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Engineering functional artificial hybrid proteins between poplar peroxiredoxin II and glutaredoxin or thioredoxin ☆

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Abstract

The existence of natural peroxiredoxin–glutaredoxin hybrid enzymes in several bacteria is in line with previous findings indicating that poplar peroxiredoxin II can use glutaredoxin as an electron donor. This peroxiredoxin remains however unique since it also uses thioredoxin with a quite good efficiency. Based on the existing fusions, we have created artificial enzymes containing a poplar peroxiredoxin module linked to glutaredoxin or thioredoxin modules. The recombinant fusion enzymes folded properly into non-covalently bound homodimers or homotetramers. Two of the three protein constructs exhibit peroxidase activity, a reaction where the two modules need to function together, but they also display enzymatic activities specific of each module. In addition, mass spectrometry analyses indicate that the Prx module can be both glutathiolated or overoxidized in vitro. This is discussed in the light of the Prx reactivity.

Keywords: Glutaredoxin; Hydroperoxide; Peroxiredoxin; Poplar; Thioredoxin

In plants, the thioredoxin peroxidase family includes four types of peroxiredoxins (Prx), called 1-Cys Prx, 2-Cys Prx, Prx Q, and Prx II, and many glutathione peroxidase (Gpx) isoforms, which use thioredoxin (Trx) as an electron donor instead of glutathione (GSH) [1–4]. All these groups differ by the number and the position of conserved catalytic cysteines [1]. The Prx II class is unique as it is able to use glutaredoxin (Grx) and sometimes Trx, although with a lower efficiency, as electron donors [5–7]. Grx and Trx are small disulfide reductases involved in dithiol–disulfide exchange using a catalytic CxxC/S active site, but Grxs possess also a more specific function in glutathiolation/deglutathiolation processes using only the first of the two active site cysteines [8]. Prxs II possesses two cysteines, the so-called peroxidatic cysteine, common to all Prxs and a putative resolving cysteine, 25 amino acids downstream, which is not conserved in all plant sequences. Mutagenesis studies indicated that the peroxidatic cysteine of Prx II and only the first cysteine of the Grx active site are absolutely essential [7], suggesting that either Grx or GSH is able to directly attack the sulfenic acid formed on the peroxidatic cysteine of Prx II during catalysis.

Interestingly, natural fusion proteins, found in many bacteria, are constituted of Prx II followed by Grx modules. These enzymes are able to use GSH or sometimes glutathione amide as electron donor and various oxidized substrates; i.e., hydrogen peroxide or various alkyl and lipid hydroperoxides as electron acceptor [9–12]. The three-dimensional structure of the fusion found in *Haemophilus influenzae* has been obtained by cristallography and indicates that the enzyme is a tetramer [13], in which each

☆ *Abbreviations:* DHA, dehydroascorbate; DTT, dithiothreitol; Grx, glutaredoxin; Prx, peroxiredoxin; Trx, thioredoxin.

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monomer is constituted of Grx and Prx subdomains connected by a flexible linker. The different subunits are linked together mostly through electrostatic interactions implicating Prx–Prx, Grx–Grx, and Prx–Grx subdomain interactions. Moreover, Kim and coworkers have identified some amino acids responsible for the Prx–Grx interaction.

In order to get a better understanding of the structural determinants of the interactions between these enzymes, we have engineered and purified artificial hybrid proteins containing poplar PrxII and two identified donors, poplar Grx C4 or poplar Trx h3. These three protein components were taken from the same plant (poplar) where they are located in the same cellular compartment (cytosol) and, in addition, we have shown that they function biochemically well together. All the chimeric recombinant proteins are soluble, correctly folded and active, but display sometimes different efficiencies compared to the isolated recombinant proteins. In addition, the Trx and Grx modules are active in the fusion enzymes as judged by specific activity tests.

Materials and methods

Plasmids and strains

Plasmids used for expression in *Escherichia coli* BL21(DE3) were pET-3d and pSBET which carry the resistances for ampicillin and kanamycin, respectively [14]. The cloning strain was *E. coli* DH5α.

ORF cloning in fusion

The method used to fuse the two proteins is similar to the PCR site-directed mutagenesis strategy. It is based on successive PCR amplifications, a first step is to amplify two overlapping DNA fragments, which are then used in a second step to synthesize the full-length open reading frame. Three different constructions, which encode artificial fusions between poplar Prx II and poplar Grx C4 or Trx h3 modules (respective GenBank Accession Nos.: AAL90751, AAL90750, and BU822062), have been engineered [6,15,16]. These various constructions differ in particular by the structure of the linker sequence. This linker, identical to the one of *Neisseria meningitidis* PrxGrx sequence (GenBank Accession No.: NP_273984), contains the sequence **APDWKA** for PrxGrx1 (PG1) and PrxTrx (PT) or **APDWKAQES** for PrxGrx2 (PG2) (Table 1 and Fig. 1). The two PrxGrx constructions also differ from one another by the N-terminal sequence of the Grx module introduced. PG1 possesses the entire poplar Grx C4 sequence (linker in bold and Grx sequence underlined) (**APDWKA**AGSPEA), whereas PG2 contained a shortened N-terminus part of the Grx module, mimicking the size of the *N. meningitidis* Grx sequence (**APDWKAQES**IVIFSK). Cloning and fusion oligonucleotides are described in Table 1. The amplification of the Prx II module including the linker sequence was the same for the three constructions and was done using the primers called Prx II for and Prx(G)Trx rev. The second overlapping fragment is different in the three constructions and necessitated the use of primers PrxTrx for and Trx h3 rev for the PrxTrx construction, PrxGrx1 for and Grx C4 rev for the PrxGrx1 construction, and PrxGrx2 for and Grx C4 rev for the PrxGrx2 construction.

