Physiological Characterization of *Haemophilus influenzae* Rd Deficient in Its Glutathione-dependent Peroxidase PGdx*

Received for publication, November 3, 2003

Frederik Pauwels, Bjorn Vergauwen, and Jozef J. Van Beeumen‡

From the Laboratory of Protein Biochemistry and Protein Engineering, Ghent University, K. L. Ledeganckstraat 35, 9000 Gent, Belgium

The chimeric peroxidase PGdx of *Haemophilus influenzae* Rd belongs to a recently identified family of thiol peroxidases capable of reducing hydrogen peroxide as well as alkylhydroperoxides by means of glutathione redox cycling. In the present study, we constructed a *H. influenzae* Rd strain, deficient in its PGdx encoding gene (open reading frame HI0572). The mutant was shown by disk inhibition and liquid culture growth assays to exhibit increased susceptibility to organic hydroperoxides. The hampered growth was restored by complementing the interrupted gene on the genome with a replicating plasmid bearing an intact copy of the gene, hereby rejecting the possible influences of polar effects. Elevated levels of hydrogen peroxide scavenging activity, due to the catalase HktE, were measured in the absence of a functional pgdx gene rendering the mutant more resilient against hydrogen peroxide. On the other hand, after initiation of the stationary phase, aerobic cultures of the pgdx mutant were practically devoid of living cells, whereas wild-type counterparts retained viability. This observed feature was alleviated by complementation with the functional gene or with the addition of catalase.

Organisms inhabiting aerobic environments unavoidably encounter the downside effect of oxygen. That is, inadequate metabolic reduction of molecular oxygen to water results in the production of reactive oxygen species, including the superoxide anion radical (O2•−), hydrogen peroxide (H2O2), and the hydroxyl radical (OH•). Furthermore, these species are able to react with other cellular components causing damage and/or generating oxidizing derivatives such as alkylhydroperoxides.

The fastidious organism *Haemophilus influenzae*, etiological agent of infections such as meningitis and *otitis media*, not only has to endure oxidative stress resulting from its own aerobic metabolism, but together with macrophages in which it may reside (1), neighboring strains of peroxidogenic *Streptococci* (2) also add in the continuous exposure of the bacterium to reactive oxygen species.

Recently, we reported the initial characterization of *H. influenzae* Rd PGdx† (encoded by open reading frame HI0572) as a glutathione-dependent peroxidase capable of reducing both H2O2 and alkylhydroperoxides (3). *H. influenzae* PGdx belongs to a family of thiol-dependent peroxidases which are typified by a chimeric structure consisting of an N-terminal peroxiredoxin domain and a C-terminal glutaredoxin domain (3, 4, 5). Previously, we have also shown that glutathione and the only catalase of *H. influenzae* Rd against respiratory-generated H2O2 (6). To investigate whether this GSH-based mitigation of H2O2 is mainly attributable to PGdx, we constructed a knock-out in the pgdx gene.

Physiological studies, including growth assays and pro-oxidant sensitivity assays, allow us to conclude that PGdx acts as a major antioxidant in vivo. We also show that PGdx plays an essential role during stationary-phase growth of the bacterium, as observed in overnight grown aerobic cultures.

**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 T4 DNA ligase (Promega, Madison, WI). Plasmid DNA was prepared by the alkaline lysis method on either a small scale (7) or a 30-ml scale using the Qiagen plasmid purification kit. H2O2, tert-butyl hydroperoxide (t-BOOH), cumene hydroperoxide (COOH), methyl viologen and bovine liver catalase were obtained from Sigma.

