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# Glutathione peroxidase 4-catalyzed reduction of lipid hydroperoxides in membranes: The polar head of membrane phospholipids binds the enzyme and addresses the fatty acid hydroperoxide group toward the redox center

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ABSTRACT

GPx4 is a monomeric glutathione peroxidase, unique in reducing the hydroperoxide group (-OOH) of fatty acids esterified in membrane phospholipids. This reaction inhibits lipid peroxidation and accounts for enzyme's vital role. Here we investigated the interaction of GPx4 with membrane phospholipids. A cationic surface near the GPx4 catalytic center interacts with phospholipid polar heads. Accordingly, SPR analysis indicates cardiolipin as the phospholipid with maximal affinity to GPx4. Consistent with the electrostatic nature of the interaction, KCl increases the $K_D$. Molecular dynamic (MD) simulation shows that a -OOH posed in the core of the membrane as 13 - or 9 -OOH of tetra-linoleoyl cardiolipin or 15 -OOH stearoyl-arachidonoyl-phosphaphatidylcholine moves to the lipid-water interface. Thereby, the -OOH groups are addressed toward the GPx4 redox center. In this pose, however, the catalytic site facing the membrane would be inaccessible to GSH, but the consecutive redox processes facilitate access of GSH, which further primes undocking of the enzyme, because GSH competes for the binding residues implicated in docking. During the final phase of the catalytic cycle, while GSSG is produced, GPx4 is disconnected from the membrane. The observation that GSH depletion in cells induces GPx4 translocation to the membrane, is in agreement with this concept.

## 1. Introduction

Non-heme peroxidases use thiols to reduce hydroperoxides to corresponding alcohols. They encompass two distinct classes of gene products, glutathione peroxidases and peroxiredoxins. While peroxiredoxins usually employ cysteine (Cys) catalysis, glutathione peroxidases (GPx) use either Cys or selenocysteine (Sec) [1]. Among the 8 homologs of GPx in vertebrates, four contain Sec (GPx1, GPx2, GPx3, GPx4) and three Cys (GPx5, GPx7, GPx8) and one (GPx6) Sec in humans only [2].

GPx1, the archetype of vertebrate GPx, has the quaternary homotetrameric structure present also in GPx2, GPx3, GPx5 and GPx6. GPx4, GPx7 and GPx8, are monomeric instead [2]. Thus, GPx4 is unique for both, being monomeric and relying on selenium catalysis. In GPx homologs, the monomeric nature confers a facilitated access of substrates to the flat depression surrounding the Sec/Cys catalytic center. Hence GPx4, and possibly the other monomeric homologs, catalyze reduction of hydroperoxide substrates that are not accepted by the tetrameric enzymes, regardless of presence of Sec or Cys at the catalytic center. These substrates include complex hydroperoxides such as those of membrane phospholipid [3,4]. Accordingly, GPx4 was discovered as a cytosolic protein inhibiting iron-dependent lipid peroxidation, a process primed by radicals produced by decomposition of membrane PLOOH [5]. It was pointed out later that *GPx4* translation yields three distinct products, catalyzing similar reactions but differing by specific localization sequences at the 5′ end of the mRNA: the so-called nuclear (nGPx4), mitochondrial (mGPx4) and cytosolic GPx4 (cGPx4) [6], cGPx4 being the sole vital gene product [7,8]. Thus, apparently both, structure and location, lends to cGPx4 a vital role. In cells dying due to missed GPx4 activity, common ground is membrane

*Abbreviations:* BSO, buthioninesulfoximine; Cys, cysteine; DOPC, 1,2-dioleoyl-*sn*-glycero 3 phosphocoline; DEM, diethyl maleate, DTT, dithiotreitol; cGPx4, cytosolic GPx4; CL, cardiolipin; GPx, glutathione peroxidases; -OOH, hydroperoxide group; MD, molecular dynamics; mGPx4, mitochondrial GPx4; nGPx4, nuclear GPx4; PC, phosphatidyl choline; PCOOH, phosphatidylcholine hydroperoxide; RMSD, root mean square deviation; SAPC, 1-stearoyl, 2-arachidonoyl-*sn*-glycero 3 phosphocoline; Sec, selenocysteine; SPR, surface plasmon resonance; TLCL, 1,1′2,2′ tetra-linoleoyl cardiolipin; TOCL, 1,1′2,2′-Tetra-oleoyl cardiolipin; VMD, visual molecular dynamics

