced in microsomal membranes by NADPH or ascorbate and iron-ADP, where preexisting lipid hydroperoxides are not required for the initiation. We suggested that in this case also the peroxidase activity of this enzyme, in cooperation with a free radical scavenger in the membranes, gives an account of the observed protection on the basis of the following experimental evidences: i) Microsomal lipid peroxidation, subsequent to the formation of the first hydroperoxides, proceeds through free radicals generated by their decomposition (secondary initiations)[8]; ii) the protection efforted by PHGPX and glutathione requires a normal content of vitamin E in the membranes[22], and iii) a cooperation between PHGPX and vit E has been observed in a simplified peroxidation system where liposomes were peroxidized by iron ascorbate[22]. Vitamin E would decrease the peroxidation initiation rate, but in a short time, its scavenging capacity would be exhausted due to the progressive rise of free radical generation rate from hydroperoxide breakdown. In the presence of PHGPX these secondary initiations are prevented and the scavenging capacity of

vitamin E, continuously recycled by ascorbate[23] is maintained.

Although PHGPX is dramatically more potent than any other "antiperoxidant" enzyme (superoxide dismutase, catalase, GPX) in the prevention of microsomal lipid peroxidation, induced in vitro by NADPH or ascorbate and chelated iron, the extrapolation of a role in vivo is not direct, mainly because the mechanism and the extent of lipid peroxidation in vivo are not known. However, the evidence that the major peroxidation products in vivo are the hydroxy, instead of the hydroperoxy derivatives of phospholipids[24], strongly suggests that the above mechanism could be effective in vivo as well as in vitro. It has been reported recently that hydroxy derivatives of phospholipids are produced in mammalian aortas, apparently by reduction of the corresponding hydroperoxides[25]. Since these hydroperoxides are involved in the activation of eicosanoid biosynthesis, and in the inhibition of the prostacyclin pathway, the catalytic capacity of this enzyme seems to be implicated in the regulation of metabolic pathways, the alteration of which might lead to important pathologies such as thrombosis and atherosclerosis.

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