An atypical catalytic mechanism involving three cysteines of thioredoxin
Cha San Koh, Nicolas Navrot, Claude Didierjean, Nicolas Rouhier, Masakazu Hirasawa, David B. Knaff, Gunnar Wingsle, Razip Samian, Jean-Pierre Jacquot, Catherine Corbier

To cite this version:
Cha San Koh, Nicolas Navrot, Claude Didierjean, Nicolas Rouhier, Masakazu Hirasawa, et al.. An atypical catalytic mechanism involving three cysteines of thioredoxin. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 2008, 283 (34), pp.23062-23072. 10.1074/jbc.M802093200. hal-02667186

HAL Id: hal-02667186
https://hal.inrae.fr/hal-02667186
Submitted on 31 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
An Atypical Catalytic Mechanism Involving Three Cysteines of Thioredoxin

Received for publication, March 17, 2008, and in revised form, June 13, 2008. Published, JBC Papers in Press, June 14, 2008, DOI 10.1074/jbc.M802093200

Cha San Koh†§¶, Nicolas Navröt†¶, Claude Didierjean†, Nicolas Rouhier†, Masakazu Hirasawa**, David K. Knaff**, Gunnar Würgler†, Razip Samian§, Jean-Pierre Jacquot§, Catherine Corbier¶², and Eric Gelhaye†²

From the †LCM3B, Equipe Biocristallographie, UMR 7036 CNRS-Université Henri Poincaré, §Unité Mixte de recherche INRA-UHP 1136, Interactions Arbres/Micro-organismes, ¶URAFFPA, Equipe PB2P, Faculté des Sciences et Techniques, Nancy Université, BP 239, 54506 Vandoeuvre Cedex France, the **Department of Chemistry and Biochemistry, and Center for Biotechnology and Genomics, Texas Tech University, Lubbock, Texas 79409-1061, the ¶School of Biology, Université Sains Malaysia, Minden, 11800 USM, Penang, Malaysia, and the †Department of Forest Genetics and Plant Physiology, Faculty of Forestry, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden

Unlike other thioredoxins h characterized so far, a poplar thioredoxin of the h type, PtTrx h, is reduced by glutathione and glutaredoxin (Grx) but not NADPH:thioredoxin reductase (NTR). PtTrx h contains three cysteines: one localized in an N-terminal extension (Cys4) and two (Cys58 and Cys61) in the classical thioredoxin active site (WC58GPC61). The property of a mutant in which Cys58 was replaced by serine demonstrates that it is responsible for the initial nucleophilic attack during the catalytic cycle. The observation that the C4S mutant is inactive in the presence of Grx but fully active when dithiothreitol is used as a reductant indicates that Cys4 is required for the regeneration of the first and second Trxs h subgroups contain a conserved WCGPC catalytic site. The first cysteine is the one involved in the nucleophilic attack on disulfide bonds present in target proteins, leading to the formation of a disulfide bond between the target protein and Trx. This intermolecular disulfide is then reduced by the second cysteine, leading to the release of reduced target protein and oxidized Trx.

It is only recently that members of plant Trx h subgroup have been detected and characterized, with much of the evidence coming from studies on poplar (8, 9). The poplar thioredoxin PtTrx h, which belongs to this subgroup, contains a typical WCGPC catalytic site but differs from previously characterized Trxs in being reduced in vitro by glutaredoxins but not by NTR (8). This unique feature raised several questions about the reaction mechanism of PtTrx h. For example, as most characterized Trxs have a redox midpoint potential of about ~290 mV, whereas Grx is more electropositive (about ~200 mV), questions about the thermodynamic favorability of reduction of a Trx-like molecule by Grx naturally arise.

The structural and redox properties of animal, bacterial, and some plant Trxs have been studied extensively, but little structural information about higher plant Trx h is available (10, 11). In addition, there is no subgroup 3 Trx h structure solved to date. Because members of this subgroup exhibit an N-terminal extension containing a conserved cysteine in the fourth position that is absent in other subgroups, questions concerning the role of this extension and its additional cysteine arise. It is of particular interest to know how this extension is positioned with respect to the conserved Trx fold and more importantly to understand why this protein does not react with its traditional reducing partner, NTR. We show here that, in contrast to other Trxs, three cysteines rather than two are involved in the catalytic mechanism of PtTrx h. Two of these cysteines are present in the classical Trx catalytic site (WC58GPC61), whereas the third one is localized in the N-terminal extension (Cys4). From
the kinetic and structural data, a new catalytic mechanism is proposed for this Trx isoform.

