Hexameric oligomerization of mitochondrial peroxiredoxin PrxIIIF and formation of an ultrahigh affinity complex with its electron donor thioredoxin Trx-o

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Abstract

Mitochondria from plants, yeast, and animals each contain at least one peroxiredoxin (Prx) that is involved in peroxide detoxification and redox signalling. The supramolecular dynamics of atypical type II Prx targeted to the mitochondrion was addressed in pea. Microcalorimetric (ITC) titrations identified an extremely high-affinity binding between the mitochondrial PsPrxIIIF and Trx-o with a $K_D$ of $126\pm14$ pM. Binding was driven by a favourable enthalpy change ($\Delta H = -60.6$ kcal mol$^{-1}$) which was counterbalanced by unfavourable entropy changes ($\Delta S = -47.1$ kcal mol$^{-1}$). This is consistent with the occurrence of large conformational changes during binding which was abolished upon site-directed mutagenesis of the catalytic C59S and C84S. The redox-dependent interaction was confirmed by gel filtration of mitochondrial extracts and co-immunoprecipitation from extracts. The heterocomplex of PsPrxIIIF and Trx-o reduced peroxide substrates more efficiently than free PsPrxIIIF suggesting that Trx-o serves as an efficient and specific electron donor to PsPrxIIIF in vivo. Other Trx-s tested by ITC analysis failed to interact with PsPrxIIIF indicating a specific recognition of PsPrxIIIF by Trx-o. PsPrxIIIF exists primarily as a dimer or a hexamer depending on the redox state. In addition to the well-characterized oligomerization of classical 2-Cys Prx the results also show that atypical Prx undergo large structural reorganization with implications for protein–protein interaction and function.

Key words: Binding, mitochondria, peroxiredoxin, plant, redox, thioredoxin.

Introduction

Peroxiredoxins (Prx) catalyse the reduction of hydrogen peroxide, alkylhydroperoxides, and peroxinitrite. They play an important role in antioxidant defence and signalling (Baier and Dietz, 1999; Rabilloud et al., 2002), and affect cell processes such as proliferation (Prospéri et al., 1993), differentiation (Rabilloud et al., 1995), apoptosis (Zhou et al., 2005), and photosynthesis (Lamkemeyer et al., 2006). In eukaryotes, peroxiredoxins localize to diverse subcellular compartments such as the nucleus, mitochondrion, cytosol, and plastid. Depending on the structure and location of the catalytic cysteinyl residues, plant Prx are grouped into four subfamilies; 2-Cys Prx, 1-Cys Prx, type II Prx, and Prx Q (Horling et al., 2003). The latter two occasionally are also classified as atypical 2-Cys Prx.
The plant mitochondrial PrxIIIF, as well as the human Prdx5, belong to the type II Prx subfamily. The Arabidopsis PrxIIIF is implicated in tolerance to stressors such as Cd, salt, and cold (Finkemeier et al., 2005; Barranco-Medina et al., 2007; Gama et al., 2007) while the human Prdx5 protects mitochondrial mtDNA from oxidative damage induced, for example, by exogenous addition of H$_2$O$_2$ (Bannmeyer et al., 2005). AtPrxIIIF is reduced by glutathione, mitochondrial Trx-o, and an atypical glutaredoxin (GRX) (Finkemeier et al., 2005). None of other tested Trx, i.e. cytosolic Trx-h1, -h3, and the mitochondrial Trx-h2, was effective in reducing poplar PrxIIIF and the authors proposed Trx-o as putative electron donor for PrxIIIF (Gama et al., 2007). As in the case of AtPrxIIIF, GSH and, more efficiently, GSH in combination with cytosolic GrxC4, regenerated oxidized PrxIIIF of poplar (Gama et al., 2007). The interaction of Trx with Prx is considered to be reversible. Covalent trapping of Prx as Trx- and Grx-partners was achieved using site-directed mutagenized mono-cysteinic Trx- and Grx variants lacking the resolving Cys (Motohasi et al., 2001; Balmer et al., 2004; Rouhier et al., 2005). However, the heterocomplexes generated under these conditions are artificial and do not necessarily reflect donor–acceptor relationships in vivo.

Prx oligomerization has been described for At2-Cys Prx which forms tightly associated dimers or decamers depending on the redox state similar to other 2-Cys Prx that form octamers, decamers, or dodecamers (Wood et al., 2003). For example, Mycobacterium tuberculosis AhpC Prx, a 2-Cys Prx, forms an oligomer consisting of six dimers (Guimaraes et al., 2005). 2-Cys Prx transition from dimer to oligomer is fostered by overoxidation of the catalytic cysteiny1 residue and causes a functional change. It was suggested that the oligomeric 2-Cys Prx acts as a chaperone, protecting cell structures from oxidative damage (Moon et al., 2005). Oligomeric states of type II Prx have not often been reported or analysed in detail. Recent crystallization of poplar cysteic type II Prx revealed the presence of a non-covalent homodimer as the unit structure (Echalar et al., 2005). With only 33% sequence similarity the mitochondrial PrxIIIF is distantly related to cysteic Prx II. The PsPrxIIIF has been crystallized (Barranco-Medina et al., 2006) and its diffraction pattern is in the process of deconvolution. An extensive biochemical description of this enzyme has been published recently (Barranco-Medina et al., 2007), including first indications for the existence of dimers and hexamers. The hexamer formation was disabled by mutation of the peroxidatic cysteine, whereas the resolving cysteine did not seem to be directly implicated in the oligomerization.