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lipid peroxidation [9]. Recently, a medicinal chemistry approach, aimed to find compounds that selectively kill cancer cells, focused GPx4 as the master regulator of a specific sub-routine of controlled cell death relying on iron-dependent membrane peroxidation, and therefore named ferroptosis [10,11]. Unequivocally, the vital function of cGPx4 is linked to reduction of membrane hydroperoxides. Yet, whether this reaction is relevant as well for the monomeric *GPx* gene products, whose deletion is not lethal, i.e. nGPx4 and mGPx4 [12–14], or for GPx7 [15,16], has not been specifically addressed so far.

Although GPx4 reduces hydroperoxides of different lipids in membranes or lipoproteins [17,18], for standardized routine activity measurements and kinetic analysis, the physical form of the substrate of GPx4 was phosphatidylcholine hydroperoxide (PCOOH) in a mixed micellar form with Triton x - 100 [19]. This permitted indeed the careful calculation of the rate constants for the oxidative and reductive steps of the catalytic cycle under steady-state condition independently from the mechanism of the interaction with the membrane. In these studies, it has been demonstrated that the catalytic Sec residue is oxidized by PCOOH at a much faster rate than the formation of an enzyme-substrate complex. In consequence, the reaction of GPx4 with PCOOH, like that of GPx1 on $H_2O_2$, does not fit the kinetic model of Michaelis–Menten, i.e. the enzymes are not saturated by substrate and, consequently, $K_m$ and $V_{max}$ are infinite [4]. This kinetic conclusion has been recently further supported by a quantum mechanical analysis of the catalytic cycle, showing that the extremely fast oxidation of selenium is accounted for by a proton shuttling leading to a charge separation, which, in the presence of the hydroperoxide, evolves to a barrier-less formation of the products of the redox transition [20].

The interfacial nature of GPx4 catalysis highlights, therefore, a new independent constraint of the efficiency of the enzyme: How does the peroxidase interact with membranes and how is this interaction compatible with the progression of the catalytic cycle? The elucidation of the relationship between the interaction of GPx4 with membranes and the peroxidatic cycle, is in fact needed for unraveling the molecular aspects linking GPx4 activity with cell life. This issue is definitely challenging, as cGPx4 is seen as a suitable drug target aiming either to prime cell death, as in some forms of cancer, or to support survival, as in degenerative diseases [21,22].

In this study, we investigated the interaction of GPx4 with bilayers mimicking biological membranes by a SPR technology, corroborated by molecular docking and dynamics. We obtained evidence that GPx4, through specific electrostatic interaction, binds to the polar head of phospholipids. Cardiolipin, which has the maximal affinity, has been analyzed in detail as a prototype of acidic phospholipid. Moreover, molecular docking and dynamics, for the first time, allowed a reasonable description of the multistep dynamics of GPx4 catalytic cycle on membranes. Finally, we could in essence confirm the conclusions derived from in vitro and in silico studies at the cellular level.

## 2. Materials and methods

### 2.1. Materials

Phospholipids: 1,2-Dioleoyl-*sn*-glycero 3 phosphocholine (DOPC), 1,1′2,2′-Tetra-oleoyl Cardiolipin (TOCL) were all from Avanti Polar Lipids, USA. Octyl Glucoside, isopropanol, Hepes, Tris, GSH, bovine serum albumin and GPx1 (from bovine erythrocyte) were from Sigma. GPx4 was purified from rat testis cytosol as previously described [23]. All solutions were freshly prepared and filtered through a 0.22 μm filter.