**Bacterial Strains, Media, and Growth Conditions**—*Escherichia coli* MC1061 and TOP10 (Invitrogen, Paisley, UK) were used as hosts for cloning. All *E. coli* strains were cultured at 37 °C in Luria-Bertani medium on an orbital shaker rotating at 200 rpm. When appropriate, 100 μg of carbenicillin, 25 μg of chloramphenicol, or 25 μg of kanamycin were added per ml of *E. coli* culture media. *H. influenzae* Rd (RW20) was obtained from ATCC (Manassas, VA; number 51907). *H. influenzae* Rd was grown at 37 °C under a 3% CO2 atmosphere (candle extinction jar method) on an orbital shaker rotating at 180 rpm (unless otherwise stated). *H. influenzae* Rd medium consisted of brain-heart infusion (BHI) liquid (Difco) supplemented with β-NAD and hemin (Fluka, Glos sop, UK). When appropriate, 2 μg of chloramphenicol or 7 μg of kanamycin were added per ml of *H. influenzae* Rd media. Solid media for all strains were prepared by adding agar to the liquid medium to a final concentration of 1.8%. See Table I for list of bacterial strains and plasmids.

**Construction of pgdx Mutant**—A 4.0-kb DNA fragment, including the pgdx gene (HI0572) and its 1.4-kb upstream and 2.0-kb downstream region, was amplified by PCR from *H. influenzae* Rd genomic DNA (prepared as described elsewhere (8)) using Expand Long Template DNA polymerase (Roche Applied Science) and the following primers: forward primer (5′-CGC GGA TCC TGC CTG AAC TTT CGC GTA-3′) and reverse primer (5′-CGC GGA TCC TGT TGT TTG ATT TGG CGG ATG TA-3′), both containing a BamHII (underlined) restriction site. The obtained PCR product was purified and cloned into the TOPO-XL vector (Invitrogen), generating plasmid pSG4.0. The

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*This work was supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT, Grant 3072) and by the Fund for Scientific Research-Flanders (FWO, Grant 3G0053601) to J. J. V. B. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Laboratory of Protein Biochemistry and Protein Engineering, Ghent University, K. L. Ledeganckstraat 35, 9000 Gent, Belgium. Tel.: 32-9-264-51-09; Fax: 32-9-264-53-38; E-mail: Jozef.vanbeeumen@UGent.be.

‡ The abbreviations used are: PGdx, chimeric peroxiredoxin/glutaredoxin peroxidase; t-BOOH, tert-butyl hydroperoxide; COOH, cumene hydroperoxide; BHI, brain-heart infusion; sBHI, supplemented brain-heart infusion; CamR, chloramphenical acetyltransferase resistance gene.
chlamydropenic acetyltransferase (Cam') gene was amplified from pACYC184 (New England Biolabs) together with its native constitutively expressed promoter using AccuPrime Taq DNA polymerase (Invitrogen), the forward primer 5'-AAC TGC AGT AGA CAG CTC AAA AAC ACC ATC ATA ACA C3' and the reverse primer 5'-AAT ACG TAG TCT ACC AGG GTT TTA AGG GCA CCA ATA ACT-3'. Both primers contain an AccI (underlined) restriction site. The amplified resistance gene was cloned into pGEM-T (Promega, Madison, WI) prior to digestion with AccI. The digested fragment was inserted into the AccI-digested construct pSG4.0, yielding plasmid pSGKO in which the pgdx gene was disrupted by the Cam' gene. The pgdx::Cam' cassette was amplified by PCR from pSGKO and used to replace the functional pgdx gene by homologous recombination (transformation of H. influenzae Rd was performed as described elsewhere (8), and positive transformants were scored by chloramphenicol resistance).

Functional Complementation—Functional complementation of pgdx was accomplished as follows. A fragment containing the complete pgdx gene and its upstream and downstream region was generated by PCR and the following primers: forward primer 5'-CAG TTA ATG AGA CAG GGA AAG ATG TCT AAT AAG AAT GGC CTG GTT CTA ATA ATT GAT CC-3' containing a HindII restriction site (underlined), reverse primer 5'-AAA ACT GCA GCA ACC GTA GGC TTA TAT GG-3' containing a PstI restriction site (underlined). After digestion with both HindII and PstI, the fragment was ligated into a HindII/PstI linearized pACYC177 (New England Biolabs) vector producing the complementation vector pCOMP. This complementation vector was transformed into a pgdx mutant of H. influenzae Rd, and positive transformants were selected by kanamycin resistance.