Expression and purification of the recombinant enzymes

Escherichia coli strain BL21(DE3) containing the helper plasmid pSBET [14] was transformed with the various recombinant plasmids. LB cultures of 2.6 L were grown at 37 °C and induced in exponential phase by 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside). After 3 h induction, the bacteria were harvested by centrifugation for 15 min at 5000*g* and resuspended in buffer A (30 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 200 mM NaCl). Bacteria were disrupted by sonication and centrifuged for 30 min at 30,000*g* to discard the insoluble fraction. The soluble part was then precipitated with ammonium sulfate successively up to 40% and 80% of the saturation.

PrxGrx1 precipitated mainly between 40% and 80%, whereas PrxGrx2 and PrxTrx between 0% and 40%. The fractions of interest were first separated onto an ACA 44 gel filtration column (Biosepra) equilibrated with buffer A, dialyzed and then onto a DEAE Sepharose column (Sigma) equilibrated with buffer A without NaCl. The proteins were eluted using a 0 to 0.4 M NaCl gradient, dialyzed again, concentrated, and finally stored in buffer A at −30 °C until further use. SDS–PAGE was used to check the homogeneity of the proteins.

Enzymatic assays

Insulin reduction. The reduction of insulin was measured at room temperature using a Cary 50 spectrophotometer (Varian) by following changes in absorbance at 650 nm, in 500 μL cuvettes containing 50 mM phosphate buffer, pH 7, 500 μM DTT, 8 U recombinant human insulin, and 5 μM of each protein tested.

Dehydroascorbate reduction. Dehydroascorbate (DHA) reduction (500 μM) was tested either in the presence of the Grx system (180 μM NADPH, 0.4 U glutathione reductase (GR), 500 μM GSH) for poplar GrxC4, PrxGrx1, and PrxGrx2 or in the presence of the Trx system (180 μM NADPH, 1 μM NADPH thioredoxin reductase (NTR)) for poplar Trx h3 and PrxTrx fusion. The reaction in 500 μL TE buffer (30 mM Tris–HCl, pH 8, EDTA 1 mM) was initiated after 1 min incubation by adding 50 nM to 1 μM enzyme.

Hydroperoxide reduction. Two different tests were used to measure hydroperoxide reduction activity of the various fusions. The first one is coupled to NADPH oxidation. When using the Trx reducing system, the reaction medium in 500 μL was the same as the one used for DHA reduction measurement except that DHA was replaced by 250 μM H_2O_2. Concentrations used were 5 μM of PrxTrx or 5 μM Trx h3 in combination with 5 μM Prx II. When the Grx reducing system (NADPH, 0.4 U GR and 1 mM GSH) was used, 500 μM *t*-BOOH was used instead H_2O_2 to

Table 1
Cloning primers

Name	Nucleotidic sequence	Amino acid sequence
Prx II for	5′ GGGGCCATGGCCCCGATTGCTGTTGGT 3′	MAPIAVG
GrxC4 rev	5′ GGGGGGATCCTTAAAAGTCATCTTTCTGCTC 3′	EQKDDF*
Trx h3 rev	5′ CCCCGGATCCTCAAGCAGAAGCAGTAGC 3′	ATASA*
PrxGrx1 for	5′ **GCTCCCGATTGGAAG**GCTGGCAGCCCTGAAGCT 3′	**APDWKA**AGSPEA
PrxGrx2 for	5′ **GCTCCCGATTGGAAGGCTCAAGAGTCT**ATCGTCATCTTCTCCAAG 3′	**APDWKAQES**IVIFSK
PrxTrx for	5′ **GCTCCCGATTGGAAG**GCTGCTGAAGATGGACAAGTG 3′	**APDWKA**AEDGQV
Prx(G)Trx rev	5′ AGCCTTCCAATCGGGAGCAAGATCCTTGAGGATATC 3′	DILKDL**APDWKA**

Cloning restriction sites, *Nco*I or *Bam*HI are underlined. In bold the linker sequence between Prx and Grx or Trx modules, either APDWKA for PrxGrx1 and PrxTrx fusions or APDWKAQES for the PrxGrx2 fusion.

* Corresponds to stop codons.