EXPERIMENTAL PROCEDURES

Cloning and Mutations of PtTrxh4—The procedures for cDNA isolation of PtTrxh4 and its subsequent cloning are described in Ref. 8. The PtTrxh4 mutants C4S, C58S, and C61S were generated by PCR using cloning and mutagenic oligonucleotides shown below (Ncol and BamHI sites are underlined and mutagenic bases are in bold): PtTrxh4 direct, 5’-CCCTCCATGGGACTTTGCTTTGAGGATC-3’; and PtTrxh4 reverse, 5’-CCCTCCATGGGACTTTGCTTTGAGGATC-3’; PtTrxh4C4S direct, 5’-CCCCCATGGGACTTTAGTGGATAGCAT-3’; and PtTrxh4C4S reverse, 5’-TTTACGATCCACATGGGAGTGGTCTTGTAGACAG-3’; PtTrxh4C58S reverse, 5’-CTCTTGTAGACAG-3’; and PtTrxh4C58S reverse, 5’-CGGTGCAATCATGGTGTGGTCCTAGTGGATAGCAT-3’. The mutated PCR products that contained the restriction sites have been cloned into expression plasmid pET-3d, yielding constructions pET PtTrxh4C4S, pET PtTrxh4C58S, and pET PtTrxh4C61S. The mutations of the recombinant plasms were verified by DNA sequencing.

Expression and Purification of the Recombinant Proteins—All procedures for the expression and purification of Arabidopsis thaliana NTR B (AtNTRB), poplar PrxQ (PtPrxQ), and Grx (WT and mutants) are described elsewhere (12–15). All the PtTrxh4 proteins have been expressed in Escherichia coli strain BL21(DE3), which was also co-transformed with the plasmid helper pSBET as described in Ref. 16. The [SeMet]PtTrxh4 gene was amplified from a P. tremula × tremuloides cDNA library. The gene was inserted in the pET-3d expression plasmid, between Ncol and BamHI sites. Recombinant plasmids carrying the gene were electroporated into the mid, between Ncol and BamHI sites. Recombinant plasmids containing the restriction sites have been cloned into expression plasmid pET-3d, yielding constructions pET PtTrxh4C4S, pET PtTrxh4C58S, and pET PtTrxh4C61S. The mutations of the recombinant plasmids were verified by DNA sequencing.

Oxidation-reduction titrations were carried out as described previously using the fluorescein of the monocromobimane-modified form of the reduced protein to monitor the extent of the reduction of the protein (18, 19). Ambient potentials (E0) were established using mixtures of oxidized glutathione (GSSG) and reduced glutathione (GSH) and the PtPrxh4 samples were incubated at these defined E0 values for 2 h to reach redox equilibrium. The oxidation-reduction midpoint potential (E1/2) value was shown to be independent of the total concentration (GSSG and GSH) present in the redox equilibration buffer over the range from 2 to 5 mM. The E1/2 was calculated by fitting the data to the Nernst equation for a two-electron process as described previously (20).

ProxQ Activity Measurement—The reduction of H2O2 by poplar PrxQ in the presence of PtPrxh4 was followed spectrophotometrically, using a Cary 50 spectrophotometer, by monitoring the decrease in absorbance arising from NADPH oxidation in a coupled enzyme assay system. The reaction mixture (500 μl) contained 30 mM Tris-HCl, pH 8.0, 1 mM EDTA, 200 μM NADPH, 0.5 IU glutathione reductase, 1 mM GSH, 6 μM PtGrx C4, 16 μM PtTrxh4 WT and mutants, 50 μM H2O2, and 2 μM PtPrxQ. Alternatively, H2O2 disappearance was followed directly. The reaction mixture (100 μl) contained 30 mM Tris-HCl, pH 7.0, 500 μM DTT, 4 μM PtPrxQ, and 36 μM PtPrxh4. The reaction was started by adding 500 μM H2O2. After given incubation times, 5 μl were mixed with 495 μl of FOX1 (ferrous oxidation in xylene orange) reagent (21). The absorbance was then read at 560 nm after 1-h incubation.

Crystallization—Three different samples were crystallized, one with Se-Met (WT PtPrxh4) and two with regular methionines (WT and PtPrxh4C61). Crystallization conditions were screened extensively at 20 °C with the microbatch method. Drops used for the initial crystallization trials consisted of 2 μl of the protein solution (20 mg/ml) mixed with 2 μl of various crystallization solutions. The [SeMet]PtPrxh4 crystals were grown in 1.0 M sodium/potassium phosphate buffer, pH 6.9, (Hampton SaltRx Screen 2, solution 54), whereas the WT PtPrxh4 was obtained by using the JBS screen 2 solution D2 (30% PEG 4000, 0.1 M NaHEPES, pH 7.5, 0.2 mM CaCl2).