Despite the recent progress our principal understanding of PrxIIIF function is still inadequate. Here, the structural and functional dynamics and the nature of the electron donor were addressed in a multidisciplinary approach using micror calorimetric, immunological, and biochemical techniques. The interaction proved to be extraordinarily strong suggesting permanent complex formation, and facilitated the PsPrxIIIF reduction by thioredoxin. Another structural peculiarity of PsPrxIIIF is its ability to form hexamers. The results add new features on structural and functional flexibility to a particular member of the peroxiredoxin family.

**Materials and methods**

**Plant material and isolation of mitochondria**

Pea seeds were germinated in moist vermiculite and the plants grown for 14–21 d in a growth chamber. Mitochondria were isolated from pea tissues according to Finkemeier et al. (2005). After sonication, matrix and membrane were separated by centrifugation at 100 000 g.

**RNA isolation and cDNA synthesis**

Total RNA was isolated from 2 g of young pea leaves using the RNA isolation kit (Roche, Mannheim, Germany). cDNA was synthesized from total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA) and an oligo dT$_{20}$ primer.

**Isolation of a cDNA encoding Trx-o from Pisum sativum**

The cDNA encoding Trx-o was isolated by RT-PCR using two primers designed from the heterologous Arabidopsis thaliana Trx-o (AF396650 and At2g35010), i.e. primer TrxA, 5'-TGGTGTGGGACCTTGAGG-3' (encoding WCGPCR) and reverse primer TrxB, 5'-AAGAAAATGCAATGTTGGCACA-3' (encoding AVPT-LHFF). The 3’ end was obtained with TrxC, 5'-TCTGTGCCCAACAC-TGCAATTTCTT-3’, and an oligo dT reverse primer. The 5’ end was cloned by 5’ RACE using two PCR reactions: (i) primer TrxD (5’-AAACTGCAACCTGCTCAATG-3’) and an oligo dT with a sequence in the 5’ end (5’-CCCCATCAAAGTCC-CCATAGA CACTC-3’); (ii) a reverse primer complementary to the sequence and a homologous primer TrxE (5’-CCTGCAA-GTTCACACCA-3’). The PCR were performed at 60 °C, the products were gel purified, cloned in pGEM-T (Promega, Madison, USA), and sequenced.

**Cloning, expression, and purification of recombinant Trx-o**

The mature Trx-o encoding DNA sequence was amplified by RT-PCR using cDNA from Pisum sativum with a forward primer 1-Trx-F (5'-CAC/CATGGGCTTAT-CCTGTAAATTCTGC-3’) and a reverse primer 2-Trx-R (5’-CCGGATCTGACAGATCCCATGAGAAGGTTC-3’) (45 cycles of 30 s at 94 °C, 30 s at 65 °C, and 60 s at 72 °C). Each primer contained a recognition site for EcoRI or BamHI (underlined), used to digest and ligate the product into the pET-3d plasmid. The sequence of the pET-Trx-o construct was verified by sequencing.

Escherichia coli strain BL21 (DE3) was transformed with pET-Trx-o. Expression was induced with 0.4 mM IPTG at 37 °C for 6 h. Cells were harvested by centrifugation and stored at −70 °C until use. Frozen cells were suspended in 20 mM TRIS-HCl/pH 8 and disrupted with a French press.

Recombinant Trx-o was solubilized from the bacterial lysate by heating at 80 °C for 10 min. After centrifugation, the supernatant was fractionated with (NH$_4$)$_2$SO$_4$ (40–85% saturation) and the...
pellet dissolved in 30 mM TRIS-Cl pH 7.9, 300 mM NaCl and subjected to size exclusion chromatography on Sephacryl S-200 using a FPLC system (GE Healthcare, UK).

**Isolation of a cDNA encoding a type II Prx from *P. sativum***

The cDNA encoding a type II Prx was isolated by RT-PCR. Two heterologous primers were designed from the primary structure of type II Prx enzymes in higher eukaryotes: primer PrxIIA, 5′-CATGGCTCCAAATTGCTTGGC-3′, that encodes the peptide sequence MAPAVG and primer PrxIIB, 5′-CTTAAAGACGCTTGAGGAT-TC-3′, used as the reverse primer that encodes the peptide sequence DILKAL.

The reaction mixture for PCR contained in a final volume of 25 μl: 0.4 μM primers, 0.4 mM dNTPs, 2 mM MgCl₂, and 1U Eco Taq Plus (Ecogen, Barcelona, Spain). The PCR was performed using the cDNA from pea leaves obtained from RNA by reverse transcription.

