Molecular and catalytic properties of a peroxiredoxin–glutaredoxin hybrid from *Neisseria meningitidis*

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Abstract A hybrid protein from *Neisseria meningitidis*, which contains both a peroxiredoxin and a glutaredoxin domain, has been isolated. The enzyme was active in the reduction of various peroxides and dehydroascorbate in the presence of reduced glutathione. These findings suggest that both the peroxiredoxin and glutaredoxin domains are biochemically active in the fusion. Moreover, when expressed separately, the glutaredoxin domain was catalytically active and the peroxiredoxin domain possessed a weak activity when supplemented with exogenous glutaredoxin.

Key words: Glutaredoxin; Glutathione; Hydroperoxide; Peroxiredoxin; *Neisseria*

1. Introduction

Reactive oxygen and nitrogen species are generated in response to oxidative stress in biological materials [1]. Among the enzymatic systems capable of degrading peroxides, a group of heme-devoid peroxidases, peroxiredoxins (Prx), catalyze alkyl hydroperoxide reduction via reactive cysteines [2]. Phylogenetic and biochemical analyses indicate that animal, yeast and plant cells contain several Prx with different subcellular localization and molecular properties [3–5].

All Prx contain one conserved catalytic cysteine residue which upon catalysis is transformed into a sulfenic acid, subsequently regenerated by a thiol reductant, in general thioredoxin (Trx) [2,6]. Trx is maintained reduced via the action of NADPH and thioredoxin reductase in the cytosol or via ferredoxin and ferredoxin-thioredoxin reductase in the chloroplast [7]. One notable exception is a type II Prx from poplar, formerly known as type C, which can be reduced by Trx but even more efficiently by glutaredoxin (Grx), a related catalyst with a modified active site (in general CPYC instead of CGPC) [8,9]. Grx is reduced via NADPH, glutathione reductase (GR) and reduced glutathione (GSH). Unlike Trx, Grx can be active as a monocysteinic enzyme [10].

Very interestingly, the human homolog of poplar type II Prx, Prx V (40% identity), can use only Trx as a donor and Grx is inefficient [11]. Comparing the 3D structures of the two Prx and matching them with those of Grx and Trx is likely to provide molecular details explaining the differences in interaction. The X-ray structure of human Prx V has already been published and these studies are under way for poplar type II Prx and Grx [12–14].

Another approach to understand these interactions is to study hybrid enzymes which contain both Grx and Prx modules. Two recent papers describe such fusions in *Chromatium gracile* or *Haemophilus influenzae* and provide evidence that the enzyme is functional with glutathione (amide) as a donor [15,16]. Another paper describes the 3D structure of the *H. influenzae* enzyme and details the contacts between the two modules [17]. We report here complementary biochemical data concerning a related natural fusion in *Neisseria meningitidis*, which indicate that the enzyme is also active with GSH as a donor. In addition, we present evidence that both isolated modules possess biological activity and identify the cysteine residues necessary for catalysis and protein–protein interaction.

2. Materials and methods

2.1. Plasmids and strains

Plasmids used for expression were pET-3d and pSBET which carry the resistances for ampicillin and kanamycin respectively [18]. The *Escherichia coli* expression strain was BL21(DE3) and the cloning strain was DH5α.

2.2. Cloning, expression and purification of the recombinant enzymes

The oligonucleotides used for cloning the full-length construction have the following sequences (*Nco*I and *Bam*HI restriction sites underlined): forward 5′-CCCCCCATGGCTTTGCAAGATCGTACCGTT-3′ and reverse 5′-CCCCGGATCCTCAGTTTTTAGCCAAGTAAGC-3′. Additional oligonucleotides for the short constructions were devised so that the N-terminus of the Grx domain would be MAQESVA and the C-terminus of the Prx domain MLQFVA. They had the following sequences: forward Grx 5′-CCCCCCATGGCTCAAGAGTCTGTGGCA-3′ and reverse Prx 5′-CCCCGGATCCTTAAGCAACGAATTGCAGCAT-3′. The mutagenic oligonucleotides were as follows: forward C185S 5′-CCAGGTTGCCAATTC**A**GCGCTAAAGCCAAA-3′ and reverse C185S 5′-TTTGGCTTTAGCGC**T**GAATTGGCAACCTGG-3′. The mutagenic bases are in bold characters. An aliquot of genomic DNA of *N. meningitidis* was used as a template. The polymerase chain reaction fragments were inserted into the expression plasmid pET-3d yielding the constructions pET-NmPrxGrx, pET-NmGrx, pET-NmPrx, pET-NmPrxGrx C185S and pET-NmGrx C185S.