For PtPrxh4C61S protein, orthorhombic crystals were obtained by using the Hampton SaltRx Screen condition 55 buffer (1.0 M sodium/potassium phosphate, pH 8.2). The drop was formed by mixing 25 mg/ml protein with crystallization solution in a 1:1 ratio. Crystals were cryoprotected and flash-cooled in liquid ethane at 100 K.

Data Collection and Processing, Structure Solutions, and Refinements—Information and statistics of data collection and processing of the three crystals are presented in Table 1. The
Glutaredoxin-dependent Thioredoxin

Models of the wild type and the PtTrxh4C61S mutant were solved by molecular replacement (MOLREP) (32) using [SeMet]PtTrxh4 as the search model. The final structure of the WT PtTrxh4 was obtained after manual rebuilding of many parts of the structure (mainly near the active site and the α-helix 3 regions) and refinements using CNS and REFMAC5. Concerning the PtTrxh4C61S mutant, 82% of the model was built automatically using ARP/wARP. The final mutant model was obtained using the same procedure used for the WT protein. Throughout the model building and refinement processes, qualities of all models were assessed using the program PROCHECK (33). Refinement statistics are summarized in Table 1. Figures were prepared with PyMOL (34).

Structure Superimpositions—Superimpositions of the present structures with their homologous structures obtained from the Protein Data Bank were performed using the LSQMAN program from the DEJAVU package (35), and the Lsqkab (superpose) program of the CCP4 suite with default parameters proposed by the authors.

Protein Data Bank Accession Codes—Atomic coordinates and structure factors have been deposited in the Protein Data Bank. Accession codes are 3D21 (WT PtTrxh4) and 3D22 (PtTrxh4C61S).

RESULTS
PtTrxh4 belongs to the third Trx h subgroup and exhibits the classical WCGPC active site. In addition to these two cysteiny1 residues, members of this subgroup contain one additional con-
served cysteine found in the fourth position in an N-terminal extension (comprising the 24 most N terminus residues) (Fig. 1). Sequence analysis using several localization prediction programs suggests that the N-terminal extension does not correspond to a signal peptide and that this protein is likely to be located in the cytosol.

**Thiol Content Determination**—To investigate the putative role of the three conserved cysteines in the catalytic mechanism of PtTrx₄₄, site-directed mutagenesis has been used to produce the full-length recombinant proteins in *E. coli*: PtTrx₄₄C₄S, PtTrx₄₄C₅₈S, and PtTrx₄₄C₆₁S. Thiol content of these proteins has been determined both in reducing and non-reducing conditions. A summary of these data is shown in Table 2. Nearly three thiols per protein are titrated for the reduced WT protein, whereas approximately one SH group is present in the unreduced WT protein. Under reducing conditions, thiol contents of the PtTrx₄₄C₄S, PtTrx₄₄C₅₈S, and PtTrx₄₄C₆₁S mutants were all close to 2 SH/mol in good agreement with the expected theoretical values. In the absence of reducants, the PtTrx₄₄C₆₁S mutant is fully oxidized (no titrated thiol group), whereas PtTrx₄₄C₄S and PtTrx₄₄C₅₈S mutants are only partially oxidized (*i.e.* thiol content values lower than 1 SH/mol were measured).

SDS-PAGE was performed under reducing and non-reducing conditions (see supplementary data Fig. S1). In the absence of DTT, both monomers and dimers were detected for PtTrx₄₄WT and C₆₁S with monomers being the dominant species for both proteins. Although thiol titration results are most consist-

---

**TABLE 2**

| Thiol content of PtTrx₄₄ under nonreducing or reducing conditions |
|---------------------------------------------------------------|
| **Thiols were titrated using DTNB as described under “Experimental Procedures.”** | **Nonreducing conditions** | **Reducing conditions** |
| **Data are expressed in mole of SH/mole of enzyme. The S.D. is typically ≤ 0.2 SH/mol of SH.** | | |
| **WT PtTrx₄₄** | 0.75 | 2.88 |
| PtTrx₄₄C₄S | 0.93 | 1.64 |
| PtTrx₄₄C₅₈S | 0.64 | 1.93 |
| PtTrx₄₄C₆₁S | 0.05 | 1.55 |