**Expression and purification of recombinant cytosolic type II Prx and generation of antibodies**

A cDNA fragment that encoded type II Prx was obtained by RT-PCR. Two primers were designed: a primer (5′-CACGGCTCCAAATTGCTTGGC-3′) with an NcoI restriction site (underlined) indicates the position of cleavage) and a reverse primer (5′-CGG/GATCCTTAAAGACGCTTGAGGAT-TC-3′) with a BamHI restriction site (underlined). PCR products were purified and cloned in the pET3d expression vector. The resulting construct was used to transform BL21 *E. coli* (DE3). Transformed cells were cultured at 37 °C for 6 h with 0.1 mM IPTG. Cells were harvested by centrifugation and stored at −70 °C until use. Frozen cells were resuspended in 20 mM TRIS-Cl (pH 8.0) and disrupted with a French press.

Recombinant type II Prx was purified by ammonium sulphate fractionation (40−95% saturation) and two sequential chromatographic steps on FPLC (Amershams Bioscience, Uppsala, Sweden): Sephacryl S-200 gel filtration and Mono Q HR 5/5 ion exchange. Elution of type II Prx was followed by a DTT-dependent peroxidase assay and SDS/PAGE analysis.

The polyclonal antibody against mature type II Prx was raised in rabbits by five consecutive subcutaneous injections of 250 μg pure recombinant protein each with at least 2 week intervals. The first injection contained complete Freund’s adjuvant and subsequent injections contained incomplete Freund’s adjuvant. The serum was taken 2 weeks after the last injection.

**Mutagenesis and purification of variants**

The Cys59Ser and Cys84Ser variants were synthesized and purified as described by Barranco-Medina et al. (2007).

**Expression and purification of mitochondrial NTRA2**

Mitochondrial NTRA2 from *A. thaliana* was expressed in BL-21 (DE3) containing the plasmid pSBET (Reichheld et al., 2007). Induction was carried out with 1 mM IPTG for 5 h. Cells were disrupted with French press and protein was purified from supernatant with Ni²⁺ NTA Sepharose column.

**Protein characterization**

The recombinant PsPrxII (AJ717306) and the variants lacking His-tag and transit peptide were expressed in *E. coli* (Barranco-Medina et al., 2006). Peroxidase activity of recombinant proteins was measured as described by Thurman et al. (1972). For the study of oligomerization, the Superdex-200 HR 10/30 column was calibrated with standard proteins (Bernier-Villamor et al., 2004). The protein concentration of the loaded sample ranged between 100 μM and 200 μM. If required, PsPrxII protein was maintained in the reduced state by the addition of dithiothreitol at concentrations between 1 mM and 10 mM. Denaturing SDS-PAGE was performed with a 6% (w/v) stacking and 15% (w/v) resolving polyacrylamide gel. Proteins were electrotransferred onto a nitrocellulose membrane. Immunoreaction was carried out by using rabbit serum against mature PsPrxII or Trx-o diluted 1:7000 or 1:2000, respectively, in TBS containing 1% skimmed milk. Antibodies against PsPrxII were obtained as described by Barranco-Medina et al. (2007), while antibodies against the Trx-o carboxyterminal peptide (ARLNHTEIKLFK) were generated by SIGMA-ALDRICH. The detection on the membrane was performed using a chemiluminescence method (Perkin-Elmer, Boston, USA) following the supplier’s manual.

**Co-immunoprecipitation**

Isolated pea mitochondria were treated with H₂O₂ (20 mM) or DTT (1 mM) at room temperature for 2 h. Prior to immunoprecipitation the mitochondria were lysed and iodoacetamide was added at 100 mM final concentration to block all free SH groups. 2.5 μl of anti-Trx-o antibody were added to 200 μg of protein in TRIS buffered saline (TBS) and incubated for 2 h on a shaker. Afterwards, 75 μl protein A Sepharose was added and the mixture incubated under shaking for 15 min. The incubation mix was loaded on a sucrose/water (40%) solution and spun at 10 000 g for 2 min. Pellets were washed twice with a solution containing 50 mM TRIS-Cl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and 0.1% SDS and once with 125 mM TRIS-Cl pH 6.8. Sample proteins were separated on a 16% SDS-PAGE and detected by western blot with antibodies against PsPrxII in the first and anti IgG (peroxidase-conjugated) in the second one. Bound antibodies were visualized by chemiluminescence.

