Purification and characterization of a chimeric enzyme from *Haemophilus influenzae* Rd that exhibits glutathione-dependent peroxidase activity

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Summary

While belonging to the same family of antioxidant enzymes, members of the peroxiredoxins do not necessarily employ one and the same method for their reduction. Most representatives become reduced with the aid of thioredoxin, whereas some members use AhpF, tryparedoxin or cyclophilin A. Recent research on a new peroxiredoxin isoform (type C) from *Populus trichocarpa* has shown that these particular types may also use glutaredoxin instead of thioredoxin. This finding is supported by the occurrence of chimeric proteins comprised of a peroxiredoxin and glutaredoxin region. A gene encoding such a fusion protein is enclosed in the *Haemophilus influenzae* Rd genome. We expressed the *H. influenzae* protein, denoted here as PGdx, in *Escherichia coli* and purified the recombinant enzyme. *In vitro* assays demonstrate that PGdx, in the presence of DTT or glutathione, is able to protect supercoiled DNA against the metal-ion catalyzed oxidation-system. Enzymatic assays did, indeed, characterize PGdx as a peroxidase, requiring the glutathione redox cycle for the reduction of hydrogen peroxide \( (k_{\text{cat}}/K_m = 5.01 \times 10^6 \text{ s}^{-1}.\text{M}^{-1}) \) as well as the small organic hydroperoxide tert-butyldihydroperoxide \( (k_{\text{cat}}/K_m = 5.67 \times 10^4 \text{ s}^{-1}.\text{M}^{-1}) \). Enzymatic activity as function of the glutathione concentration deviated from normal Michaelis-Menten kinetics, giving a sigmoidal pattern with an apparent Hill coefficient of 2.9. Besides the formation of a disulfide-linked PGdx dimer, it was also shown by mass spectrometric analysis that cysteine 49, which is equivalent to the active site cysteine of the peroxiredoxins, undergoes glutathionylation during purification under non-reducing conditions. Based on these results we propose a model for the catalytic mechanism.
Introduction

Aerobic organisms intrinsically encounter reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), the superoxide anion radical (O₂·⁻) and the hydroxyl radical (OH·), during some stage of the four-electron reduction of O₂ to water, or following exposure to environmental factors (1,2). The unrestrained accumulation of these species gives rise to oxidative stress and can lead to cell damage, mutations or even death. One issue of the ROS detoxification concerns the decomposition of hydroperoxides. Most pro- and eukaryotic cells rely on the action of heme-containing enzymes called catalases, which disproportionate H₂O₂ to water and O₂. Some eukaryotic cells may also use glutathione peroxidases to remove H₂O₂ as well as organic and lipid hydroperoxides. In addition, research over the past decades has led to the characterization of a new family of peroxidases, collectively called ‘peroxiredoxins’ (Prxs) (3). They decompose organic hydroperoxides and H₂O₂ by means of thiol-containing electron donors such as thioredoxin (Trx), AhpF, cyclophilin A, tryparedoxin, or, as recently reported, also the redox protein glutaredoxin (Grx) (4-8).

*Haemophilus influenzae* is an important, opportunistic, gram-negative human pathogen. The bacterium resides in the upper respiratory tract of humans where it generally grows aerobically, although facultative anaerobic growth is also possible (9). Besides oxidative stress from its aerobic respiratory metabolism, or as a result of the high O₂ tension at the nasopharynx, *H. influenzae* may also be exposed to high levels of oxidants produced by the host’s immune system, which uses the destructive power of ROS to eliminate bacterial infections (10). Moreover, experimental data indicate that *H. influenzae* has to deal with H₂O₂ secreted by peroxidogenic *Streptococci* (11). While the existence of a H₂O₂-inducible catalase (HktE) has been described in *H. influenzae* Rd, the enzyme seems to be redundant (12,13). As yet, no other
antioxidant enzyme has been identified that acts against hydroperoxides, making the ways in which the bacterium deals with hydroperoxide stress an interesting topic for future research.

Previously, we described a glutathione amide-dependent peroxidase from the phototrophic purple sulfur bacterium *Chromatium gracile*, capable of reducing both H$_2$O$_2$ and tert-butylhydroperoxide (t-BOOH) at comparable high rates (14). By means of a BLAST-search using its deduced amino acid sequence, we were able to identify several homologs in different bacterial species, including one encoded by an open reading frame (HI0572) enclosed in the *H. influenzae* Rd genome. The comparisons revealed the fusion of an N-terminal Prx region to a C-terminal Grx region, a unique feature typical for this novel family of homologs. This structure suggests that a thioltransferase reaction by the Grx moiety may be involved in the reduction of the Prx moiety (8,15,16). Grxs are small, ubiquitous thioltransferases that are specifically designed to use glutathione (GSH) for their reduction (17,18). They catalyze the reduction of protein disulfide groups and GSH-containing mixed disulfide groups either via a dithiol or monothiol mechanism (18).