The cotransformed *E. coli* cells were multiplied to a final volume of ca. 5 l at 37°C. 100 μM isopropyl-β-D-thiogalactose was added in the exponential phase and the bacteria were harvested by centrifugation for 15 min at 5000×*g*. The cells were resuspended in TE buffer (Tris–

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Abbreviations: DHA, dehydroascorbate; DTT, dithiothreitol; Grx, glutaredoxin; GR, glutathione reductase; GSH, reduced glutathione; Prx, peroxiredoxin; Trx, thioredoxin

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HCl 30 mM pH 8.0, EDTA 1 mM, 20 mM dithiothreitol (DTT). All subsequent chromatographic steps were effected in the same buffer except that DTT was omitted. The recombinant proteins were purified by ammonium sulfate fractionation (all proteins precipitated between 40 and 90% of the saturation except for the NmGrx module which precipitated between 0 and 40%), ACA 44 gel filtration (NmPrxGrx, NmPrxGrx C185S and NmPrx) or G50 gel filtration (NmGrx and NmGrx C185S) and DEAE Sephacel chromatography. After dialysis and concentration, proteins were stored frozen at −20°C.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), formation of heterodimers and thiol titration

The formation of heterodimers was as in [9] except that H_2O_2 was used as an oxidant instead of diamide. Thiol content titration of the proteins using 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) was as described in [9].

2.4. Biochemical assays

The manner of obtaining recombinant poplar type II Prx, Grx and Trx h1 is described in [8,19,20]. Glutathione amide and glutathione amide reductase were gifts of Dr. Vergauwen. Dehydroascorbate (DHA) and hydroxyethyldisulfide (HED) reduction were performed as in [10]. Hydroperoxide reduction was followed in two different ways. First, peroxides were quantified using the FOX method with DTT as a reductant [21]. Alternatively, NADPH oxidation was followed at 340 nm in 500 μl cuvettes in the presence of 30 mM Tris–HCl pH 8.0, 1 mM EDTA buffer, 250 μM NADPH, 1 mM GSH, 0.5 unit GR, and Prx and Grx as needed. The reaction was started by adding H_2O_2 after 1 min incubation at 30°C to allow initial reduction of the system.

3. Results

3.1. Sequence analysis

Fig. 1 shows a sequence comparison which includes the *N. meningitidis* fusion protein, poplar Grx and type II Prx and selected fusion homologs. All fusion enzymes are prokaryotic sequences, two photosynthetic organisms belonging to cyanobacteria and purple sulfur bacteria, (*Nostoc* sp. PCC 7120 and *Chromatium gracile*) and four non-photosynthetic pathogenic bacteria (*N. meningitidis*, *Haemophilus influenzae*, *Yersinia pestis*, and *Vibrio cholerae*). All the fusion sequences share the same organization, the Prx module is present in the