**Isothermal titration calorimetry (ITC)**

ITC measurements were performed on a VP-Microcalorimeter (MicroCal, Northampton, MA, USA) at 25 °C. PsPrxIIF, C59S, C84S, Trx-x, and Trx-y were dialysed against 25 mM TRIS-Cl (pH 7.5) supplemented with 0.1 mM DTT. To study binding after oxidation, PsPrxIIF and PsTrx-o were oxidized with 20 mM H₂O₂ at room temperature for 2 h and dialysed against 25 mM TRIS-Cl (pH 7.5). Protein concentrations were determined using the Bradford assay by reading the absorbance at 280 nm (Nano-Drop). Each titration involved 1.6 μl injections of PsPrxIIF, as well as its C59S or C84S variants (each at 65 μM) into 1.4 ml of 2 mM Trx-o (reduced or oxidized), Trx-x or Trx-y. The mean enthalpies measured from injection of Prx into the buffer were subtracted from raw titration data prior to data analysis with the ORIGIN software. Titration curves were fitted by a non-linear least squares method to a function for the binding of a ligand to a macromolecule (Wiseman et al., 1989). From the curve fit, the parameters ΔH (reaction enthalpy) and Kₐ (binding constant, Kₐ = 1/Kᵢ) were determined. From the values of Kₐ and ΔH, the changes in free energy (ΔG) and entropy (ΔS) were calculated with the equation: ΔG = −RT lnKₐ = ΔH − TΔS, where R is the universal molar gas constant, and T the absolute temperature.

**Determination of midpoint redox potential**

100 μg recombinant protein was incubated in MOPS buffer (100 mM) containing 2 mM total DTT at varying ratios of the reduced to the oxidized form in a volume of 500 μl. After 3 h at room temperature, monobromobimane was added at a final concentration of 10 mM. The samples were prepared for fluorescence analysis as described by Hirasawa et al. (1999).
Results

Oligomerization state of recombinant PsPrxIIF

Purified recombinant PsPrxIIF eluted in two peaks after size exclusion chromatography (Fig. 1A). The first peak of 119 kDa (Stokes radius of 41.8 Å) tentatively corresponds to a hexamer as observed in the crystal structure (Barranco-Medina et al., 2006) while the second peak with a molecular mass of 39 kDa (Stokes radius of 28.1 Å) represented the dimer. Site-directed mutated variant C84S lacking the resolving Cys also formed hexamers and dimers while C59S formed dimers only (data not shown). Apparently, the presence of the catalytic Cys59 affects the dimer interface needed for hexamer formation. The hexamer converted to the 20 kDa monomer in reducing denaturing PAGE (Fig. 1B,1), but two bands each at 38/40 kDa and 19/20 kDa, respectively, appeared under oxidizing conditions. The results indicate that the two dimeric forms correspond to two monomers linked by intermolecular disulphide bonds since the addition of DTT abolished the doublet band at 38/40 kDa. The hexameric C84S variant (Fig.1B,3) without DTT showed different electrophoretic mobility in SDS-PAGE. Only a single band each appeared at 38 kDa and 20 kDa. The band at 38 kDa corresponded to the lower mobility band (Fig. 1B,1). The dimer of C59S (Fig. 1B,2) without DTT showed two bands, one at 38 kDa that corresponded to the faster mobility of the wild type and another one at 20 kDa. Since both mutants have only one Cys there only exists the possibility to form intermolecular disulphide bonds. Based on the identity of the dimeric bands present in PrxIIF and its variants C59S and C84S, it is concluded that the dimeric PrxIIF forms of fast and low mobility correspond to intermolecular disulphide bonds between Cys84-Cys84 and Cys59-Cys59, respectively. This issue implies the coexistence of two different intermolecular disulphide bonds in the hexamer of wild type PrxIIF. The assignment of the lower and upper bands at 38/40 kDa was confirmed in several electrophoretic separations. The low mass bands (Fig. 1B,1) possibly represent the monomers with and without an intramolecular disulphide bond between Cys 59 and Cys 84. Due to a more compact structure, oxidized polypeptides with intramolecular disulphide bridges often show increased electrophoretic mobility compared to their dithiol form (Chae et al., 1994; Finkemeier et al., 2005; Ströhler and Dietz, 2006). The band intensities of the monomers were low compared to the dimers. As expected, only one monomer appeared in non-reducing SDS-PAGE in the case of C59S and C84S (Fig. 1B,2, 3). In addition to the monomers and dimers, oligomers with lower band intensity were detected at about 60 and 100 kDa as revealed by silver staining and western blot analysis with antibody directed against recombinant PsPrxIIF (data not shown).

The proportion of the hexameric fraction increased in H₂O₂-oxidized PsPrxIIF where only a residual dimeric fraction was detected (Fig. 2A). The hexamers dissociated to dimers upon reduction, as seen by a chromatographic re-run on Superdex-200 (not shown). Variation of the ionic strength by addition of NaCl at concentrations between 0.15 M and 2 M and altered pH between 6.5 and 8.0 (adjusted with phosphate and TRIS buffers) did not affect the oligomerization state. However, at pH 9.9 in carbonate buffer, the dimeric form increased and the hexamer disappeared (not shown). PsPrxIIF oligomers were also observed in electropherograms of pea mitochondria (Fig. 2B). Four bands of molecular masses similar to those obtained with purified PsPrxIIF were detected by western blotting in extracts from leaf mitochondria, while only two appeared in root mitochondria. The pattern was unchanged under reducing conditions. All aggregates dissociated to monomers and separated as a single band in the presence of 6 M urea only (Fig. 2B). Despite the similarity of the pattern in recombinant and native leaf mitochondrial solutions.