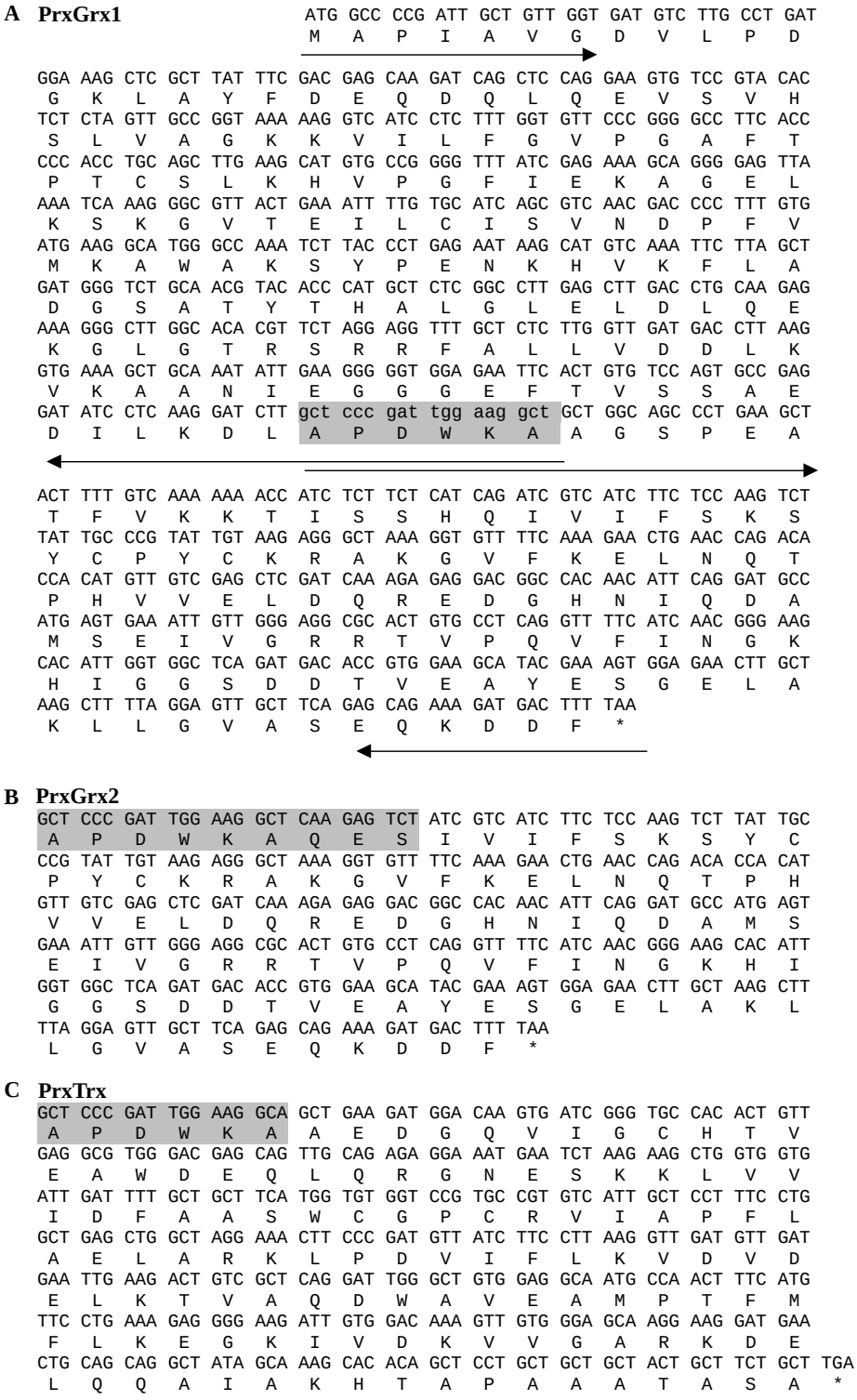

Fig. 1. Hybrid protein sequences. The linker sequence introduced was shaded for each fusion and oligonucleotides described in Table 1. (A) Artificial fusion protein (PrxGrx1) between poplar Prx II and GrxC4 modules. The entire Grx sequence was added downstream to the Prx II sequence using a linker sequence homolog to the one of the natural fusion enzyme from *Neisseria meningitidis*. Cloning primers are indicated by arrows. (B,C) C-terminal end of PrxGrx2 and PrxTrx, the Prx module until the linker sequence is similar to the one in PrxGrx1.

prevent direct reduction of H_2O_2 by ionised GSH. Concentrations used were 1 μM PG1 or PG2 and 1 μM GrxC4 plus 1 μM Prx II when using the separate catalysts. Background activity in these conditions was negligible.

In the second test used, H_2O_2 consumption was measured directly in the presence of DTT or GSH using the FOX (ferrous oxidation in xylenol orange) colorimetric detection method [17]. The 50 μL reaction medium contained 30 mM Tris–HCl, pH 8.0, 500 μM DTT or GSH, 500 μM H_2O_2, and 5 μM of enzymes. The reaction was stopped by adding 5 μL of the reaction mixture to 495 μL of the FOX reagent.

Conformational characterization

Circular dichroism spectra were recorded at 25 °C between 190 and 260 nm on a Jobin-Yvon CD6 spectro-dichrograph with a 2 s integration time for each 0.5 nm step and a bandwidth of 2 nm. Two spectra of each protein at a concentration of ca 50 μM in 10 mM Tris–HCl, 0.3 mM EDTA buffer were averaged and corrected from the baseline for buffer solvents. Analysis was performed using K2D program (http://www.embl-heidelberg.de/~andrade/k2d/) described by Andrade et al. [18].