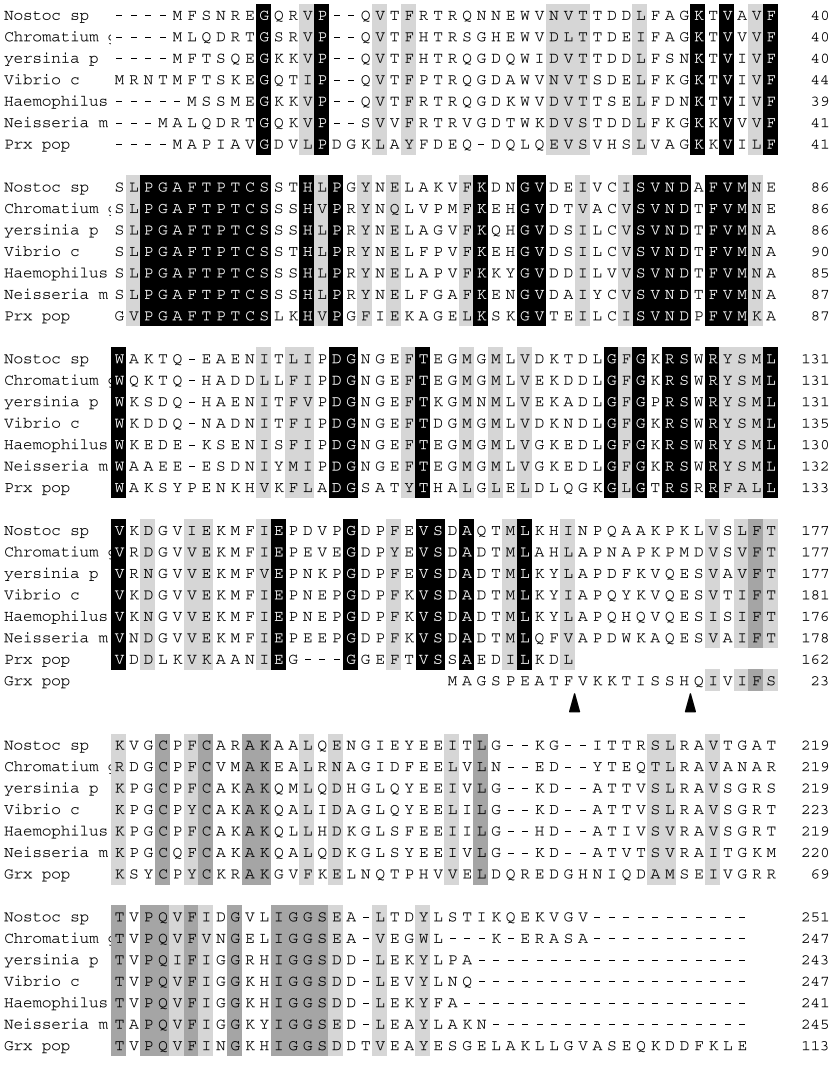

Fig. 1. Amino acid sequence comparison of NmPrxGrx with other hybrid enzymes and poplar Prx and Grx. Alignment was performed using ClustalW. Accession numbers or reference of the various sequences are as follows: *Nostoc* sp. PCC 7120 NP_485581, *Chromatium gracile* [17], *Yersinia pestis* NP_407361, *Vibrio cholerae* NP_232265, *Haemophilus influenzae* NP_438729, *Neisseria meningitidis* NP_273984, *Populus×interamericana* Prx AAL90751, *Populus×interamericana* Grx AAL90750. Shaded in black: strictly conserved residues with poplar Prx; dark gray: strictly conserved residues with poplar Grx; light gray: functionally conserved residues. Arrows indicate the position of the linker peptide and the limits of the modules.

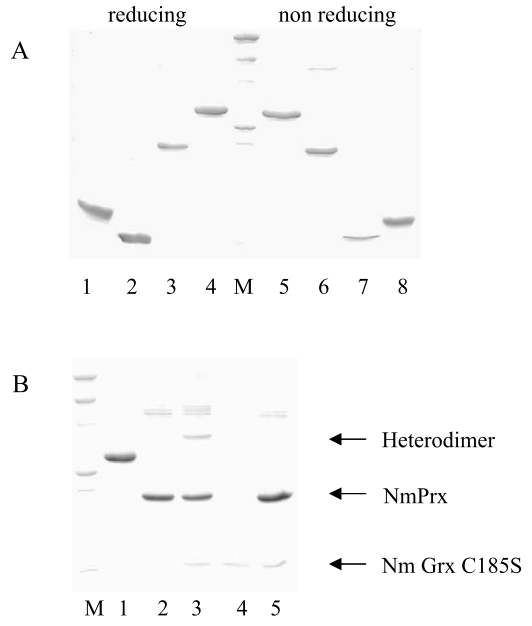

Fig. 2. SDS–PAGE of the recombinant proteins and covalent heterodimer formation. A: NmGrx C185S (lanes 1, 8), NmGrx wild-type (2, 7), NmPrx (3, 6) and NmPrxGrx (4, 5) in reducing (1–4) or non-reducing conditions (5–8). B: Heterodimer formation between NmPrx and NmGrx C185S. Lane 1: NmPrxGrx, 2: NmPrx, 3: NmPrx and NmGrx C185S no reductant, 4: NmGrx C185S, 5: NmPrx and NmGrx C185S plus DTT. M: Molecular mass markers, top to bottom: 50, 37, 25, 20 and 10 kDa.

N-terminus of the protein and the Grx module in the C-terminal part. The fusion sequences are closely related and display between 59 and 80% identity. The *N. meningitidis* Prx and Grx modules are respectively 38% and 32% identical to poplar type II Prx and Grx. When the fusion is compared to Prx and prokaryotic Grx sequences which are functional but shorter than eukaryotic ones, it is apparent that there is a ca. 8 amino acid long linker peptide that connects the two modules, with the sequence APDWKAQ. None of these amino acids is involved in catalysis or in contact between the modules [12]. As the poplar Grx sequence presented here is elongated on the N-terminal side [10], the linker peptide is not visible in the configuration of Fig. 1.