Southern Blot Analysis—Purified H. influenzae Rd chromosomal DNA was overnight digested with MfeI and ScaI and subjected to agarose gel (0.7%) electrophoresis. The DNA was transferred to nitrocellulose paper and probed by Southern blot analysis as previously described by Ref. 9. Digoxigenin-labeled DNA probes were constructed by PCR using the forward primer Probe A 5'-TTT TTA ATG ATA TGG CTG GTT CTA ATA ATT GAT CC-3', forward primer Probe B 5'-AAC ATC TCT TAT CCA AGA AA-3', and reverse primer Probe B 5'-TTC TTC TTG ATG TTA AAG TTG TT-3'.

Western Blot Analysis—Determination of H2O2 Scavenging Activity—H2O2 scavenging activity in whole-cells was either measured in the presence of low concentrations (1.2 μM) or high concentrations (20 mM) of H2O2. For low concentrations H. influenzae Rd strains were grown aerobically to an OD600 of 0.15. Cells were pelleted in a microcentrifuge, washed twice, and resuspended in 1 ml of room temperature phosphate-buffered saline at an OD600 of 0.15. H2O2 was added to a final concentration of 1.2 μM. Reactions were terminated at regular intervals by removing cells using a Millix-GV13 0.22-μm pore size filter unit (Millipore Products Division, Bedford, MA). Remaining H2O2 in solution was assayed using the Amplex Red Catalase Assay Kit (Molecular Probes, Eugene, OR).

For the experiments with high concentrations of H2O2 cells were grown aerobically, pelleted during different stages of growth, washed twice with phosphate-buffered saline, and resuspended at an OD600 of 0.14. H2O2 was added to a final concentration of 20 mM and the decrease in absorbance was monitored at 240 nm (ε240 43.6 M−1 cm−1).

Catalase Activity Staining—Cells extracts of aerobically grown mid-exponential phase wild-type and mutant strains (15 μg) were electrophoretically separated on a native polyacrylamide gel. Catalase activity was measured, using a ferricyanide-negative stain, as follows: the gel was soaked in 0.003% H2O2 for 15 min, rinsed with distilled H2O, and stained with a 50:50 solution of 2% K3Fe(CN)6 and 2% FeCl3•6H2O.

RESULTS

Construction of a Mutant Deficient in PGdx Expression—To investigate the in vivo relevance of PGdx as an antioxidant enzyme, we made a pgdx mutant utilizing the integrative disruption method (Fig. 1A). A 4.0-kb subgenomic fragment, encompassing the gene for pgdx (HI0572) and a 1.4-kb upstream

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**Table I**

Bacterial strains and plasmids

| Strain or plasmid | Genotype or relevant characteristics | Source or reference |
|-------------------|------------------------------------|--------------------|
| **H. influenzae** strains | | |
| Rd KW20 | Wild-type avirulent strain | ATCC no. 51907 |
| Rd KW20 Δpgdx | Chloramphenicol-resistant pgdx mutant constructed by interrupting the pgdx gene in Rd KW20 by a Cam' cassette | This study |
| Rd KW20 Δαppgdx | Kanamycin/chloramphenicol-resistant Rd KW20 Δpgdx strain containing the wild-type pgdx gene obtained by transformation with vector pCOMP | This study |
| **E. coli strains** | | |
| K12 MC1061 | F' araD139 Δ(ara-leu)7697 ΔlacX74 galK16 galE15 λ- e14 - mcrA- relA1 rpsL150(Str') spoT1 mcrB- hsdR2 | CGSC no. 6649 |
| K12 TOP10 | F' mcrA Δmrr-hsdRMS-mcrBC d380lacZAM15 ΔlacX74 deoR recA1 araD139 | Invitrogen |
| **Plasmids** | | |
| pACYC177 | Cloning vector, Kan’ Amp’ | New England Biolabs |
| pACYC184 | Cloning vector, Cam’ Tet’ | New England Biolabs |
| pGEM-T | Cloning vector, Amp’ | Promega |
| TOPO-XL | Cloning vector, Kan’ Zeo’ | Invitrogen |
| pSG4.0 | TOPO-XL vector with a 4.0-kb subgenomic fragment of chromosomal DNA from Rd KW20 that contains the H. influenzae pgdx gene; Kan’ Zeo’ | This study |
| pSGKO | pSG4.0 vector containing a Cam’ cassette in the Accl site of the pgdx gene; Cam’ Kan’ Zeo’ | This study |
| pCOMP | pACYC177 vector with a 1441-bp HincII/PstI fragment of chromosomal DNA from Rd KW20 that contains an intact pgdx gene, inserted into the Amp’ gene of the vector; Kan’ | This study |