Gel filtration chromatography experiments

The apparent molecular sizes of the three fusion proteins were determined by calibration against a standard curve generated using proteins of known molecular mass : chymotrypsinogen A, myoglobin, bovine serum albumin, and chicken egg ovalbumin (all from SIGMA) using an ACA 44 gel permeation chromatography (Biosepra).

In vitro glutathiolation and overoxidation experiments

In 50 μL reaction mixtures containing 30 mM Tris–HCl, pH 8.0, and 1 mM EDTA, 50 μM of PG1, PG2, PT, Trx h3, Prx II, and Grx C4 were incubated for 10 min with 1 mM DTT before adding 5 mM oxidized glutathione or 5 mM H_2O_2 for 30 min. Before Electrospray Mass Spectrometry analyses, samples were diluted 20-fold in 50:50 acetonitrile:0.1% formic acid solution. Acquisition of the electrospray ionization (ESI) mass spectra were performed using a Micromass Q-Tof Ultima (Waters Micromass MS Technologies) hybrid tandem mass spectrometer, equipped with a nanoflow electrospray source. The samples were infused into the mass spectrometer using nanoflow capillaries (Proxeon Biosystems, Denmark). The needle voltage was ~1800 V, and collision energy was 10 eV for MS analyses. Data analysis was accomplished with a MassLynx data system and Transform deconvolution software supplied by the manufacturer (Waters Micromass MS Technologies).

Results and discussion

Engineering artificial fusion proteins

One remarkable feature of poplar PrxII is its ability to use both Grx and Trx as electron donor for its regeneration. In order to study the structural interactions between poplar Prx and Grx or Trx, stable covalent interactions have been obtained, using cysteinic mutants of the active site of the different partners [7] but we were not able to further purify these heterodimers. Another approach was to characterize fusion enzymes naturally present in many bacteria. However, we only found PrxGrx but no PrxTrx prototypes. Here, we describe the construction of both PrxGrx or PrxTrx fusions using poplar enzymes. Based on the recent characterization of peroxiredoxin-glutaredoxin hybrid enzymes in several bacteria (*N. meningitidis*, *H. influenzae*, *Vibrio cholerae*, and *Chromatium gracile*) three different artificial fusion proteins between a poplar Prx II and two of its efficient electron donors, poplar Grx C4 or Trx h3 have been constructed by using a strategy described in Materials and methods. When comparing the fusion of *N. meningitidis* with the non-fused Prx and Grx, one detects the presence of a linker sequence APDWKA or even APDWKAQES when considering shorter bacterial Grx sequences as references [11]. Fig. 1 displays the three different hybrid sequences created here, called PrxGrx1 (PG1), PrxGrx2 (PG2), and PrxTrx (PT). The two different PrxGrx fusions (PG1 and 2) differ first by the length of the incorporated linker (APDWKA or APDWKAQES, respectively). Another difference is the length of the Grx module. Bacterial Grxs are generally shorter (75 to 90 amino acids) compared to eukaryotic sequences (110 to 140 amino acids). Thus, in PG1 the full-length poplar Grx sequence (109 amino acids) was introduced, whereas a shorter Grx (92 amino acids), mimicking bacterial Grxs, was introduced in PG2. In this shorter version, the first α-helix of poplar glutaredoxin was truncated (D'ambrosio et al., unpublished results). Indeed, most of the eukaryotic Grxs possess an additional α-helix in the N-terminus part, but it is not known whether it is required for biological activity. A PrxTrx fusion was also engineered by fusing the entire poplar Trx h3 sequence to Prx II and using the linker APDWKA. This construction is particularly interesting to understand the molecular contacts between Prx II and Trx since there are no homologous sequences in any database. Overall, the different constructions (PG1, PG2 and PT) contain respectively 276, 263, and 288 amino acids.

Purification and conformational organization

The three fusions were expressed in *E. coli* as folded and soluble proteins and purified to homogeneity with high yield (20, 35, and 73 mg protein can be obtained in a highly homogeneous state from 1 liter culture, respectively, for PT, PG2, and PG1). In order to determine whether these fusions were properly folded, they were analyzed by circular dichroism. Fig. 2 shows CD spectra recorded between 190 and 260 nm for each fusion. All spectra are indicative of structured proteins with a positive absorption around 195 nm and negative peaks around 208 and 222 nm.