---

**FIGURE 1.** Multiple alignment of poplar Trx₄₄-related sequences. Amino acid sequences were aligned using CLUSTALW software. Accession numbers are as follows: Pt₁, *Populus tremula × Populus tremuloides* (AAL99941); Sec, *Secale cereale* (AF159386); Ta, *Triticum aestivum* (AF438359); Hv, *H. vulgare* (AF435815); Hb, *Hordeum bulbosum* (AF159385); Lp, *Lolium perenne* (AF159387); Pc, *P. coerulescens* (AF159388); Os, *Oryza sativa* (AF435817); Zm, *Zea mays* (AF435816); Ls, *Lactuca sativa* (TC9985); Vv, *Vitis vinifera* (CB004453); Gm, *Glycine max* (CA798951); Mc, *Mesembryanthemum crystallinum* (CA838461); Pt₄, *P. tremula × P. trichocarpa* (P85801); At9, *A. thaliana* (At3g08710). The asterisk corresponds to strict identity, the colon corresponds to functional homology, and the period corresponds to structural homology. Amino acids that compose hydrophobic sites 1 and 2 are highlighted in dark gray and light gray, respectively. Three cysteines proposed to be involved in the atypical catalytic mechanism of PtTrx₄₄ (see text for details) are well conserved in its orthologs (shown in “black”).
Glutaredoxin-dependent Thioredoxin

![Graphs showing activity of PtPrxQ in the presence of PtTrxhC4(a) or with DTT (b)]

FIGURE 2. Activity of PtPrxQ in the presence of PtTrxhC4 (a) or with DTT (b). For assay in a, PtPrxQ (2 mM) was incubated with either PtTrxhC4WT or different mutants (16 mM) in the presence of the GSH/Grx system (200 mM NADPH, 1 mM GSH, 0.5 mM glutathione reductase, 6 mM PtGrx C4, 500 mM H2O2). NADPH oxidation was measured at 340 nm as absorbance change per min. For assay in b, the reduction of H2O2 by PtPrxQ (5 mM) was measured by following the disappearance of 500 mM H2O2 using 1 mM DTT in the presence of either PtTrxhC4WT or different mutants (30 mM). Depletion of H2O2 was measured at 560 nm as absorbance change per min.

ent with the formation of an intramolecular disulfide bond, the presence of reducible dimers for the C61S mutant indicates that Cys58 and/or Cys4 could be also involved in intermolecular disulfide bonds. Only monomers were observed for the C4S mutant in the absence of DTT, suggesting that an intramolecular disulfide bond is formed between Cys58 and Cys61. In the case of the C58S mutant, oligomeric forms (disappearing under reducing conditions) were present in high abundance, which is consistent with the presence of intermolecular disulfide bonds involving Cys4 and/or Cys61.

Redox Potential Determination—The redox titrations of PtTrxhC4 and of its three C/S variants have been carried out at pH 7.0 over the potential range of −50 to −250 mV. Each result represents the average of at least two determinations and the average deviations suggest that the experimental uncertainty in Em is between 5 and 10 mV. The data obtained for the PtTrxhC4 and PtTrxhC4C61S give a good fit to the curve expected for a single two-electron process with an Em value of −165 ± 10 and −178 ± 10 mV, respectively. Data from redox titrations of PtTrxhC4C4S and PtTrxhC4C58S could not be fitted to the Nernst equation for a single two-electron process but did give good fits for the sum of the Nernst equation for two separate two-electron processes. In the case of PtTrxhC4C4S, the Em values for the two components are −140 ± 10 and −200 ± 10 mV and for PtTrxhC4C58S the Em values of the two components are −130 ± 10 and −180 ± 10 mV. The two-electron nature of these redox couples, the Em range over which they titrate and the fact that the experiments rely on a thiol-specific reagent, monobromobimane, to monitor the course of the titrations leave little doubt that the components being titrated are dithiol/disulfide couples.

As the redox titration were carried out using the GSH/GSSG couple for redox buffering, it may be possible that some of the components of the redox titration arise from protein-GSH adducts. To investigate the susceptibility of PtTrxhC4 to glutathionylation, the protein was reduced by DTT, incubated with a large excess of GSSG, and analyzed by quadrupole time-of-flight mass spectrometry. It showed the formation of a low-amplitude peak with a mass of 15,761.00 Da (around 35% of the total preparation) in addition to the peak corresponding to the oxidized recombinant PtTrxhC4 (15,455.125 Da). This additional peak is compatible with the addition of one GSH molecule (305.5 Da). Concerning the mutants, a large amplitude additional peak (around 90% of the total preparation) corresponding to a GSH adduct was detected with PtTrxhC4C58S; PtTrxhC4C61S was glutathionylated to a lesser extent (around 35% of the total preparation); whereas PtTrxhC4C4S showed no detectable glutathionylation. To identify the glutathionylation site(s) of the WT protein, we performed tryptic digestion of fully reduced or GSSG-oxidized protein and analyzed the tryptic fragments by mass spectrometry. The data indicated that Cys4 is the glutathionylation site on PtTrxhC4, a result consistent to the absence of GSH adduct formation with PtTrxhC4C4S.