In this paper, we expand our knowledge of the chimeric enzyme from *H. influenzae* Rd (aptly named PGdx as in Peroxiredoxin/Glutaredoxin) by demonstrating its abilities to protect supercoiled DNA from oxidative damage and to catalyze the *in vitro* reduction of H$_2$O$_2$ and t-BOOH using GSH as electron donor. Furthermore, we noticed the formation of a homodimer and a glutathionylated monomer during purification under non-reducing conditions. Surprisingly, kinetic studies of PGdx revealed sigmoidal kinetics with respect to GSH, normally attributed to the phenomenon of cooperativity.
Experimental procedures

Materials – Restriction endonucleases were obtained from New England Biolabs (Beverly, MA). DNA purification from gel or solution was carried out using either the Qiaquick DNA Extraction or PCR Purification Kit (Qiagen, Crawley, UK). Ligations were performed using Ready-To-Go T₄ DNA ligase (Amersham Biosciences). Plasmid DNA was prepared by the alkaline lysis method on either a small scale (19) or a 30-mL scale using the Qiagen plasmid purification kit. Chromatographic protein purification steps were performed on an ÄKTA-design FPLC system (Amersham Biosciences) with chromatographic equipment from the same manufacturer, or with materials from BioRad (Hercules, CA). Spectrophotometric measurements were taken using a Uvikon 943 double beam UV-visible spectrophotometer (Kontron Instruments, Watford, UK). Reduced GSH, H₂O₂, t-BOOH, DTT, ascorbate and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). GSH-reductase (GR; type IV from bakers’ yeast) was from Fluka (Glossop, UK). E. coli Trx and Trx-reductase (TR) were from Sigma-Aldrich (St. Louis, MO).

Bacterial Strains, Media and Growth Conditions – Haemophilus influenzae Rd [KW20] was obtained from ATCC (Manassas, VI; N°51907). E. coli MC1061 and E. coli B834(DE3) were used as host for cloning and expression of PGdx, respectively. All E. coli strains were cultured at 37°C in Luria-Bertani (LB) medium on an orbital shaker rotating at 200 rpm. H. influenzae Rd was grown at 37°C under a 3% CO₂ atmosphere (candle extinction jar method) on an orbital shaker rotating at 100 rpm. H. influenzae Rd medium consisted of brain heart infusion liquid (Difco Laboratories, Detroit, MI) supplemented with β-NAD and hemin (Fluka, Glossop, UK). Solid media for all strains were prepared by adding agar to the liquid media to a final
concentration of 1.8%. When appropriate, 100 µg of carbenicillin (Cb) per mL was added to the 
*E. coli* culture media.

*Cloning of H. influenzae PGdx* – The gene HI0572 (GenBank accession AAC22230) was amplified by the polymerase chain reaction (PCR) from *H. influenzae* Rd genomic DNA (prepared as described elsewhere; 20) using Gold Star DNA polymerase (Eurogentec, Seraing, BE) and the following primers: forward primer (5’-TC CAT ATG TCT AGT ATG GAA GG-3’) containing an *NdeI* (underlined) site and the initiation codon (boldface), reverse primer (5’-CGC GGA TCC TTA TGC AAA GTA T-3’) containing a *BamHI* site (underlined) and the stop codon (boldface). The PCR product obtained was purified and subcloned into a pGEM-T vector (Promega, Madison, WI) prior to digestion with *NdeI/BamHI*. The digested fragment was cloned into an *NdeI/BamHI* digested pET-11a expression vector (Novagen, Madison, WI).