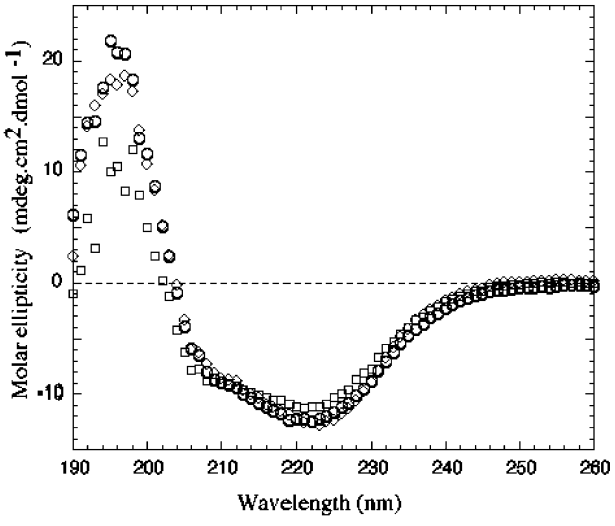

Fig. 2. Far UV circular dichroism spectra of the fusion proteins. PrxGrx1 fusion (open circles); PrxGrx2 fusion (open squares); PrxTrx fusion (open diamond). Spectra were recorded for each species at 5 μM and 25 °C. The negative absorption at 208 and 222 nm are indicative of structured species.

Deconvolution of each spectrum using K2d software is in agreement with the presence of more than a third of helical secondary structures in each protein. Gel filtration chromatography unambiguously indicates that in the absence of reductant, PG2 elutes as a tetramer, whereas PG1 and PT behave as dimers (Fig. 3). This result is very interesting when compared to the only known 3D structure of *H. influenzae* PrxGrx, which is a tetrameric protein either in reducing or oxidizing conditions [13]. Indeed, this indicates that the interactions between the subdomains in PT and PG1 are probably different.

In addition, previous reports on bacterial PrxGrx fusions are somewhat ambiguous. Indeed, the X-ray structure and analytical ultracentrifugation results clearly indicate that *H. influenzae* PrxGrx is a tetramer [13]. On the other hand, mass spectrometry and SDS–PAGE analyses demonstrated that *H. influenzae* PrxGrx exists either as a monomer in the glutathiolated form or as a dimer linked by a disulfide bridge [9,10]. In the same way, the PrxGrx fusions from *N. meningitidis* and *V. cholerae* generally exist as monomers under reducing or non-reducing conditions, but lines of evidences indicated that *V. cholerae* PrxGrx can also form an intramolecular disulfide bridge between the two cysteines of the Prx module [9,11]. In order to analyze the nature of the contacts within our oligomers, we performed SDS–PAGE separation both in reducing or non-reducing conditions (Fig. 4). The three hybrid proteins migrated as monomers under both conditions, indicating that these oligomers are not linked by disulfide bridges but more likely by charge interactions as described for *H. influenzae* PrxGrx [13].

Catalytic properties and overoxidation

The peroxidase activities of the fusions were measured and compared to those of the isolated modules mixed together. Using a spectrophotometric assay coupled to NADPH oxidation, we measured PT activity in the presence of NTR (NADPH thioredoxin reductase). The initial velocity of PT is comparable to the one observed in the presence of the two isolated proteins, but the activity decreases rapidly, presumably because of a higher sensitivity to inactivation (Fig. 5A). Incidentally, these results indicate that NTR is able to recognize and access the Trx module within the fusion and that Trx is able to reduce the Prx module and subsequently to support its H_2O_2 reducing activity. We do not know presently if the electrons are transferred from Trx to Prx within a single fusion subunit, but the presence of a dimeric organisation rather suggests a head-to-tail organisation in which the Prx module could be reduced by a Trx from another subunit. PG2 was only slightly active within the range of concentrations tested, whereas initial velocity for PG1 was faster when compared to the activity obtained when mixing together the individual poplar proteins (Fig. 5B). Nevertheless, PG1 also seemed to be inactivated rather quickly by *t*-BOOH. One possibility to explain the inactivation is the formation of sulfinic or sulfonic acid intermediates. The formation of overoxidized Prx forms was checked by mass spectrometry after reaction with high concentrations (5 mM) of H_2O_2 (Table 2). After H_2O_2 treatment, the molecular mass of Prx II increased by 48 Da, a value which can be interpreted as resulting from the formation of a sulfonic acid. A similar mass increment was also found in PG1 and PT but not in PG2, in which the increase in mass was only about 32 Da (presumably arising from a sulfinic form). Although these overoxidized forms can also be formed on Prx II, it is likely that a lower rate of regeneration of Prx by the Trx or the Grx modules occurs, when engaged in the fusion. This suggests that the Prx sulfenic acid reduction or the subsequent steps are less efficient in the fusions compared to the isolated modules. Indeed, it has already been demonstrated for some enzymes (Prx or

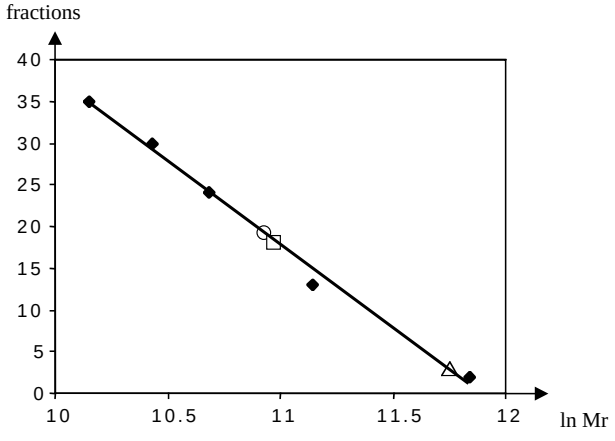

Fig. 3. Gel filtration analysis of the fusions. The molecular sizes of PG1 (open square), PG2 (open triangle), and PT (open circle) were determined using an ACA 44 exclusion size chromatography and proteins of known molecular weight (closed diamonds). Log of the molecular mass is reported as a function of the elution volume.