Due to the possibilities of extensive glutathionylation and the multiplicity of possible disulfides (both intra- and intermolecular), the potential redox values obtained are actually the average values of a mixture of disulfides. Hence, it is not yet possible to provide a unique assignment of each Em component in these two-component titrations. Nevertheless, we can conclude that the redox midpoint potential values for all of the couples involved are more positive than −200 mV, a value that is very much more electropositive than the redox potential of typical Trxs (about −300 mV).

PrxQ Activity—The activity of the recombinant proteins was tested using a non-physiological PrxQ-based system involving PtPrxQ, GSH, and PtGrx (type C4 with an active site CPYC) (8). Both PtTrxhC4C4S and PtTrxhC4C58S are totally inactive in this system, whereas the mutant PtTrxhC4C61S retained some of the activity exhibited by WT PtTrxhC4 (Fig. 2a). Taking into account the data reported from previously characterized Trxs, Cys58 is most likely the catalytic residue. As sequence comparisons suggest that Cys58 and Cys61 are the active site residues (with Cys58 and Cys61 making the initial nucleophilic attack characteristic of Trxs), it is not surprising that converting either of these cysteine residues to serine results in loss of activity. The most interesting feature of PtTrxhC4, based on the observed total loss of activity
for the PtTrxh4C4S mutant, is that three cysteines seem to be involved in the catalytic mechanism.

The interactions between PtTrxh4 and several PtGrx mutants have also been investigated using this assay. The PtGrx C27S mutant, in which the catalytic cysteine of PtGrx C4 has been replaced to serine, is not active. In this system, mutations of residues surrounding the Grx catalytic cysteines (Y26A and Y29F) altered the reduction efficiency of Grx (36) and thus the PtTrxh4 reduction by these mutants. In contrast, Grx C30S (in which the second cysteine of the active site is removed) was still able to reduce PtTrxh4, suggesting that monothiol Grx may be able to interact with this kind of Trx.

The interactions between Trxs and PrxQ were also investigated by measuring H2O2 disappearance in the presence of DTT (Fig. 2b). Again, the mutant C58S is totally inactive in this system, consistent with the hypothesis that Cys58 is the catalytic cysteine. The PtTrxh4C61S mutant exhibited a much lower activity than PtTrxh4, showing the importance of Cys61 in target protein reduction by PtTrxh4. In contrast to the results obtained when glutaredoxin was used as the reductant, the PtTrxh4C4S mutant is fully active when DTT is the donor.

Overall Description and Structure Comparison of Wild Type PtTrxh4 and PtTrxh4C61S—The two PtTrxh4 structural models presented in this study contain intramolecular disulfide bonds involving the catalytic Cys58 and either Cys61 (WT PtTrxh4, despite the crystallization in the presence of DTT) or Cys4 (PtTrxh4C61S mutant). Here, unless indicated otherwise, structure A refers to the WT PtTrxh4 (Fig. 3a) and structure B to the PtTrxh4C61S mutant (Fig. 3b). Generally, both forms of PtTrxh4 structures retain the overall thioredoxin fold, which is shared by all members of the thiol-disulfide oxidoreductase family, with a five-stranded β sheet surrounded by four α helices in a βαβαβα topology (Fig. 3, a and b).

In structure A, the electron density is well defined along the main chains and for most of the side chains except that the 23 most N-terminal and the 5 most C-terminal amino acids of the WT enzyme could not be located due to weak electron densities, suggesting a high degree of structural flexibility of these parts of the molecule. Hence, this structural model comprises residues 24–134 of PtTrxh4. Nevertheless, the full-length of the protein was verified by mass spectroscopy. The oxidized form A is present as two monomers in the asymmetric unit with active site regions facing each other. The active site architecture is similar to that of other known Trxs (37–40). One side of the redox-active disulfide bond is flat and rather hydrophobic (formed by residues Ala55, 59Gp60, 99ATP101, 116VG118; named as hydrophobic site 1; Fig. 3c), whereas the other side is more shielded with a pitcher-like predominantly hydrophobic cleft (this cleft is formed by Val26, Leu70, Asn85, Ser89, 64IAp66cleft, 69YF69, Val82, Phe103, Leu115, and Leu123, named as hydrophobic site 2; Fig. 3d). At the edge of the pitcher-like cleft lies a positively charged patch, 38RW57 and Lys62. The hydrophobic site 2 accommodates hydrophobic residues from the symmetry-related second monomer.