Fig. 1. Oligomerization of recombinant PsPrxIIF. (A) Elution profile of wild-type PsPrxIIF after size exclusion chromatography through Superdex S-200. The column was equilibrated with 50 mM TRIS-Cl (pH 7.5) without DTT containing 150 mM NaCl at a flow of 0.5 ml min⁻¹. 100 µl of purified recombinant proteins at 100 µM concentration were diluted 30 min before the run. (B) Electrophoretic mobility of wild type, as well as C59S and C84S variant proteins. SDS-PAGE plus/minus DTT of the collected PsPrxIIF hexameric (H) or dimeric (D) peaks from (B1) PsPrxIIF wild-type protein, (B2) C59S and (B3) C84S visualized by silver staining.
PsPrxII, the binding strength in oligomers of native PsPrxII seemed stronger than in recombinant PsPrxII since only strong denaturing agents such as urea dissociated them.

The mitochondrial Trx-o

Following catalytic turnover, oxidized Prx needs to be regenerated by electron donors such as mitochondrial Trx-o (Laloi et al., 2001). Trx-o was cloned as cDNA from *Pisum sativum* and comprised a 546 bp open reading frame (AM235208). The deduced protein of 181 amino acids has a molecular mass of 20.064 Da and a theoretical pI of 9.58. The N-terminal 70 amino acids encode a mitochondrial transit peptide (MITOPROT www.expasy.org) predicted to be cleaved upon import. The processed protein has a molecular mass of 12 613.5 Da and a pI of 6.3. Typical cysteine residues are found at positions 33 and 36 of the mature protein as part of a conserved motif. Similar to other Trxs, Trx-o has a highly conserved active site WCGPC. Glycine and proline are positioned between the two cysteine residues and contribute to keep the conformation of the active site and to realize the negative reduction potential of Trx. Trp 32 of the mature protein is exposed to the bulk phase and influences the conformation of the active centre. Pro and Thr at positions 78 and 79 directly interact with the catalytic site. The roles of Pro 41, Gly 86, and Gly 94 are structurally important since they produce a turn of the α2 helix stabilizing the active site located at the beginning of this helix. Figure 3 shows the amino acid sequence alignment of PsTrx-o with Trx-o1 (AAC12840) and Trx-o2 (AF396650) from *A. thaliana*. Identity between PsTrx-o and AtTrx-o1 and AtTrx-o2 is 58% and 50%, respectively, while both enzymes of *A. thaliana* share 65% identity.

A cytosolic type II Prx from pea and analysis of its amino acid sequence

In order to test the specificity of PrxII-antibodies recognition, the recombinant cytosolic type II Prx from pea was expressed, purified, and the protein used to produce polyclonal antibodies. The cloning of a cDNA sequence (identification number FM162015) encoding a pea type II Prx is reported for the first time. The primary structure of the known type II Prx was used to design oligonucleotides to obtain a cDNA fragment encoding a type II Prx. The cDNA sequence comprises 489 bp and its translated protein shows more than 50% similarity to known type II Prx enzymes. The open reading frame encodes 162 amino acids with a molecular mass of 17 346 Da and a theoretical pI of 5.57. The cytosolic localizations of the isolated pea Prx was predicted using PSORT and TargetP, the latter calculating a probability of 0.87 of a cytosolic protein. The protein presented two conserved cysteine residues in positions 51 and 76 and showed the highly conserved peptide regions around the conserved cysteine residues.
cysteine residues: VPGAFTPTCS and CISVNDPFV. The antibody directed against Ps-PrxIIF did not react with the cytosolic Ps-PrxII (data not shown).

**PsPrxIIF/Trx-o interaction in vitro and in situ**

Gel filtration analysis revealed a stable interaction between wild-type PsPrxIIF and Trx-o. The heterocomplex eluted at 13.5 ml (Fig. 4A). The mutant variants C59S and C84S and some wild-type PsPrxIIF eluted as dimers at 15.3 ml. Western blot analysis confirmed the presence of both proteins, i.e. Trx-o and PsPrxIIF, in the eluate at 13.5 ml (Fig. 4B). No stable interaction was detected between Trx-o and the C59S and C84S PrxIIF variants, respectively.

The PsPrxIIF/Trx-o interaction was detected in mitochondrial matrix extracts fractionated by size exclusion chromatography in the presence (Fig. 5A) or absence (Fig. 5B) of DTT. An ELISA with reduced protein fractions confirmed that the two peaks at 13.5 and 15.3 ml contained PsPrxIIF protein and, likewise, the fractions at 13.5 and 18.8 ml the Trx-o protein (Fig. 5A). Thus the fraction collected at 13.5 ml contained the heterocomplex PrxIIF-Trx-o of 92.7 kDa. Under non-reducing conditions, the two additional peaks at 12.9 ml and 16.6 ml corresponded to hexameric PrxIIF and dimeric Trx-o, respectively (Fig. 5B). Western blot analysis confirmed the presence of PrxIIF and Trx-o in the fractions (Fig. 5C).