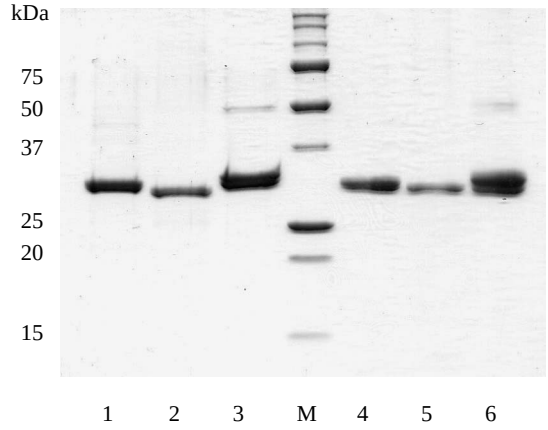

Fig. 4. Reducing or non-reducing SDS–PAGE of the recombinant proteins. 2.5 μg of PrxGrx1 (lanes 1 and 4), PrxGrx2 (lanes 2 and 5), and PrxTrx (lanes 3 and 6) were separated in reducing (10 mM DTT) (lanes 1–3) or non-reducing (lanes 4–6) conditions. M, molecular weight marker is the protein unstained standard marker from Bio-Rad. Band sizes (kDa) are indicated on the left.

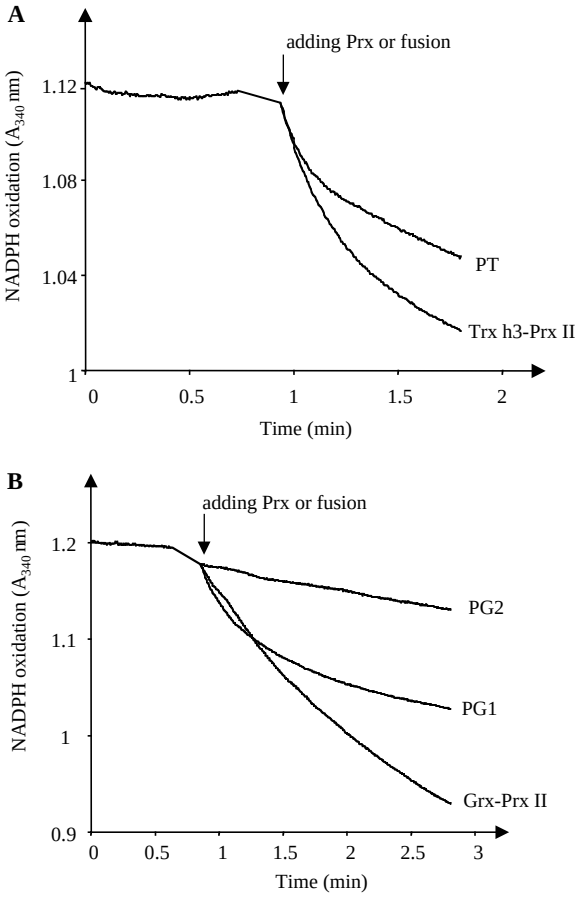

Fig. 5. Time course NADPH dependent reduction of peroxides. (A) Activity of PrxTrx (5 μM), or the isolated modules Trx h3 (5 μM), and Prx II (5 μM) in the presence of the Trx reduction system (180 μM NADPH, 1 μM NTR) and 250 μM H_20_2. (B) Activity of PrxGrx1 (1 μM), PrxGrx2 (1 μM), or the isolated modules GrxC4 (1 μM) and Prx II (1 μM) in the presence of the Grx reduction system (180 μM NADPH, 0.4 U GR, 500 μM GSH) and 500 μM *t*-BOOH.

methionine sulfoxide reductases) which use sulfenic acid chemistry for their catalysis, that the sulfenic acid reduction or rather the subsequent reduction steps by Trx are the rate-determining steps [19,20].

Fig. 6 shows the time-dependent H_2O_2 consumption by the three fusion enzymes using either DTT or GSH as reductants. With DTT as a donor, the reduction is independent of the Trx or Grx module and we thus measure the ability of the Prx module, either isolated or within the fusion, to directly reduce H_2O_2. All the constructions were able to reduce H_2O_2, but PG1 is more active than PG2 and PT which present similar efficiencies. Thus, either the Prx module of PG1 is more accessible to DTT or PG2 and PT are inactivated faster by H_2O_2. This second proposal is supported by the fact the peroxide reduction is not complete and tends to decline and stop with time.