*Expression and Purification of Recombinant PGdx* – An *E. coli* B834(DE3) expression strain was transformed with the expression construct, cultured overnight in 100 mL of LB medium supplemented with Cb, and then transferred to fresh medium up to the ratio 1/100. Cells were grown to an optical density at 600 nm of 0.8, after which isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After induction for 4 h, cells were harvested by centrifugation, resuspended in 10 mM Tris-HCl buffer, pH 7.5, containing the Complete Protease Inhibitor mix (Roche, Brussels, BE), and stored at –80°C. Frozen cells were thawed and then disrupted by sonication. The supernatant was cleared by centrifugation at 14,000 rpm for 30 min. Clear supernatant was loaded onto a Q-Sepharose Fast Flow column (10 by 200 mm) equilibrated with 10 mM Tris-HCl, pH 7.8. Proteins were eluted
with a continuous gradient of 0 to 1 M NaCl, at a flow rate of 3 mL/min. Fractions containing the protein were pooled and dialyzed against 50 mM phosphate buffer, pH 6.8. The sample was applied to a CHT-2 Ceramic hydroxyapatite column from BioRad (Hercules, CA) equilibrated with 50 mM sodium phosphate buffer, pH 6.8. The column was washed with equilibration buffer before eluting the protein with increasing concentrations of phosphate, pH 6.8. Protein fractions were pooled and concentrated using a Millipore (Bedford, MA) concentrator. For the ultimate purification step we used a MonoQ column. Purified PGdx was stored at -80°C until further use. Aliquots of the peak fractions were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 15%) performed as described by Laemmli (21). Protein concentration was measured in 20 mM phosphate buffer (pH 6.5)/6 M guanidine hydrochloride on basis of the molar extinction coefficient computed from the amino acid composition using software at ExPASy (http://www.expasy.org; \( \varepsilon_{280} = 23,470 \ M^{-1} \text{cm}^{-1} \)).

**Mass Spectrometric Analysis of PGdx and PGdx Peptides** – Mass determinations were performed on a hybrid quadrupole-time of flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK), equipped with a nano-electrospray source. For precise measurements, the samples were first desalted by ultrafiltration on a Millipore 0.5 mL concentrator (Bedford, MA), with a molecular mass cut-off of 5-kDa. The sample was then dissolved and diluted in 50% acetonitrile/0.1% formic acid to a final concentration of approximately 2 pmol/µL. Tryptic digestion of PGdx was carried out by first dissolving 1 µL of the purified PGdx (10 µg/µL) in 50 mM ammonium bicarbonate (pH 7.8), and then adding trypsin up to a ratio enzyme/PGdx of 1/50 (w/w). After 4 hours of incubation (37°C), 1 µL of the digested protein
was diluted in 20 µL 50% acetonitrile/0.1% formic acid and analyzed by Q-TOF mass spectrometry.

**DNA Supercoiling Assay** – The ability of PGdx to protect supercoiled DNA from oxidative degradation was assayed with either a DTT or GSH metal ion-catalyzed oxidation system (DTT or GSH/Fe³⁺/O₂; thiol MCO system) or an ascorbate metal ion-catalyzed oxidation system (ascorbate /Fe³⁺/O₂; non-thiol MCO system). Assays were done in a 20 µL reaction mixture containing 50 mM HEPES-NaOH, pH 7.3, 3 µM of freshly prepared FeCl₃, 10 µM of PGdx (except for the control where PGdx was replaced with 10 mM EDTA), and either 10 mM DTT, GSH or ascorbate. The reactions were incubated for 40 minutes before adding approximately 1 µg of pBSK plasmid DNA. After incubation for 30 min at 37°C, samples were analyzed on a 1% agarose gel stained with ethidium bromide.

**Enzyme Assays** – GSH/GR/NADPH-dependent peroxidase activity of PGdx was determined using a continuous assay. Assays were performed at 25°C in a 0.1 M Na/K phosphate buffer, pH 7.8, with 0.15 mM NADPH, 0.1 mM EDTA, and the following products each in turn added as last component, with the others already having been added: 50 nM PGdx, 10 mM GSH, 100 µM hydroperoxide, and 3 units GR. Trx/TR/NADPH-dependent peroxidase activity was assayed by adding 100 µM of hydroperoxide to a reaction mixture containing 50 nM PGdx, 8.5 µM Trx and 2 µM TR in 0.1 M Na/K phosphate buffer, pH 7.8, with 0.15 mM NADPH and 0.1 mM EDTA. Final reaction volumes were 500 µL each. The decrease in NADPH absorbance was continuously monitored at 340 nm.
Velocity versus substrate curves for both GSH, H$_2$O$_2$ and $t$-BOOH were determined using a coupled assay consisting of 0.15 mM NADPH, 3 units GR and 50 nM PGdx in 0.1 mM Na/K phosphate buffer, pH 7.1, with 0.1 mM EDTA. Concentrations of GSH were varied while keeping those of $t$-BOOH or H$_2$O$_2$ constant, and vice versa. Hydroperoxides were added last. Peroxidase activity was monitored at 340 nm against a blank containing no enzyme, and expressed as µM NADPH oxidized per min using the molar extinction coefficient of 6,200 M$^{-1}$ cm$^{-1}$ for NADPH. All measurements were done in triplicate, and the mean, and the standard error of the mean (SEM), calculated. Data were analyzed by fitting them to the Michaelis-Menten equation ($v = V_{max}[S] / K_m + [S]$), the Hill equation ($v = V_{max}[S]^n / K'^n + [S]^n$) or the Eadie-Scatchard equation ($v / [S]^n = - (1/K') v + (V_{max}/K')$) using the non-linear least squares method.
Results