To study the redox-dependent dynamics of the heterocomplex, mitochondria were isolated and treated with DTT or H$_2$O$_2$ at room temperature for 2 h and subjected to a co-immunoprecipitation with antibodies against Trx-o (Fig. 6) as described in the Materials and methods. The precipitate was washed and, by western blotting, analysed for the presence of PrxIIF. The band with the size of PsPrxIIF in the immunoblot analysis with antibodies against PsPrxIIF proved successful, supporting the assumption of the existence of the heterocomplex in mitochondria (Fig. 6). A significant difference in band intensities was not observed between oxidizing and reducing treatments.

The presence of cytosolic type II Prx in the mitochondrion was excluded, based on the absence of a signal in

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Fig. 4. Heterocomplex formation between wild type PsPrxIIF, C59S- or C84S-variants with Trx-o. (A) PsPrxII or variants were incubated with Trx-o in the presence of 100 μM DTT for 12 h and separated through Superdex 200 HR. (B) Aliquots of the peaks from gel filtration were probed with antibodies against Trx-o or PrxIIF in western blots as indicated.

Fig. 5. Chromatography of pea mitochondrial matrix. Isolated pea mitochondria were lysed at pH 8.0 and separated into matrix and membrane by centrifugation. The supernatant was fractionated on Superdex 200 HR equilibrated with 25 mM TRIS-Cl pH 8.0, 150 mM NaCl in presence (A) or absence (B) of 0.1 mM DTT. Fractions of 200 µl were collected and analysed by ELISA with antibodies against PsPrxIIF (—) and Trx-o (—). (C) Western blot with Trx-o and PsPrxIIF antibodies, respectively, of the peaks identified through ELISA.
western blots with antibodies against cytosolic Prx type II confirming the purity of the mitochondrion preparations. Furthermore, isothermal titration calorimetry binding assays between PrxIIF/anti cytosolic antibodies and cytosolic-type II Prx/anti-PrxIIF-antibodies reported no interaction and only heat dilution was observed (data not shown).

Quantitative analysis of the PsPrxIIF/Trx-o interaction in vitro

The thermodynamics of the interaction between PsPrxIIF and Trx-o was studied by isothermal titration microcalorimetry (ITC) through titrating PsPrxIIF with Trx-o (Fig. 7). Strong binding was driven by extremely favourable enthalpy changes ($\Delta H = -60.6\pm0.1$ kcal mol$^{-1}$) and consequently counterbalanced by unfavourable entropy changes ($\Delta S = -47.1\pm0.1$ kcal mol$^{-1}$). Apparently the binding of both proteins was ultratight and near the detection limit of any equilibrium-based method. However, two points on the fast rising part of the titration curve permit the calculation of the binding constant which was found to be $7.9\pm0.9\times10^9$ M$^{-1}$. This corresponds to a dissociation constant of $126\pm14$ pM and a $\Delta G$ of $-13.5\pm0.1$ kcal mol$^{-1}$. Binding of C84S and C59S to Trx-o was not observed (Fig. 8A), suggesting that the Cys mutagenesis produced conformational changes which drastically modify the interaction with Trx-o.

In addition, ITC experiments were carried out to determine the specificity of thioredoxin/PrxIIF binding. Chloroplasts Trx-x and Trx-y (Fig 8B,2, 3) under reducing conditions, as well as oxidized Trx-o, failed to interact with reduced and oxidized PsPrxIIF, respectively, in ITC binding assays (Fig. 8B,1).

Peroxidase activity of PrxIIF as well as the PrxIIF-Trx-o heterocomplex

The biochemical and functional implications of the different assemblies and the heterocomplex between PrxIIF and Trx-o were addressed in an H$_2$O$_2$ reduction assay with DTT as the electron donor. The PrxIIF/Trx-o heterocomplex displayed the highest capacity to reduce H$_2$O$_2$, followed by the dimeric state or hexameric PrxIIF whereas PrxIIF had an intermediate antioxidant capacity between the dimeric and hexameric form (Fig. 9A,1). Furthermore, the peroxidase activity representation clearly shows these differences between the assemblies at an equal enzyme concentration of 0.2 M (Fig. 9A,2). The peroxidase activity of PrxIIF was also assessed in a test with all the members of the electron transport chain, i.e. NADPH, thioredoxin reductase of wheat or A. thaliana (NTR A2), Trx-o2, and PrxIIF. The complete system failed to reduce hydrogen peroxide, perhaps due to the use of thioredoxin reductase of other origin in the assay or because Trx-o, which is trapped in a large complex with PrxIIF, cannot be reduced by the NTR active site which is buried in a groove. The midpoint redox potential ($E_m$)
determined by fluorimetry was lower for PsTrx-o (–321 mV) (Fig. 9B), than for PsPrxIIF and the C84S variant that showed values of –309 and –306 mV, respectively (data not shown). These values are in good agreement with other organellar type II Prx from Arabidopsis (Horling et al., 2003). It was not possible to determine the $E_m$ of the C59S variant. The difference in redox potential should ease electron transfer from Trx-o to PsPrxIIF or C84S.