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**Construction of a Mutant Deficient in PGdx Expression**—To investigate the in vivo relevance of PGdx as an antioxidant enzyme, we made a pgdx mutant utilizing the integrative disruption method (Fig. 1A). A 4.0-kb subgenomic fragment, encompassing the gene for pgdx (HI0572) and a 1.4-kb upstream
and 2.0-kb downstream region, was generated by PCR from _H. influenzae_ Rd chromosomal DNA. The amplified and AccI-digested chloramphenicol resistance cassette (_Cam^r_) from pACYC184 was ligated into a unique AccI site within the _pgdx_ gene. The resulting _pgdx^::Cam^r_ knock-out fragment was reintroduced into the chromosome of _H. influenzae_ Rd by transformation and homologous recombination. Disruption of the chromosomal _pgdx_ gene was confirmed by PCR (Fig. 1B; the mutant amplicon was enlarged with around 1,000 base pairs of the antibiotic resistance cassette) as well as by Southern hybridization (Fig. 2 A; probes used for Southern hybridization are depicted in Fig. 1A). Western blot analysis with mouse polyclonal antibody raised against recombinant PGdx confirmed the absence of peroxidase protein in mutant whole cell extracts (Fig. 2B).

**Fig. 1. Construction of _H. influenzae_ Rd _pgdx_ mutant.** A, schematic representation of the _H. influenzae_ Rd chromosomal DNA insert in pSG4.0. Open reading frames are represented by arrows. The oligonucleotide probes (A and B) used for Southern blot analysis are represented by boxes. Inactivation of the _pgdx_ gene was accomplished by inserting a 1-kb chloramphenicol resistance (_Cam^r_) cassette into the AccI site. B, PCR analysis of chromosomal DNA from _H. influenzae_ Rd and _pgdx_ mutant.

**Phenotypic Characterization of the _pgdx_ Mutant**—The _pgdx_ mutant and its wild-type parent were cultured both aerobically and anaerobically to distinguish for possible differences in growth due to environmental oxygen. As such, no dissimilarities were recorded when plotting the data of the growth curve (data not shown).

The sensitivity of the _H. influenzae_ Rd _pgdx_ mutant to var-
ious pro-oxidants was examined by disk diffusion (Fig. 3A) and liquid culture assays (Fig. 3, B–D). Compared with the wild-type strain the mutant displayed enhanced sensitivity to t-BOOH and COOH, with inhibition zone differences ranging from 12 to 13%, respectively. When stressed in liquid culture with increasing concentrations of hydroperoxides, such as t-BOOH and COOH, we observed the expected, more rapid decline in growth rates in the case of the pgdx mutant (Fig. 3, C and D). In both assays, resistance against the organic hydroperoxides was restored in a strain complemented in trans with a copy of the intact pgdx gene (Fig. 3, A–D; replication of the complementing plasmid and expression of PGdx in the complemented strain was confirmed; results not shown). However, above observations are not akin for H2O2. Here, the mutant shows more resilience than the wild-type or the complemented strain in both the disk diffusion assays and the liquid culture assays (Fig. 3B). In the case of methyl viologen, an indirect inducer of H2O2 (11), we observed similar results (Fig. 3A).