B structure consists of 129 amino acids (residues 2–139, excluding residues from 7 to 15) and the protein crystallizes as a monomer in the asymmetric unit. Upon formation of the Cys4–Cys58 disulfide bond, the active site form of B is covered by the N-terminal region. The electron density between residues 7 and 15 was barely visible, so no residue was assigned to this assumed highly flexible loop region. Nevertheless, the disulfide bond is undoubtedly present (Fig. 3b). The N-terminal is kinked from the rest of the molecule at the double-glycine region (23GG24) and this extension is further stabilized by making extensive hydrophobic interactions that involve residues Ile19 and 21LAGG24 with the residues located at hydrophobic site 2. Apart from the hydrophobic interactions, the N-terminal extension is also stabilized by hydrogen bonding (V26N–G23O, I19N–P17O, T100N–C4O, C4N–T100O, A118N–G20I, H19O–R56N–η). Upon closure of the N-terminal region toward the core of the protein, the molecular surface properties of hydrophobic site 1 remains unchanged (just as described in structure A, see Fig. 3, c and e, for comparison). However, hydrophobic site 2 pocket is shielded by residues from the N-terminal extension (Pro17–Gly24) and therefore its depth is greatly reduced (see Fig. 3, d and f, for comparison). Indeed, half of the pitcher-like cleft wall (hydrophobic site 2) has now become more hydrophilic in the B structure (Fig. 3f).

If the first 23 amino acids are excluded, superimposition of forms A and B structures yield root mean square deviation values of 0.6 Å (for 110 common Ca atoms). The positions of the active site cysteines (or Ser61 in form B structure) remain unchanged in both structures. The A and B structures exhibit different conformations in their active site regions with the most obvious difference observed at residue Trp57. In the B structure, the position of Trp57 (χ1 value = +50.01°, residue stabilized by making a hydrogen bond with the Oε2 atom of a conserved residue Asp865) is the same as in most of other classical Trxs (χ1 values between +40° and +55°) and its side chain covers an important part of the active site (Cys4–Cys58 disulfide bond) surface. This residue has been shown to be important for the Trx-protein interaction or recognition (41, 42). In contrast, the plane of the indole ring of Trp57 (χ1 value = –81.87°) in the A structure has to “flip out” by about 90° with respect to its usual position as described in the B structure and orients toward the surface of the protein. In the A structure, the Trp57 residues of the two independent monomers are facing each other. This residue possesses an elevated average B factor (72.55 Å2) and the indole side chain lacks electron density, suggesting the flexibility of the side chain. Fig. 4 displays a superimposition of the active site region close-up view of form A PtTrxh4, form B PtTrxh4, and other Trx structures, highlighting the position and orientation of the active site Trp residue. The atypical position of Trp57 in structure A is not retained in structure B mainly due to the constraint of the N-terminal.

Apart from the flexibility of Trp57, superimposition of monomers of the A and B structures also revealed that its neighboring residue, Arg56, also possesses a certain degree of mobility. This side chain has moved in toward the molecule by about 40° when comparing the A and B structures. The movements of these two residues actually make way for the N-terminal extension of PtTrxh4 to gain access to the active site of the enzyme. Another difference between the two forms is the direction of the side chain of Ser25. Contrary to the A structure where it is more solvent-exposed, the O-γ atom of Ser25 is hydrogen bonded to...
FIGURE 3. Overall structures of the WT PtTrxh4 (blue) and the PtTrxh4C615 (orange) in schematic representation (a and b) and the electrostatic potential surface charges of the WT (c and d) and PtTrxh4C615 mutant (e and f), viewing from both sides of the molecule (rotated by 180°). Molecules in c–f correspond to the orthogonal view of the molecule in a. For a and b, the intramolecular disulfide bond in each structure is highlighted, with final 2Fo − Fc electron density (1.2 σ level) covering chosen residues for clarity. The N-terminal extension (residues 1–23) is not modeled in the WT PtTrxh4 structure (a) due to the lacking of electron density, whereas in the PtTrxh4C615 partial of the N-terminal extension (residues 2–6 and 16–24, colored in green) can be assigned to the structure (b, see text for details). In the latter structure, lacking residues (7–15) are shown in an imaginary gray loop for a better overview of the structure. Relevant residues and secondary structures are labeled. Two hydrophobic sites, site 1 (c) and site 2 (d), are circled for clarity. Corresponding sites in the mutant can be found in the bottom panel (e and f). Notice that the site 1 cleft is shallower than the pitcher-like hydrophobic site 2 (for WT PtTrxh4). The figure was prepared using PyMOL.
of Thr<sup>100</sup> is less than 4 Å away from the S-γ of the catalytic Cys<sup>58</sup>) and also near hydrophobic site 2, also suggests an important role for this residue.