Similarity of PGdx with Type C Prx and Grx3 – A BLAST-search with the deduced amino acid sequence of the *C. gracile* Prx/Grx protein (14) identified the highly homologous open reading frame HI0572 (63% identity, 79% similarity) in the *H. influenzae* Rd genome sequence database (http://www.tigr.org/; 22). This ORF, annotated as ‘hypothetical protein’ and/or ‘membrane protein’ based on its hydrophobic character, encodes a 241 amino acid polypeptide chain with a theoretical mass of 26,742.5-Da and, in particular, three cysteine residues located at the positions 49, 180 and 183.

Alignments show the N-terminal region of *Haemophilus* PGdx (amino acids 1-160) to share 30-40% identity and 50-60% similarity with members of the Prx family, particularly with members of type C. The term ‘type C Prx’ is a recently adopted nomenclature by Rouhier and colleagues (8). It includes those Prxs that are characterized by their short length (averaging 160 amino acids), limited sequence similarity with type A (2-Cys) and B (1-Cys) Prxs (except for the conserved sequence surrounding the strictly conserved N-terminal cysteines) and an extra cysteine residue located some 24 amino acids further down the N-terminal cysteine. While this extra cysteine is lacking in PGdx, a situation also shared by the *Oryza* and *Actinobacillus* homolog, identities of the N-terminal region are still higher with type C than with type A or B Prxs. Those Prxs that show the highest identity with the Prx region of PGdx are depicted in Figure 1.

Another distinguishable fact is the apparent ability of type C Prxs to use Grx instead of Trx as electron donor (8). This especially merits to be mentioned since the C-terminal region of PGdx shares strong homology (52% identity, 69% similar) with Grx3 from *E. coli*. This Grx-homologous domain contains cysteine residues 180 and 183, arranged in a characteristic CPFC
disulfide motif, and is coupled to the Prx region via a Gln rich stretch starting with a Pro. Depicted in Figure 1 are the sequences of *E. coli* Grx3 and *P. trichocarpa* Grx. The latter reduces the poplar type C Prx, which is also included in the figure.

BLAST-searches of the *H. influenzae* PGdx against the Microbial Genomes database revealed numerous as yet uncharacterized homologs, the majority of them in microorganisms implicated in human disease (Fig.1). Similarity extends over the entire sequence, especially in the Prx region, and ranges from 60 to 95% identity and from 75% to 100% similarity.

*Expression, Purification and Physical Characteristics of PGdx* – Recombinant PGdx, of which the expression level in the *E. coli* cytosol was considerably high, was purified to homogeneity as described under ‘Experimental procedures’. About 30 mg of pure PGdx was obtained from 1-liter cell culture. Apart from a protein band at monomeric migration distance, non-reducing SDS-PAGE also clearly visualized a second band migrating as a homodimer (Fig.2). This band disappeared with the addition of β-mercaptoethanol, DTT or GSH to the sample buffer, but did not with ascorbate, suggesting the possibility of two monomers being linked by a disulfide bridge. Electrospray ionization mass spectrometry (ESI-MS) measurements confirmed the existence of a completely oxidized dimer at 53216.2-Da that disappeared when 10 mM of GSH was added to the sample solutions (Table I, Fig.3).

When the non-reduced monomeric form was analyzed by ESI-MS we observed a peak with a mass of 26915.1-Da that is 305-Da higher than expected (Table I, Fig.3). This prompted us to investigate the possibility whether the enzyme was modified by glutathionylation during the overexpression and subsequent isolation from *E. coli*. Addition of GSH, indeed, did reduce the mass to that of the fully reduced monomer (Table I, Fig.3), a phenomenon that was not observed
when ascorbate was added (not shown). In order to determine the location of the GSH-moiety in
the peptide chain, we performed a tryptic digest and analyzed the digest mixture of the reduced
and non-reduced condition by mass spectrometry (spectra not shown). The mass spectrum of the
reduced PGdx digest mixture contained a peak at 2316.2-Da, corresponding to the monoisotopic
mass of the unmodified peptide T35-56R. Under non-reducing conditions, this peak was absent
and replaced by a peak at 2621.2-Da, which agrees with the monoisotopic mass of the modified
peptide T35-56R. To confirm this observation and the location of the modification, the peptide
was subjected to collision-induced fragmentation mass spectrometry. As shown in Figure 4, the
MS/MS spectrum was found to be consistent with the sequence of the peptide. This figure also
shows an increment in mass of 305-Da after Cys49 (note the shift of the y”-ions), which points to
the GSH-molecule being linked to the Cys49 residue.