Up-regulation of H2O2 Scavenging Activity in pgdx Mutant—To resolve this inconsistency we measured the total H2O2-scavenging activity of aerobically grown cells. As indicated in Fig. 4A, wild-type and mutant show similar patterns, i.e. a moderate increase in H2O2 reducing power during the

Fig. 2. Southern blot and Western blot analysis of PGdx-deficient mutant. A, chromosomal DNA preparations from wild-type and PGdx-deficient H. influenzae Rd were digested with MfeI and ScaI, resolved by agarose gel electrophoresis, and probed with either a 300-bp product derived from the Peroxiredoxin-domain (Probe A) or a 300-bp product derived from the glutaredoxin-domain (Probe B) (see also Fig. 1A). Size markers are indicated on the left. Theoretical sizes for Probe A: wild-type, 1,427-bp; mutant, 1,645-bp and for Probe B: wild-type, 1,427 bp; mutant, 760 bp. B, Western blot analysis of whole cell extracts from wild-type and PGdx-deficient H. influenzae Rd. Left side, Coomassie Blue-stained SDS-PAGE gel; right side, blot probed with polyclonal antibody raised against purified recombinant PGdx. The third lane represents partially purified PGdx sample (1 μg).
exponential phase followed by an attenuation when entering the stationary phase. However, when comparing absolute activities, the PGdx-deficient mutant exhibited around 2 orders of magnitude higher H₂O₂ detoxification throughout growth compared with the wild-type strain. The complemented strain had its activity restored to wild-type levels (Fig. 4A). To determine whether at low micromolar concentrations mutant cells continue to turn over H₂O₂ more efficiently compared with its wild-type counterpart, we analyzed scavenging activities at 1.2 μM H₂O₂. These results reflect the ones when high concentrations were assayed (Fig. 4B).

The up-regulation of scavenging activity in the mutant most probably results from an increase in catalase activity. To assess this premise, cell extracts of exponential-phase cultures were resolved by native polyacrylamide gel. The gel was subsequently stained for catalase activity using a ferricyanide-negative stain. In the case of the mutant, we observed a higher in-gel activity derived from the catalase HktE (Fig. 5).

**PGdx Is Essential for Stationary Survival**—Much to our surprise, we found that when streaked onto a plate, aerobic cultures of the pgdx mutant exhibited no or barely detectable growth after 12 h of stationary phase while the anaerobically grown culture did (Fig. 6A and Table II). The wild-type parent and complemented mutant showed a moderate decrease in plating efficiency overnight in liquid culture. In contrast, the pgdx mutant rapidly lost viability within hours (Fig. 6B).

This aerobic growth defect in the mutant was restored in the complemented strain (Fig. 6, A and B) or with the addition of catalase during the mid-exponential phase and the early stationary phase (Fig. 6A and Table II).

**DISCUSSION**

The chimeric enzyme peroxiredoxin/glutaredoxin, or PGdx, from *H. influenzae* Rd constitutes a recently characterized glutathione-based removal system that reduces H₂O₂ as well as alkylhydroperoxides at significant rates (3). Based on former work on the involvement of glutathione in oxidative stress, we already found that this low molecular weight thiol, when omitted from the growth medium, (i) has no effect on the growth rate of *H. influenzae*, (ii) increases the sensitivity of the bacterium for alkylhydroperoxides while decreasing it for H₂O₂, and (iii) induces a doubling in catalase activity (12). In addition we demonstrated that glutathione-based scavenging is crucial for metabolizing endogenously generated H₂O₂, since it complements for the absence of a functional catalase (6). Hence, from these data and from kinetic parameters (3), it was inferred that *H. influenzae* PGdx, in analogy with the NAD(P)H-dependent peroxidase Ahp from *E. coli* (13), fulfils a role as major peroxidase for low concentrations of H₂O₂. To assess the quantitative importance of this antioxidant enzyme against hydroperoxides,
we constructed a *H. influenzae* Rd knock-out in the PGdx encoding gene HI0572 and studied its effect.

**Characterization of the pgdx Mutant**—No differences in growth rate between the pgdx mutant and its wild-type parent were observed, both under aerobic or anaerobic conditions. This confirms the apparent redundancy of GSH-based scavenging during logarithmic growth. However, as will be discussed below, the absence of PGdx is actually compensated for, thus concealing the loss in antioxidant capacity.