### 2.2. Liposome preparation

Phospholipids were dissolved in chloroform/methanol/water (65:35:8) and different lipid mixtures were prepared by mixing TOCL with DOPC in the desired ratio. After chloroform and methanol have been removed by nitrogen flushing, the phospholipid mixtures were suspended in 2 mL of buffer (10 mM HEPES, 150 mM NaCl, pH 7.4, final lipid concentration 1 mM) and vigorously stirred by vortex for 2 min. A homogeneous population of uni-lamellar liposomes was finally obtained by extruding 10 times the lipid suspensions through 0.1 μm polycarbonate filters (Whatman Nuclepore Track Etched Membranes), using an extruder (LIPEX$^R$ Extruder Transferra Nanosciences Inc. Canada) at 50 °C.

### 2.3. Surface plasmon resonance (SPR) analysis

#### 2.3.1. Lipid immobilization for SPR analysis

SPR experiments were carried out at 25 °C with a Biacore T100 analytical system (Biacore, Uppsala, Sweden), using an L1 sensor chip (Biacore). All four sensor chip channels were used, immobilizing a different lipid mixture on each. Before immobilizing the lipid bilayers, the lipophilic sensor chip surface was cleaned by 40 mM octyl glucoside and isopropanol/50 mM NaOH (2:3). The deposition of the lipid bilayers onto L1 surface was carried out injecting the liposome suspensions for 25 min with 4 μL/min flow rate. 10 mM HEPES, 150 mM NaCl, pH 7.4 was used as running buffer. After the liposome injection, 10 mM NaOH was flushed for 60 s at 10 μL/min to remove the loosely bound liposomes. 0.1 mg/mL of bovine serum albumin was then injected for 300 s at 10 μL/min to seal the gap in the phospholipid layer for preventing non-specific binding. After the BSA injection a solution of 10 mM NaOH and then a solution of 2 M KCl were introduced at 10 μL/min for baseline stabilization [24,25].

#### 2.3.2. SPR analysis of GPx4 binding

To minimize presence of an autoxidized form of GPx4 containing a selenenylamide group on the catalytic center [20], the purified enzyme [23] was reduced 1 h on ice before use, by 10 mM dithiotreitol (DTT). This was removed by a buffer exchange against 25 mM Tris–HCl, pH 7.4, repeated twice. For this purpose, an Amicon ultrafiltration cell equipped with an YM10 membrane (Amicon Corp. Lexington, MA, USA) was used.

The protein concentration was determined by the Bradford method with BSA as standard protein.

GPx4 was applied to immobilized phospholipid bilayers for 180 s at a flow rate of 30 μL/min. Then the running buffer was let to flow and the protein dissociation recorded. After a dissociation period of 300–600 s, 2 M KCl and 50 mM NaOH was injected for 30 s to regenerate the phospholipid bilayer surface. GPx4 concentration ranged from 0.05 to 5 μM. The running buffer was 25 mM Tris–HCl, pH 7.4, containing, in particular experiments, 150 mM KCl or 5 mM GSH and 150 mM KCl.

Biacore T100 Evaluation Software 2.0.3 (GE) was used for data analysis, and the apparent $K_D$ was calculated by fitting the experimental data to the equation:

$$R_{eq} = R_{max} \cdot A/(K_D + A)$$

where A is GPx4 concentration, $R_{eq}$ the response at steady state at a given concentration of enzyme, and $R_{max}$ is the maximum value of the biosensor signal, extrapolated to maximal enzyme concentration. Different bilayers at different lipid compositions were used for calculations.

### 2.4. In silico analyses

#### 2.4.1. Surface electrostatic calculation and sequence alignment

A model of rat GPx4 has been obtained by homology modelling using human GPx4 (PDB code 2OBI) as template in SWISS-MODEL [26] whereas bovine tetrameric GPx1 has been downloaded from Protein Databank (pdb code 1GP1). Since precise parameters for protein-bound selenium in different redox states were not available, the catalytic selenocysteine has been substituted by cysteine to perform subsequent