PGdx Protects Supercoiled DNA from Oxidative Damage – Supercoiled DNA is prone to nicking
when exposed to oxidative radicals such as those generated by the MCO system. Therefore,
PGdx was tested for its ability to protect supercoiled DNA from degradation induced by the
MCO system in the presence of either DTT, GSH or ascorbate (Fig.5). Absence of PGdx resulted
in open coiled or nicked DNA, while addition of 10 mM EDTA completely inhibited
degradation. PGdx, in combination with DTT or GSH, was successful in protecting the DNA.
When DTT or GSH was replaced by ascorbate as electron donor, the enzyme was unable to
protect the DNA at a concentration that was sufficient to provide full protection against
degradation when a thiol was present.
The GSH/GR/NADPH System Provides Electrons for PGdx-catalyzed Hydroperoxide Reduction

– We set up a reconstitution assay by which we demonstrated that PGdx can use the GSH/GR/NADPH-system and that the reduction of hydroperoxides depends on the presence of each component. The high activity observed after addition of GR (Fig.6.A) is due to the accumulation of its substrate, GSSG. In contrast, no discernible peroxidase activity was observed when GSH and GR were replaced by Trx and TR. For both H2O2 (not shown) and t-BOOH (Fig.6.E and F) the background activity coincided with the decrease in absorbance when PGdx was present.

Kinetic Parameters of PGdx-catalyzed Peroxide Reduction – Measurements for H2O2 or t-BOOH reduction at saturating GSH concentrations gave normal Michaelis-Menten patterns with $K_m$ and $k_{cat}/K_m$ values of 2.29 µM and 5.01 x $10^6$ s$^{-1}$.M$^{-1}$ for H2O2 reduction (not shown) and 208.80 µM and 5.67 x $10^4$ s$^{-1}$.M$^{-1}$ for t-BOOH reduction (Fig.7.A). The $V_{max}$ was in the range of 25.74 µmol/min/mg of PGdx for H2O2 reduction, and 26.57 µmol/min/mg of PGdx for t-BOOH reduction.

For a kinetic analysis with GSH we chose t-BOOH as substrate, since its spontaneous reaction at physiological pH with GSH is less pronounced compared to H2O2. In order to avoid extensive background activity with GSH we also used a pH of 7.1 instead of pH 7.8, the established pH optimum for PGdx (not shown). Measurements revealed a sigmoidal substrate-velocity curve (Fig.7.B). By fitting our data into the Hill equation we obtained an apparent Hill coefficient ($n_{app}$) of 2.9, indicating a phenomenon of strong cooperativity. $K_{m,app}$ and $k_{cat}/K_{m,app}$ were 3.11 mM and 3.01 x $10^3$ s$^{-1}$.M$^{-1}$, respectively. $V_{max,app}$ was 20.98 µmol/min/mg PGdx. The insets in Figure 7
represent the data as an Eadie-Scatchard plot. When \( n \) equals 1, the \( v/[S] \) versus \( v \) is linear, as is the case for \( t \)-BOOH, but when \( n \) is greater than 1, the plot is curved, as shown for GSH (23).
Discussion

We have recently shown that a subgenomic fragment from H. influenzae Rd, bearing the ORF HI0572, is capable of complementing t-BOOH and H₂O₂ sensitivity of an Ahp- and catalase negative E. coli strain, respectively, and of delivering GSH-dependent alkyl hydroperoxide reductase activity to a naturally GSH peroxidase negative E. coli (24). It was envisioned that these properties could be attributed to the unusual primary structure of the gene product, consisting of a peroxidase region at the N-terminus, showing homology with type C Prx, and a C-terminal region, showing homology with Grx.

Studies described here demonstrate clearly that this chimeric protein of H. influenzae Rd, denoted as PGdx, effectively catalyzes the GSH/GR/NADPH-dependent reduction of both H₂O₂ and t-BOOH at high rates. The Trx/TR/NADPH-system was unable to support peroxidase activity. PGdx protects supercoiled DNA against the thiol MCO-system, suggesting that the protein can function as an effective antioxidant enzyme in vivo. Assays with t-BOOH and H₂O₂ as substrates indicated activities with specificity constants of 10⁴ s⁻¹.M⁻¹ and 10⁶ s⁻¹.M⁻¹, respectively. Interestingly, this latter value is comparable with that of the major peroxidase system of E. coli, AhpR (25). While the AhpR system reduces both organic hydroperoxides and H₂O₂ with similar kinetic efficiencies, it was proposed that organic hydroperoxides are pseudosubstrates and that the only role of AhpR in nature is the decomposition of H₂O₂ (25). Consequently, bearing in mind that PGdx could fulfill a similar role as AhpR, the two orders of magnitude lower value of κcat/κm for t-BOOH reduction compared with that of H₂O₂, at least, supports this assumption.