3.2. Expression of the fusion enzyme and of the Prx and Grx domains

Based on secondary structure predictions, three different versions of the *N. meningitidis* fusion protein were expressed in *E. coli*. A first construction (NmPrxGrx) represents the full-length protein with 245 amino acids. The second construction (NmPrx), which stretches from the N-terminus to the end of the Prx domain (C-terminal sequence MLQFVA), contains 165 amino acids. The third one, the Grx module (NmGrx), starts with MAQESVA and ends at the C-terminus of the fusion with 77 amino acids overall. In addition, C185S mutants, where the second cysteine of the active site has been removed in NmGrx and NmPrxGrx, have also been constructed. All proteins were soluble when expressed in *E. coli*. The purification procedure employed here routinely involves the addition of DTT at the breaking step and upon storage for NmPrxGrx. The final yields were 20, 17, 10, 7 and 22 mg/l for NmPrxGrx, NmPrx, NmPrxGrx C185S, NmGrx and NmGrx C185S respectively. Fig. 2A shows that all protein preparations were highly homogeneous after these purification steps. The NmPrxGrx C185S preparation is not shown in Fig. 2 but is highly homogeneous too (data not shown). In general, in SDS–PAGE, all polypeptides exhibited the expected size indicating that the fusion enzyme was not processed in *E. coli*. However, the NmGrx C185S module migrates slightly differently from the corresponding wild-type NmGrx module. This is not unusual for this type of protein, as similar behaviors have been observed for mutants of plant thioredoxins (unpublished). This may be due to local conformational changes generated by the disruption of the disulfide. The molecular mass of the fusion enzyme was estimated by comparison to standard proteins of known size on an ACA 44 gel filtration column, and the enzyme was found to be a tetramer (data not shown). Similar results have been described for the *Haemophilus* homologous enzyme using analytical ultracentrifugation [17]. On the other hand, the isolated Prx and Grx modules migrated respectively as a dimer and as a monomer (data not shown). DTNB titration of the fusion and isolated modules indicated that all recombinant proteins were in a fully reduced state (data not shown). Thus, the tetrameric form of the fusion does not involve any disulfide bond. Fig. 2B shows that when the two modules were mixed in the presence of H_2O_2, a heterodimer with an apparent slightly larger size (ca. 32 kDa instead of 27) than the fusion enzyme can be generated (lane 3). Addition of DTT results in the disappearance of this heterodimer (lane 5). This experiment proves that

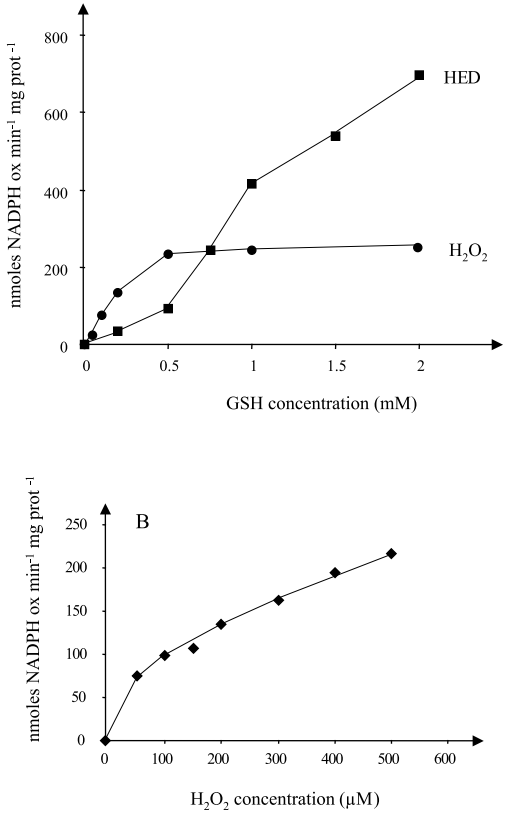

Fig. 3. Reduction of H_2O_2 and HED by NmPrxGrx. A: GSH dependence. Reactions were carried out at 500 μM H_2O_2 or 7 mM HED. Values were corrected for the GSH-dependent NADPH oxidation. B: H_2O_2 dependence. Reactions were carried out at 1 mM GSH. NmPrxGrx concentration in the assays was 4 μM or 2 μM for HED.