Our results also demonstrate that *H. influenzae* Rd, deficient in PGdx, is considerably hampered in the ability to reduce organic hydroperoxides such as t-BOOH and COOH. Polar effects can be ruled out here because sensitivities are restored when complementing *in trans* with an intact copy of the disrupted gene. However, comparison of the *in vitro* measured catalytic efficiencies for t-BOOH \( (k_{\text{cat}}/K_m = 5.67 \times 10^4 \text{ s}^{-1}\text{M}^{-1}) \) and \( \text{H}_2\text{O}_2 \) \( (k_{\text{cat}}/K_m = 5.01 \times 10^6 \text{ s}^{-1}\text{M}^{-1}) \), in terms of substrate preference, puts alkylhydroperoxides second in line after \( \text{H}_2\text{O}_2 \) (3).

When \( \text{H}_2\text{O}_2 \) challenging was tested, the pgdx mutant para-
doxically showed higher resistance compared with the wild-type and the complemented strain. However, it was shown here that this resilience is attributed to an alteration in H$_2$O$_2$ scavenging activity on behalf of the mutant strain. Similar phenotypes have already been reported in numerous cases such as E. coli (13), Xanthomonas campestris (14), Bacteroides fragilis.
PGdx-deficient Mutant of H. influenzae Rd

TABLE II
Growth characteristics on plate of stationary-phase cultures

| Strain          | Growth of aerobically grown stationary-phase culture | Growth of aerobically grown stationary-phase culture |
|-----------------|-----------------------------------------------------|-----------------------------------------------------|
| Rd KW20         | With catalase +                                    | Without catalase +                                   |
| Rd KW20 Δpgdx   | +                                                   | No or barely distinguishable growth                  |
| Rd KW20 ΔpgdxΔhktE | ND*                                              | +                                                   |

* Not determined.

(15), Pseudomonas aeruginosa (16), and Bacillus subtilis (17), where disruption of ahp causes an induction of catalase activity to compensate for its loss. Transformation with the functional gene complemented for the variation in resistance, as was also the case for Xanthomonas and Bacillus (14, 17). We can conclude that this response of H. influenzae, inherent to the loss of the enzyme, underscores the importance of PGdx in H\textsubscript{2}O\textsubscript{2} scavenging.

Induction of hktE in the Absence of PGdx—At high H\textsubscript{2}O\textsubscript{2} concentrations, whole cells of the pgdx mutant scavenged nearly twice as much H\textsubscript{2}O\textsubscript{2} than its isogenic wild-type strain, which correlates well with the determined HktE activity in GSH-devoid medium (76 ± 13 units/mg protein) compared with GSH-replete medium (36 ± 7 units/mg of protein) (12). At low concentrations the mutant continued to Scavenge at a higher rate, again reflecting our previous results when comparing a GFdx-deficient mutant scavenged nearly twice as much H\textsubscript{2}O\textsubscript{2} than its isogenic wild-type strain, when cultured anaerobically, did the PGdx-deficient mutant continue to grow after prolonged stationary growth.

Stationary-phase survival is often associated with virulence (20). In addition, a recent paper from Kraiss et al. (21) concludes that invasiveness of H. influenzae relies on the ability of the bacterium to grow aerobically. Several reports exist where stationary-phase induction of catalase plays a critical role (e.g. Caulobacter crescentus (22), Legionella pneumophila (23), B. fragilis (15)). From our observations, however, it seems that PGdx, rather than HktE, provides protection during stationary phase. This is likely because the latter becomes repressed at this point (19) and does not play a role in virulence (24). While in E. coli aerobic respiration was reported to decrease during stationary phase (18), other sources may continue to be an oxidative threat. With antioxidant power in H. influenzae being scarce due to the combined repression of catalase and the deficiency of PGdx, these threats become emphasized in the stationary phase, resulting in cells that succumb.

In short, we have demonstrated that, besides reducing organic hydroperoxides, the primary action of PGdx is to Scavenge metabolically generated H\textsubscript{2}O\textsubscript{2} as well as to safeguard the cell from oxidative stress during the stationary phase. How exactly PGdx is involved in stationary-phase survival remains to be determined. Therefore, we are currently evaluating the virulence properties of a pathogenic variant of H. influenzae defective in pgdx, as well as the mechanisms behind the regulation of the gene.

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