Rouhier and colleagues previously described the Grx-dependent reduction of a poplar phloem Prx (8,15). They suggested a mechanism where the sulfenic acid of the oxidized Prx becomes
reduced by formation of a disulfide linkage with the N-terminal cysteine residue of the CysXXCys motif from Grx. This disulfide bond then becomes reduced either through the mono- or the dithiol mechanism characteristic for Grx activity (Fig.8). On the basis of our results, it appears that Cys49 has an affinity for GSH. Therefore, we propose another possible reaction mechanism for PGdx, in which the reduction of hydroperoxides is accompanied by the formation of a glutathionylated Prx-cysteine. The GSH-mixed disulfide is subsequently reduced by the action of the C-terminal Grx region, following a monothiol pathway. The mechanism can be summarized as follows, and is schematically given in Figure 8, where E is PGdx and numbers represent the positions of the cysteine residues:

\[
\begin{align*}
E_{49}S^- + ROOH & \rightleftharpoons E_{49}SOH + ROH \quad \text{(Reaction 1)} \\
E_{49}SOH + GSH & \rightleftharpoons E_{49}S-SG + H_2O \quad \text{(Reaction 2)} \\
E_{49}S-SG & \rightleftharpoons E_{180}S-SG \quad \text{(Reaction 3)} \\
E_{180}S-SG + GSH & \rightleftharpoons E_{180}S^- + GSSG \quad \text{(Reaction 4)} \\
GSSG + NADPH + H^+ & \rightarrow 2 \text{GSH} + \text{NADP}^+ \quad \text{(Reaction 5)}
\end{align*}
\]

Reaction 1 shows the formation of sulfenic acid (Cys-SOH) at Cys49, with concomitant reduction of the hydroperoxide. In Reaction 2, GSH forms a protein mixed-disulfide with Cys49. Reaction 3 describes the dethiolation of the Prx region by Cys180 of the Grx region. In Reaction 4, PGdx becomes regenerated by GSH, forming GSSG, which in turn will be reduced by GR in Reaction 5. Glutathionylation of Prxs has already been mentioned in numerous cases (26-28) where it functions as a regulatory mechanism in which the Prx gets inactivated and protected against further oxidation of its active site cysteine into the more stable forms of sulfinic (Cys-
SO\textsubscript{2}H) or sulfonic acid (Cys-SO\textsubscript{3}H). Other examples of such protection and regulation are already known to occur in protein tyrosine phosphatases where reactivation takes place with either GSH or Grxs (29-31). Dethiolation via Grxs follows a monothiol mechanism, requiring only one Cys residue of the redox active disulfide motif. Besides the inability of monocysteinic mutants of Trx to follow a monothiol pathway (32), Grxs are also 10 times more effective, on a molar basis, than Trxs in reducing GSH-mixed protein disulfides (31,33). In addition, the C-terminal Grx region of PGdx shares strong homology with Grx3 from \textit{E. coli}, which has a higher activity as reductant of glutathionylated proteins than \textit{E. coli} Grx1 and 2 (34). Hence, from an evolutionary point of view, the mechanism we propose provides a possible explanation for the fact that Grx- instead of Trx-homologs constitute these chimeric enzymes.

Most oxidized Prxs (e.g. bacterial AhpC, yeast TSA) form disulfide-linked dimers that are subsequently reduced by a separate constituent (e.g. AhpF, Trx) (3). The existence, however, of a PGdx homodimer does not necessarily imply such a form to be involved in the catalytic mechanism. Given that PGdx is a hybrid protein with its reducing partner actually being a part of the enzyme, we are inclined to believe that the dimeric form may be an artifact of purification or a way to prevent irreversible oxidation, and consequently inactivation, in the absence of significant levels of GSH. In order to validate this premise, we are currently studying cysteinic mutants of PGdx. Note that poplar Prx forms no dimers during its catalytic cycle (8,15).

Since the precise reaction mechanism of PGdx awaits further investigation, interpretation of the sigmoidal behavior observed during kinetic measurements is not straightforward. Generally, velocity curves displaying sigmoidal kinetics reflect allosteric enzymes in which distal binding sites of an oligomeric complex communicate with each other in a cooperative manner (23,35). Besides covalent homodimers, PGdx may also form multimeric complexes like the decameric
structures formed by some Prxs (36,37). Yet, no sigmoidal kinetics were mentioned in these cases. So, although the situation of a cooperative active oligomeric PGdx complex is possible, in which binding of GSH induces an increase in peroxidase activity of the other associated enzymes, we are not convinced this is indeed the case. Rather, we believe that other factors are responsible for the sigmoidal features of the velocity curve. Hence, the Hill coefficient obtained does not relate to the number of cooperative interacting sites, but is the intrinsic result of the sigmoidal velocity curve. Substrate depletion seems unlikely because the decrease in activity sets in when GSH-concentrations are still high and, moreover, the lowest GSH-concentration used was still 12,000 times that of the total enzyme concentration.