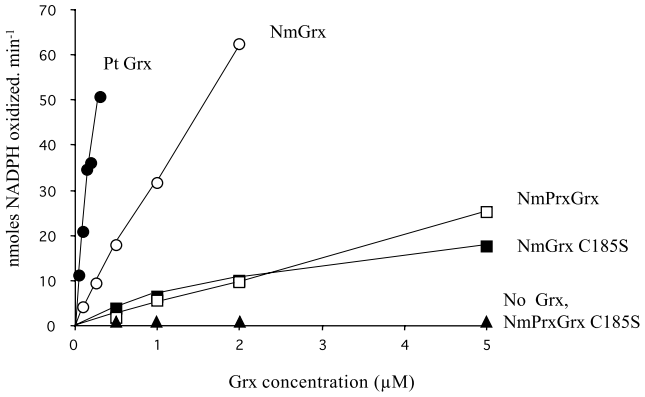

Fig. 4. Grx activity determination via DHA reduction. DHA reduction (1 mM final) was measured by following NADPH oxidation as a function of the various Grx (NmPrxGrx, NmPrxGrx C185S, NmGrx, NmGrx C185S and poplar Grx) concentrations.

the Prx domain can interact with a Grx domain that does not belong to the same subunit.

3.3. Catalytic activity of the recombinant proteins

The fusion protein is able to reduce various hydroperoxides (data not shown), and notably H_2O_2. These reactions are driven by GSH which is maintained reduced via NADPH and GR. The rate of NADPH consumption linked to peroxide or HED reduction as a function of GSH concentration is presented in Fig. 3A. With H_2O_2, the reaction saturates at about 500 μM GSH and the $S_{0.5}$ of the reaction is around 200 μM. No cooperativity was observed in this assay, but the reaction was clearly cooperative when HED was tested as an acceptor of the Grx module in the fusion enzyme. The reaction rate increased with increasing H_2O_2 concentration but did not saturate until 500 μM (Fig. 3B). Using the FOX assay (which detects directly peroxides), we determined that DTT can replace GSH as a donor, indicating that glutathiolation of the Prx catalytic cysteine is not necessary (data not shown). Glutathione amide can also replace GSH as a donor in the presence of the appropriate reductase but with lower efficiency (data not shown). This observation suggests that the *Chromatium* and *Neisseria* fusions are closely related enzymes.

The efficiency of the Grx module in the fusion enzyme and of the isolated NmGrx module was tested in both the HED (data not shown) and DHA reduction assays (Fig. 4) and similar results were generated. NmGrx displays a very good activity in the wild-type version. Removing the second cysteine of NmGrx had a pronounced effect on catalysis but the protein retained an activity level similar to that recorded with the fusion enzyme. In the same conditions, NmPrxGrx C185S was inactive.

3.4. Reconstitution of the fusion with the two separated modules

The NmPrx module was tested for the reduction of H_2O_2 in the presence of NmGrx, NmGrx C185S or of poplar Grx. Both proteins could complement NmPrx, but the efficiency of the 'reconstituted system' was clearly low compared to the NmPrxGrx fusion or to an all-poplar system used as a control (Table 1). Poplar Trx h1 was also able to reduce NmPrx (Table 1). NmGrx or NmGrx C185S could also serve as a donor to poplar Prx, while poplar Grx could also promote the catalysis of NmPrx.

4. Discussion

Two recent papers provide a 3D structure of the *Haemophilus* Prx–Grx fusion and kinetic data describing the catalytic reaction of this hybrid enzyme [16,17]. Based on the results obtained in parallel with the poplar system where the two proteins are separate but can complement each other [8,9], one can postulate a mechanism in which the reducing power of GSH promotes the regeneration of the catalytic cysteine of the Prx domain via the Grx domain. While poplar Prx contains an additional cysteine besides the catalytic one, site-directed mutagenesis has provided evidence that it is not necessary for catalysis [9]. This is confirmed in the *Haemophilus* fusion where the second cysteine is absent [16].