With GSH as a donor, the reduction is dependent on the Grx module, and this test can thus provide valuable information concerning the low catalytic activity of PG2. PT is not able to reduce H_2O_2 using GSH as electron donor. This result was expected as GSH alone cannot support the activity of poplar Prx II and cannot be used by Trx h3 [7, unpublished results]. PG1 is almost as active with DTT or GSH, although the initial velocity is slower with GSH, but with elapsing time, the consumption of H_2O_2 is quite similar. As PG2 is only active using DTT but not GSH, the low efficiency of PG2 is likely to result from an impaired GSH recognition due to the first N-terminal α-helix deletion of Grx since the major difference between PG1 and PG2 is the presence or absence of this α-helix. Thus the low catalytic activity of PG2 observed in Fig. 2 is not due to a distorted Prx active site, which might have prevented the recognition of peroxides.

The catalytic activity of the Grx and Trx modules within the fusions was then investigated by using specific tests. Only poplar Trx h3 and PT were able to reduce insuline with similar efficiency (Fig. 7A), whereas poplar GrxC4, Prx II, PG1, and PG2 were inactive, indicating that the Trx module is fully active within the fusion. Alternatively, the activity of the Grx module can be evaluated using the DHA reduction test. In this case, PG1 is as efficient as Grx C4 alone (Fig. 7B), whereas PG2 is only slightly active at very high concentration (Fig. 7B and data not shown) and Trx h3 or PT were not active at all. These results again suggest that PG2 is unable to bind GSH and support catalysis.

In vitro glutathiolation experiments

In order to further investigate the catalytic mechanism of the Prx II and of the fusions and especially to test if the catalytic peroxidatic cysteine is glutathiolated or if Grx is able to directly attack the sulfenic acid, the glutathiolation state of the various proteins was tested in vitro and detected using mass spectrometry (Table 2). As expected, GrxC4 was able to bind one GSH, whereas Trx h3 does not bind any GSH, either on its two active site cysteines, or on its additional cysteine. All other enzymes (Prx II, PT, PG1, and PG2) were also able to bind GSH, although the reaction was not always complete since some non-modified proteins were still detected. For Prx II and PrxTrx, the results suggest that the catalytic cysteine of the Prx module is able to react with GSSG and thus become modified. For PG1 or PG2, the results seem to be in accordance with those described earlier for the *H. influenzae* PrxGrx [10]. Indeed, Pauwels and colleagues have demonstrated that the PrxGrx was glutathiolated on the peroxidatic cysteine whereas the two active site cysteines of the Grx module formed a disulfide bridge. This can be expected if the mixed-disulfide Prx-SG formed primarily is reduced by the first Grx active site cysteine making the catalytic cysteine available for another glutathiolation, whereas the Grx-SG adduct is reduced by the second cysteine, preventing additional reaction. The in vitro glutathiolation of the Prx module suggests that the sulfenic acid formed during Prx catalysis can be reduced by GSH, this GSH adduct being likely reduced by Grx. Nevertheless, if Trxs are the preferred electron donors in vivo or if Grxs are not

Table 2
Glutathiolation and hyperoxidation experiments

	Theoretical mass	DTT treated protein	GSSG treated protein	GSSG dependent mass increment	H_2O_2 treated protein	H_2O_2 dependent mass increment
PrxGrx1	29962	29762.4	29762		29810.6	48.2
			30066.8	304.2		
		29964.4 (203)	29964.6		30012.4	48
			30270.9	306.3		
PrxGrx2	28607	28408.3	28408.8		28440.9	32.6
			28714.2	305.4		
		28611.3(203)	28610.6		28643.3	32
			28917.2	306.6		
PrxTrx	31086	30886.3	30886.4		30934.5	48.2
			31190.6	304.2		
		31089.3 (203)	31089.6		31137.25	48
			31394.8	305.2		
PrxII	17358	17156.5	17156.4		17204.6	48.1
			17463.3	306.9		
		17358.9 (202.4)	17358.3		17407.2	48.3
			17664	305.7		
GrxC4	12526.1	12395.9 (130.2)	12700.4	304.5	n.d.	n.d.
Trxh3	13345	13142.9 (202.2)	13143	0	n.d.	n.d.

The theoretical mass was calculated from the primary nucleotide sequence. The analysis revealed that the initial N-terminus methionine is cleaved for Grx C4 (mass decrease of 130.22 Da), the methionine and an alanine are cleaved for Trx h3 (mass decrease of 202.22 Da), whereas we have a mixture of two populations for Prx II and thus for the three fusions, one without cleavage and a second with methionine and alanine cleaved (mass decrease around 203 Da). After reduction, around 50 μM proteins were treated with 5 mM GSSG or 5 mM H_2O_2 as described in the method section and analyzed by mass spectrometry. n.d., not determined.