Kinetic measurements were performed with a non-reduced PGdx sample, thus containing both the glutathionylated monomer and the homodimer. Although sigmoidicity remained with a protein sample purified under reducing conditions (not shown), we cannot exclude the possibility that, in the presence of low GSH concentrations, formation of dimeric PGdx through oxidation prevails. Since participation in our proposed catalytic mechanism requires PGdx to remain monomeric, the homodimer formed needs to be reduced. Hence, low GSH concentrations may lead to a lower availability of active monomeric species and, therefore, to lower activity.

Given the proposed reaction scheme (Fig.8), the unproductive side reaction in the monothiol mechanism of a Grx may also be considered to be responsible for the sigmoidicity. When concentrations of reduced GSH are running low, the equilibrium shifts towards the formation of a disulfide bridge between the Cys residues of the active motif, with concomitant release of GSH from the N-terminal cysteine. Such an event renders a Grx inactive, up to the moment the disulfide bridge becomes reduced again by GSH, a situation which may also apply to PGdx. At low GSH-concentrations only a small fraction of the total enzyme population remains reduced.
and active. Enzyme activity gradually increases with increasing GSH-concentrations until the side reaction (reaction 6 in Figure 8) becomes insignificant. Although further experiments are needed to prove this hypothesis, initial kinetic measurements with a cysteinic 183 mutant of PGdx revealed total absence of sigmoidicity (data not shown). A full characterization of the cysteinic mutants of PGdx is being carried out.

In conclusion, our initial characterization of the chimeric H. influenzae PGdx provides a basis for future studies of other homologs. Not only is PGdx the first heme-independent hydroperoxidase in H. influenzae ever characterized, it is also, to our knowledge, the first prokaryotic peroxidase effectively using GSH for its reduction, albeit in a manner different from eukaryotic GSH peroxidases. Besides a more in depth investigation of the catalytic role behind each of its three cysteine residues and their roles in catalysis, regulatory as well as physiological studies are currently undertaken to gain more insight into its in vivo functions. Studies with the separate regions of the PGdx enzyme will provide new insights into the catalytic mechanism and the interaction between its two regions.
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Footnotes

1 The abbreviations used are: ROS, reactive oxygen species; Prx, peroxiredoxin; Trx, thioredoxin; Grx, glutaredoxin; DTT, dithiothreitol; GSH, reduced glutathione; GR, glutathione reductase; LB, Luria-Bertani; Cb, carbenicillin; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MS, mass spectrometry; ESI, electrospray ionization; MCO, metal-catalyzed oxidation; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SEM, standard error of the mean; GSSG, glutathione oxidized; H$_2$O$_2$, hydrogen peroxide; TSA, thiol specific antioxidant protein; t-BOOH, tertiary butyl hydroperoxide; ORF, open reading frame; AhpR, alkyl hydroperoxide reductase; AhpC, a 21-kDa component of AhpR; AhpF, a 57-kDa component of AhpR; TSA, thiol-specific antioxidant; pBSK, plasmid BlueScript.

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Figure legends

Fig. 1.: Multiple sequence alignment of *H. influenzae* Rd PGdx with prokaryotic homologs and members of the Prx and Grx family.

The alignment was performed using CLUSTALX software. Strictly conserved residues are marked with ‘*’, functional homology with ‘:’ and structural homology with ‘.’. Symbols above the PGdx sequence represent conserved positions for the Prx and Grx alignment, symbols beneath the PGdx sequence represent conserved positions for the alignment of the PGdx homologs. Redox active cyssteines are shaded gray, gaps are represented with a dash. Accession numbers or references for the sequences aligned are: *Brara*: Brassica rapa, AF133302; *Poptr1*: *Populus trichocarpa* Prx, Rouhier et al. (9); *Orysa*: Oryza sativa, AF203879; *Homsa*: Homo sapiens putative peroxisomal antioxidant enzyme, P30044; *Escco*: Escherichia coli glutaredoxin 3, P37687; *Poptr2*: *Populus trichocarpa* Grx, AI166603; *Haein*: Haemophilus influenzae PGdx, AAC22230; *Chgr*: Chromatium gracile, Vergauwen et al. (15); *Borpe*: Bordetella pertussis, NC_002929; *Actac*: Actinobacillus actinomycetemcomitans, NC_002924; *Yerpe*: Yersinia pestis, CAC93382; *Vibch*: Vibrio cholerae, AAF95778; *Haedu*: Haemophilus ducreyi, NC_002940; *Pasmu*: Pasteurella multocida, NP_246286; *Neime*: Neisseria meningitidis, NP_273984.