A noticeable difference between PtTrx<sub>4</sub> and other Trx structures is that Val<sup>82</sup> of PtTrx<sub>4</sub>, which is situated in the hydrophobic site 2 mentioned earlier (see previous section) is replaced by a lysine residue in all other Trxs (corresponding to Lys<sup>57</sup> in <i>E. coli</i> Trx). In all plant Trxs h of subgroup 3, a hydrophobic and relatively small residue is always present. This substitution underlines the hydrophobicity of site 2, whereas in other Trxs a positively charged residue (Lys<sup>57</sup>) changes the properties of the cavity. In classical Trxs, the corresponding cavity is more solvent-exposed, whereas in PtTrx<sub>4</sub>, Ser<sup>54</sup> is the most hydrophilic and polar residue embedded at the bottom of the hydrophobic site 2. All these results suggest that the “hydrophobic cavity” at hydrophobic site 2 may be involved in regulating the flipping of the N-terminal extension of PtTrx<sub>4</sub>. Indeed, site 2 is complementarily covered by the N-terminal extension in form B structure.

Another peculiarity of the active site of PtTrx<sub>4</sub> is the absence of a buried carboxylate behind the active site cysteines. Instead of the common aspartate residue, the corresponding position is occupied by Asn<sup>52</sup> in PtTrx<sub>4</sub> with an equivalent conformation. The buried Asp<sup>26</sup> carboxyl (using the <i>E. coli</i> Trx numbering) and Lys<sup>57</sup> ε-amino groups mentioned, which are in equivalent positions of residues Asn<sup>52</sup> and Val<sup>82</sup> in PtTrx<sub>4</sub>, are suggested to have significantly modulating the pK<sub>a</sub> values of the active site thiolis, thereby enhancing the rates of thiol-disulfide reactions at physiological pH (45). In other words, the substitution of these two highly conserved residues of Trxs in PtTrx<sub>4</sub> and its orthologs may be responsible for the deviation observed in the more electropositive redox potentials. The substitution of the conserved Asp (but above mentioned Lys remains conserved) by another residue in PtTrx<sub>4</sub>, has also been reported for <i>Anabaena</i> Trx-2 (replaced by Tyr<sup>56</sup>) (46) and for <i>Trypanosoma</i> Trx (replaced by Trp<sup>24</sup>) (47). However, no pK<sub>a</sub> values of the active site thiolis of these proteins have been determined experimentally.

**DISCUSSION**

**Uniqueness of PtTrx<sub>4</sub>**—All plant Trxs characterized up to now are reduced by either NTR or ferredoxin/thioredoxin reductase except for poplar thioredoxin PtTrx<sub>4</sub> (6). To date, PtTrx<sub>4</sub> is the only characterized Trx that is reduced by the GSH/Grx system <i>in vitro</i>. It was thus of interest to examine the reasons for the original properties of this Trx. Three conserved cysteines are present in PtTrx<sub>4</sub>, two found in the classical Trx active site (WCGPC) (Cys<sup>58</sup> and Cys<sup>61</sup>, respectively) and one localized in the N-terminal part of the protein (Cys<sup>5</sup>). Cys<sup>5</sup>, shown to play a role in the catalytic mechanism in this study, is a well conserved residue found at the same position in all other Trxs of subgroup 3 to which PtTrx<sub>4</sub> belongs (8).

**Proposed Catalytic Mechanism for an Atypical Thioredoxin**—Based on the biochemical and structural data described here, we propose a four-step mechanism for the reaction of reduced PtTrx<sub>4</sub> with an oxidized target protein (e.g. PrxQ) and its regeneration (Fig. 5). As for other Trxs, catalysis proceeds through the formation of a heterodimer between the target pro-
Glutaredoxin-dependent Thioredoxin

FIGURE 5. A proposed four-step disulfide cascade mechanism for Grx-dependent PtTrx4 catalysis. (1) Cys\textsuperscript{58} attacks initially the S-atom of an oxidized target protein; 2) the heterodisulfide is then reduced by the active site recycling cysteine (Cys\textsuperscript{4}); 3) the resulting disulfide bond (Cys\textsuperscript{58-Cys\textsuperscript{58}}) is further reduced by the second recycling cysteine, Cys\textsuperscript{4}; and 4) a glutathione molecule (GSH) reduces the Cys\textsuperscript{4-Cys\textsuperscript{58}} disulfide bond prior to, 4i, the final nucleophilic attack of the Trx-SG adduct by the catalytic Cys of monothiol or dithiol Grx.