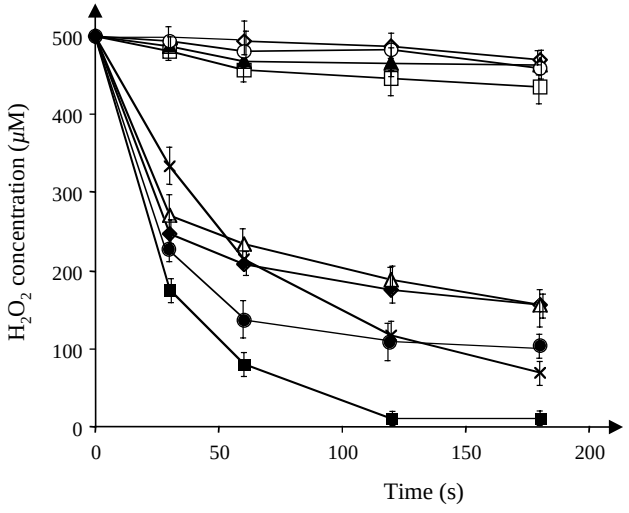

Fig. 6. DTT or GSH dependent reduction of H_2O_2 GSH dependent activity : (□) control, (○) PT, (×) PG1, (▲) PG2 or DTT dependent activity : (◊) control, (■) PG1, (♦) PG2, (●) Prx II, (△) PT. Five micromolar enzyme was used.

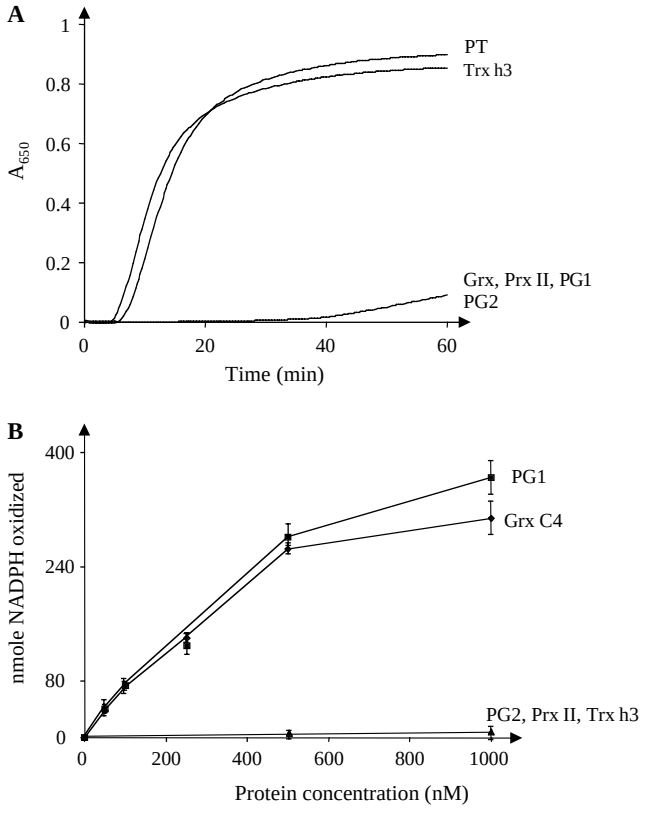

Fig. 7. Specific Trx or Grx activity tests. (A) Insulin reduction was followed at 650 nm in the presence of 500 μM DTT and the various proteins at 5 μM. (B) DHA reduction activity in function of protein concentration was measured by following NADPH oxidation at 340 nm in a coupled reaction system.

expressed in the same subcellular compartment, glutathiolation could be a way to transiently inactivate the protein but also to prevent overoxidation of the reactive cysteine.

Conclusion

These experiments were initiated to study the molecular contacts involved in Prx–Trx or Prx–Grx interactions, but

the prerequisite, for which we present evidence here, was to produce and purify folded and active enzymes. It is remarkable that all three chimeric constructions folded properly and could be purified to homogeneity with high yield. Out of the three chimeric enzymes, only the PT and PG1 constructs yielded enzymes that possessed catalytic efficiencies comparable to the combination of the individual poplar proteins. Our results suggest that in the PG2 construct, the truncation of the N-terminal part of the glutaredoxin module is responsible for the drop in activity of the fusion presumably because GSH recognition and binding is impaired. This observation strongly suggests that the N-terminus extension found in plant glutaredoxins and absent in many other species plays an important role in determining the cognate properties of this protein and is required for efficient catalysis. On the other hand, it is promising that the PrxTrx and PrxGrx1 fusions were correctly folded, active and organized as dimers rather than tetramers, which confirm that the molecular contacts between the partners should be different from those described in the known PrxGrx structure from *H. influenzae* [13]. In this enzyme, Prx–Prx, Prx–Grx, and Grx–Grx interfaces were described but the Prx module of one monomer is certainly reduced by the Grx module of another monomer. One question which thus remains to be elucidated is whether the electrons are transferred from the Trx or Grx modules to the Prx module within a monomer in electron channelling or not. Indeed, the dimeric organization could be suggestive of a head-to-tail orientation, in which the N-terminal Prx domain of one subunit is interacting with the C-terminal Trx or Grx domain of another subunit. Nevertheless, we cannot at this point rule out the possibility that the two N-terminal Prx domains and the two C-terminal domains face, and that the linker peptide is flexible enough to allow the two intra-chain domains to interact directly with one another. In order to solve this ambiguity, we are currently trying to solve the 3D structure of the artificial fusions. Finally, we also had evidence that Prx II can be glutathiolated or overoxidized as was already demonstrated for some eukaryotic Prx [21–24]. This kind of post-translational modification could thus represent a signalling process in plants as suggested in other organisms [25].

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