Fig. 2.: SDS-PAGE analysis of purified recombinant PGdx from *H. influenzae* Rd.

Lane 1: Coomassie stained 15% polyacrylamide gel with purified recombinant protein in non-reducing sample buffer; lane 2: PGdx with 5 mM ascorbate; lane 3: PGdx with 5 mM GSH; lane 4: PGdx with 5 mM DTT; lane 5: molecular mass marker (Protein Ladder, 10-200-kDa, MBI Fermentas). Lanes 1-4 contain about 3 µg of protein.
Fig. 3.: Mass spectrometric analysis of the monomeric and dimeric form of PGdx.
ESI-MS spectra of the PGdx protein under non-reducing conditions (upper trace) and after reduction with 10 mM GSH (lower trace). The relative intensities of the peaks are shown against the mass.

Fig. 4.: MS/MS spectrometric analysis of the tryptic fragment T35-56R of non-reduced PGdx.
The peptide has a theoretical molecular mass of 2621.2-Da. Note the shift of 305-Da due to a molecule of GSH covalently linked to the cysteine residue at position 15 (position 49 of the protein).

Fig. 5.: Protection of supercoiled DNA against oxidative degradation by PGdx.
Experiments were performed as described in ‘Experimental procedures’ and subsequently analyzed on a 1% agarose gel stained with ethidium bromide. OC: open coiled DNA, SC: supercoiled DNA. OC forms are more distinctively present in the absence of PGdx, or in the presence of PGdx and the non-thiol MCO system. In the case of the DTT-MCO system (lane 2) one can even observe a DNA smear.

Fig. 6.: Continuous assay evaluating the GSH/GR/NADPH- or Trx/TR/NADPH-dependent PGdx activity.
NADPH oxidation is coupled by GSH/GR or Trx/TR to the PGdx mediated reduction of t-BOOH. Reactions were performed as described in ‘Experimental Procedures’. Oxidation was measured at 340 nm, the arrow indicates the addition of product. In the case of E (background)
and F, t-BOOH was added as the last component to a reaction mixture containing the complete Trx/TR/NADPH-system.

**Fig. 7.: Velocity versus substrate curves of PGdx catalyzed t-BOOH reduction.**

A: PGdx shows Michaelis-Menten kinetics with respect to t-BOOH.

B: PGdx shows positive cooperativity with respect to GSH.

The solid line represents the best fit through all data points using either the Michaelis-Menten (A) or the Hill (B) equation. The assays were performed in triplicate, ± SEM are shown. *Insets*, Eadie-Scatchard plot.

**Fig. 8.: Proposal for the mechanism of the GSH-dependent reduction of PGdx.**

Dotted lines represent steps as originally proposed by Rouhier *et al.* (16) for a Grx reducible poplar Prx, but adapted here for PGdx; see text for a detailed discussion of each reaction (1-5).

The top black part of the letter E corresponds to the N-terminal Prx region, whereas the bottom grey part of the letter is the C-terminal Grx region.
Tables

Table I. Mass spectrometric analysis of purified PGdx

Predicted masses are those for PGdx lacking the N-terminal Met (NM = 26742.5-Da - 131.2-Da), as confirmed by NH₂-terminal amino acid sequencing (data not shown). # Determined from a tryptic digest analyzed using mass spectrometry.

| Enzyme         | Experimental mass | Calculated mass | Comment                                                                 |
|----------------|-------------------|-----------------|-------------------------------------------------------------------------|
| Non-reduced PGdx | 26915.05-Da ± 1.42-Da | 26914.6-Da      | NM + GSH (307.3-Da) - 2-Da (disulfide bridge between GSH and Cys49) # - 2-Da (disulfide bridge between Cys180 en 183) |
|                | 53216.17-Da ± 5.01-Da | 53216.6-Da      | (NM) x 2 - 6-Da (3 disulfide bridges)                                   |
| Reduced PGdx   | 26610.67-Da ± 0.89-Da | 26611.3-Da      | NM                                                                      |
|                | 26915.83-Da ± 1.13-Da | 26914.6-Da      | NM + GSH (307.3-Da) - 2-Da (disulfide bridge between GSH and Cys49) # - 2-Da (disulfide bridge between Cys180 en 183) |
Fig. 2
Purification and characterization of a chimeric enzyme from Haemophilus influenzae Rd that exhibits glutathione-dependent peroxidase activity
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