Pauwels et al. [16] have produced a *Haemophilus* fusion enzyme in an oxidized form where the catalytic cysteine is glutathiolated. Based on this observation, they propose that enzyme catalysis generates a sulfenic acid that can be glutathiolated. The deglutathiolation could then be performed via the Grx module and the catalytic cysteine regenerated. DTNB titrations indicate that the *Neisseria* enzyme preparations obtained in this study are in a reduced form. It was reported that the *Haemophilus* fusion enzyme exhibits cooperativity vs. GSH. This was not observed with NmPrxGrx when H_2O_2 is the acceptor, but cooperativity was clearly apparent with HED as an exogenous acceptor of the Grx module. It is thus likely that the discrepancy results from the nature of the electron acceptor. The results presented here do not contradict those in [16] but bring additional information. As we produced not only the fusion enzyme but also the isolated Prx and Grx modules, we have shown unambiguously that the regeneration of the catalytic sulfhydryl is indeed catalyzed by the Grx module. While the isolated Grx module is active in a monocysteinic form in the DHA reduction (Fig. 4), the two cysteines of Grx are required in the fusion enzyme for peroxide or DHA reduction (data not shown and Fig. 4). This is not unusual as it is generally recognized that Grx can function as either monocysteinic or bicysteinic enzymes, depending on the nature of the substrate. Also, it should be emphasized that the Grx module purified here is extremely short (only 77 amino acids) and its active site is a non-canonical one (CQFC instead of CPYC). These particularities do not prevent this

Table 1
Reconstitution of peroxide catalysis with isolated *N. meningitidis* Prx and Grx modules

	Activity (nmol NADPH oxidized/min/ml)
NmPrxGrx	26.4
NmPrx, NmGrx	20.2
NmPrx, NmGrx C185S	11.2
NmPrx, PopGrx	24.6
PopPrx, NmGrx	24
PopPrx, NmGrx C185S	22.7
PopPrx, PopGrx	192
NmPrx, PopTrx h1	16.6

Concentrations used: row 1: NmPrxGrx 4 μM; row 2: NmPrx 40 μM, NmGrx 25 μM; row 3: NmPrx 40 μM, NmGrx C185S 25 μM; row 4: NmPrx 40 μM, PopGrx 25 μM; row 5: PopPrx 2.5 μM, NmGrx 10 μM; row 6: PopPrx 2.5 μM, NmGrx C185S 20 μM; row 7: PopPrx 2.5 μM, PopGrx 2.5 μM; row 8: PopTrx h1 25 μM, NmPrx 40 μM. The rates with GSH alone as a donor (minus Grx, ca. 4 nmol NADPH oxidized/min/ml) have been subtracted from the values of the table.

protein of being functional, it can even serve as a donor to poplar type II Prx (Table 1). The DHA activity of the isolated Grx module is higher than that recorded with the fusion enzyme, presumably because its active site is not as readily available as in the isolated domain.

In general, our results confirm the working hypothesis by demonstrating that the Grx module possesses a Grx activity, that Prx and Grx can be reassembled with partial restoration of catalysis and furthermore that GSH is an excellent donor to the fusion protein for reduction of H_2O_2 or other peroxides. It is likely that the low efficiency of the reconstituted system can result from the loss of the contacts between the Prx and Grx units and thus of the tetrameric interactions when we used the isolated modules. While the sequence of events leading to the regeneration of the thiol group of the catalytic cysteine is getting clear, how the Grx and Prx domains interact in the fusion is still a matter of debate. From the primary sequence analysis and the 3D structure, there is a short linker peptide that connects the two modules in a single subunit. One hypothesis could be that it is flexible enough to allow the two modules to interact with one another. A second possibility is related to the quaternary structure of the enzyme reported in [17]. In the crystallographic structure, Kim et al. have pointed out that the Grx module of one subunit is in close contact with the Prx module of another subunit. In our study, the native fusion enzyme migrates as a tetramer in non-denaturing conditions, and in addition, the amino acids involved in the Prx–Grx interaction area are conserved in the *Neisseria* sequence (E91 and E92 of the Prx module and K177 and R213 of the Grx module). This supports the idea that the fusion enzyme is not likely to function as a monomeric but rather as a multimeric enzyme. The residues involved in GSH binding and Grx–Grx and Prx–Prx interaction domains are also essentially conserved. However, in order to test whether the electron transfer between the Prx and Grx domains is intra- or inter-subunit, we have analyzed the electrophoretic pattern of the NmPrxGrx C185S mutant after addition of diamide. No dimer or tetramer was formed suggesting that the transfer could be intra-subunit (data not shown). Elucidating the 3D structure of this mutant will help to determine its catalytic behavior.

N. meningitidis is a human pathogen inducing hearing loss, mental retardation and death in the most severe cases. The presence of the pathogen in the nasopharynx is likely to expose it to high tensions of oxygen and oxidants. The PrxGrx function could be to protect the bacterium against these dangerous molecular species. Thus, a detailed analysis of the fusion enzyme absent in human cells could be one basis for fighting this dangerous pathogen.

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