**Discussion**

This report describes features of PrxIIF that add a new dimension to our understanding of the versatility of peroxiredoxins in general. It is shown that PsPrxIIF functions as a low activity peroxidase but a versatile and high affinity protein–protein interactor.

The binding between PrxIIF and Trx-o was demonstrated with recombinant proteins and in extracts from isolated mitochondria by three different methods, ITC, molecular filtration, and co-immunoprecipitation. The thermodynamic characteristics of the interaction differed significantly from normal values measured in protein–protein interaction. The first special feature is the interaction affinity ($K_D=126\pm14$ pM) which is amongst the tightest protein–protein interactions reported so far (Stites, 1997). The affinity is about 50 000 times higher than the interaction in other electron transfer systems such as the binding of cytochrome c oxidase to ferri-cytochrome c (Kresheck et al., 1995; Lesch et al., 2000). The second unusual feature of this interaction is the mode of binding characterized by extremely favourable enthalpy changes ($\Delta H = -60.6\pm0.1$ kcal mol$^{-1}$) which are counterbalanced by very unfavourable entropic contribution ($T\Delta S = -47.1\pm0.1$ kcal mol$^{-1}$). The enthalpy change is $\sim$7 times larger than for other protein–protein interactions ($-8.6\pm13.6$ kcal mol$^{-1}$). Enthalpy changes are attributed to the extent of ligand interaction. Extreme enthalpy
changes are unlikely to be caused by bond formation, rather they may be linked to large conformational changes of the interacting proteins as, for example, documented for the binding of the CDA receptor and antibodies to the HIV surface protein gp120 (Myszka et al., 2000; Kwong et al., 2002). Therefore, binding of PsPrxIIF and Trx-o may be accompanied by significant conformational changes. The ultratight interaction may stimulate the in vivo PsPrxIIF activity through Trx-o, which is in agreement with the greater activity observed for PsPrxIIF-Trx-o heterocomplex in the peroxidase assay. The results imply a role of Trx-o as the physiological electron donor to PsPrxIIF. In addition, the redox potential hierarchy as determined for the recombinant proteins will ease the electron transfer from PsTrx-o with the more negative $E_m$ ($\text{-321}$) to PsPrxIIF ($\text{-309}$).

Binding between PrxIIF and Trx-o depended on the presence of both catalytic Cys residues and on the redox state. Altered intrinsic Trp fluorescence of the Cys-free variants (not shown) indicates changes in the environment of the tryptophan residues during conformational rearrangement or disrupted Trp-Cys interaction in mutant forms. To test whether the Trp residues of PrxIIF are in the vicinity of C59 and C84, a three-dimensional model of the protein was constructed using program Geno3D (not shown) (Combet et al., 2002) with poplar PrxII as the template (Echalier et al., 2005) which shares 33% sequence identity with PsPrxIIF. In this structural model the cysteine residues were distant to both tryptophan residues by more than 5 Å excluding direct interaction. Thus, global conformational changes in both mutant proteins appear to inhibit the interaction with Trx-o. Oxidized PsPrxIIF and Trx-o did not interact in ITC assay (Fig. 8B,1) underlining the significant importance of the redox state to allow for the in vitro heterocomplex formation. The results differ from those obtained in vivo by means of gel filtration (Fig. 5) and co-immunoprecipitation (Fig. 6) where the heterocomplex was present in both reduced and oxidized mitochondria. The result from the immunoeassay is a valuable confirmation of the in vivo interaction between PsPrxIIF and PsTrx-o. In addition, the binding of PsPrxIIF seems to be very specific for PsTrx-o since other plastid Trx proteins tested by ITC were unable to interact in a similar manner (Fig. 8B,1, 2).

The high affinity characteristics of the PsPrxIIF and Trx-o interaction as determined by ITC agrees with the stability of the complex during gel filtration of the recombinant proteins or the mitochondrial extract. In vivo formation of heterocomplexes is assumed because PsPrxIIF and Trx-o are unlikely to interact after dilution into the large volume of extraction buffer. The molecular mass of the heterocomplex estimated from gel filtration (92.7 kDa) is higher than the theoretical value for a complex of a PsPrxIIF dimer with two Trx-o (about 60 kDa). Thus, either the subunit stoichiometry may be different from 2 + 2 or the complex does not behave like an ideal globular protein. For example, the decamer of 2-Cys Prx with 220 kDa has an atypical behaviour in gel filtration: although its Stoke’s radius was similar to that derived from its structure, a molecular mass of 338 kDa was estimated by gel filtration (Bernier-Villamor et al., 2004). PsPrxIIF was strongly retained in a phenylsuperose column (data not shown) and eluted only with 6 M urea, showing very high hydrophobicity. The additional bands in the leaf sample are not understood but could indicate additional binding partners in mitochondria from photosynthetic cells. The exclusive detection of the monomeric band after urea treatment of leaves/roots proteins excludes the possibility of unspecific recognition by the anti PrxIIF antibodies. Strikingly, the addition of DTI did not change the electropherogram underlining the binding stability between PrxIIF and its targets in the cell. Possibly, in addition to Trx-o, PrxIIF interacts in vivo with other electron donors such as mitochondrial glutaredoxin (Finkemeier et al., 2005).

Prx oligomerization has mostly been studied with 2-Cys Prx (König et al., 2002; Wood et al., 2003; Bernier-Villamor et al., 2004). For the cytosolic type II Prx of poplar the structural analysis revealed dimers (Echalier et al., 2005) while the aggregation state of mitochondrial PrxII is almost unexplored (Barranco-Medina et al., 2007). Since tag-free recombinant proteins were used in this work, tag extension could not affect the relative abundance of dimers and oligomers (Cao et al., 2007). This study reports the formation of PsPrxIIF dimers and hexamers in vitro and also in a rapidly diluted extract suggesting their existence in vivo. Dimers are formed in the reduced state and hexamers are favoured after oxidation. It is suggested that three dimers linked by intermolecular disulphide bonds form the hexamer (Fig. 1). Intermolecular disulphide bridge formation in type II Prx (i.e. atypical 2-Cys Prx) has been observed before, at least in subfractions of oxidized At-PrxII and At-PrxIIB (Bréhélin et al., 2003), however, in that study the intramolecular disulphide bond was predominant. Bréhélin et al. (2003) proposed that this particular dimer might have a regulatory role. MALDI TOF analysis showed the presence of disulphide bridges as an exclusively post-translational modification even after treatment with 20 mM H$_2$O$_2$. In addition, intermolecularly linked dimers were detected after slow oxidation under non-reducing conditions without any peroxide substrate. The fact that both Cys are exposed to the solvent, as observed in the titration of the SH groups (Barranco-Medina et al., 2007), may be favourable for the formation of covalently linked dimers that, in turn, assemble to hexamers. Also C59 is essential for hexamer formation since C59S only existed as the dimer. This property underlines again the structural role of Cys59 beside its implication in antioxidant activity. This is similar to the dimer/tetramer-transitions of...
triosephosphate isomerase in *Giardia lamblia* (GITIM) which also depends on the establishment of a disulphide bridge between two dimers while an intramolecular disulphide bond abolishes catalysis (Reyes-Vivas et al., 2007). Removal of the critical Cys in GITIM by site-directed mutagenesis abolishes the ability for tetramer assembly. Reducing conditions reverse multimerization and inactivation both in PsPrxIIF and GITIM (Reyes-Vivas et al., 2007).

The hexamer of PsPrxIIF could mimic the toroid structure of some decameric 2-Cys Prx. However, unlike PsPrxIIF, the oligomerization of 2-Cys Prx is stabilized by DTT (Bernier-Villamor et al., 2004). The crystallographic study on AhpC from *Mycobacterium tuberculosis* showed a toroid structure formed by the binding of six dimers (Guimaraes et al., 2005). pH in the range of 6.5 and 8.5 and ionic strength had little influence on oligomerization of PsPrxIIF excluding these parameters as regulators of oligomerization. However, during the elution of PrxIIF from the Superdex 200 columns equilibrated with carbonate buffer of pH 9.9 the enzyme dissociated into dimers. This indicates the presence of groups which protonate in this pH range and facilitate formation of the hexamer (data not shown).

As in the case of dimer-decamer transition of the typical 2-Cys Prx (König et al., 2002; Wood et al., 2003; Barranco-Medina et al., 2008), it appears that the dimer-hexamer transition brings about a functional switch. The increased peroxidase activity of the dimer suggests an antioxidant function whereas the hexamer, which represented the oxidized form of PsPrxIIF and also exhibited the lowest activity in the enzymatic assay, could be implicated in other functions such as redox signalling by binding to other structures or as a chaperone. At the concentration of 2 mM DTT used here, the activity of the heterocomplex sample doubled compared with the other samples, reaching turnover rates considerably exceeding those observed with 10 mM DTT and Trx-α/ NTRA, respectively, by Finkemeier et al. (2005). It is concluded that the heterocomplex efficiently catalyses electron transfer from DTT to H$_2$O$_2$. The most likely in vivo formed oligomers detected in leaf extracts correspond either to PsPrxIIF or heterocomplexes with other target proteins and indicate that PsPrxIIF oligomerization is of functional significance in vivo. The implication of AtPrxIIF in mitochondrion-to-nucleus signalling was hypothesized based on altered nuclear gene expression in knock-out plants (Finkemeier et al., 2005). It is tempting to assume that the dimer–hexamer transition, with the concomitant switch in activity, represents the basis for subsequent signalling. Despite the progress in understanding conformational changes of mitochondrial PrxIIF, many questions about oligomerization dynamics and function of Prx remain unanswered. Sensitive techniques such as isothermal titration calorimetry (Barranco-Medina et al., 2008) or analytical ultracentrifugation might prove to be useful tools.

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