Cys\textsuperscript{4} of PtTrx4 Acts as the Second Resolving Cysteine—From the activity assay results (Fig. 2a), we know that PtTrx4C4S is nearly as inactive as the PtTrx4C58S when the Grx/GSH system is used as a reductant. In structure B, we observed that (i) PtTrx4 can form a disulfide bond between Cys\textsuperscript{4} and Cys\textsuperscript{58}, (ii) this disulfide bond is non-artifactual because the N-terminal extension fits perfectly in the hydrophobic site 2 pocket by complementary matching involving invariant residues of sub-group 3 Trxs, (iii) Cys\textsuperscript{4} is unlikely to attack the disulfide bond because it is totally buried in the enzyme, and (iv) Cys\textsuperscript{4} is the only solvent-accessible cysteine, suggesting that it is able to interact with the electron donor. This study suggests that Cys\textsuperscript{4} acts as the second recycling cysteine by reducing the Cys\textsuperscript{58-Cys\textsuperscript{61}} disulfide bond, leading to the formation of a second intramolecular disulfide bond, i.e., Cys\textsuperscript{58-Cys\textsuperscript{58}}. The catalytic mechanism proposed for PtTrx4 is related to the one described for some peptide methionine sulfoxide reductases A (MsrA), whereupon catalysis of three cyssteines form two consecutive intramolecular disulfide bonds before the regeneration of the enzyme by Trxs (48).

PtTrx4 Is Regenerated by a GSH/Grx Reducing System but Not by the Typical NTR Reducing System—In terms of redox potential, WT PtTrx4 (E\textsubscript{m}, average value of −165 mV, see “Results”) should be reducible by the NTR reducing system as demonstrated for the vast majority of the Trxs (E\textsubscript{m} values in the −280 to −310 mV range observed for other Trxs (6, 7, 49)). Thus, there must be reasons other than thermodynamic driving force for the inability of NTR to regenerate PtTrx4. The superimposition with the structure of the Trx-NTR complex (PDB code 1F6M) (50), reveals that the N-terminal extension of PtTrx4 is preventing the interaction with NTR. In addition, structure B exhibits a highly negatively charged molecular surface around the active site that may disfavor its interaction with NTR, which also possesses a negatively charged active site surface. Clearly, the N-terminal part of PtTrx4 is not the only determinant responsible for the lack of reactivity with NTR because the [SeMet]PtTrx4C61S (truncated form, consisting of residues Asn\textsuperscript{25} to Met\textsuperscript{334}) is also not reduced either by A. thaliana or E. coli NTR.

The conformation of the side chain of Trp\textsuperscript{57} at the active site (WCXXC) may also play an important role in preventing a productive interaction between PtTrx4 and NTR. Although the major role of this residue is related to substrate/Trx recognition and interaction (41, 42, 51), it is also involved in the Trx/NTR interaction (50). In both A (WT and [SeMet]PtTrx4) and B (PtTrx4C61S) structures, the side chain of Trp\textsuperscript{57} shows a high flexibility, so that it can either be close to the disulfide bond (structure B) or somewhat distant from the disulfide bond (structure A). A similar flexibility of the corresponding tryptophan has been reported for other Trxs that do not use the common NTR reducing system: the spinach chloroplastic Trx f (short form), which uses the ferredoxin/thioredoxin reductase system for its reduction (52) and the Trx of Trypanosoma brucei (Fig. 4), which is spontaneously reduced by the trypanothione/trypanothione reductase system of this parasite (53). These interesting reports hint that Trxs with increased flexibility for this conserved Trp may most likely use regeneration system(s) other than the NTR reducing system.

One point that is still unclear is whether the Cys\textsuperscript{4-Cys\textsuperscript{58}} disulfide bond, once formed, is reduced by GSH or by Grx. In terms of redox potential, an E\textsubscript{m} value of −165 mV for PtTrx4 is fully compatible with its thermodynamically favorable complete reduction by either glutathione (GSH/GSSG, E\textsubscript{m} value of −241 mV) (54) or glutaredoxins (E\textsubscript{m} values of −233, −198 mV and −170 ± 10 mV for E. coli Grx1, E. coli Grx3, and plant Grx C4, respectively) (55–57). We show here that neither GSH nor Grx alone can complete the regeneration of the enzyme but both together are essential for the catalytic mechanism. The fact that we have not been able to obtain a complex between a mutated PtGrx C30S and the PtTrx4C61S suggests that the possibility of direct reduction by Grx is unlikely. It is interesting...
to note that PtTrx4 can be easily glutathionylated in vitro (using mass spectrometry to characterize tryptic digests of glutathionylated WT PtTrx4). Hence, we can postulate that the reaction with a GSH molecule occurs prior to the reaction with Grx in the catalytic cycle.

Physiological Considerations of PtTrx4—Orthologs of PtTrx4 are also present in different plants. Based on amino acid sequence comparisons, it seems quite reasonable to assume that these proteins should, like PtTrx4, be able to use Grx as an electron donor. This assumption is consistent with the report that the Phalaris coerulescens ortholog is not reduced by NTR (9). It has been shown that ribonucleotide reductase cannot use monothiol Grx as donor, whereas other reactions catalyzed by Grx require either monothiol or dithiol Grxs (55, 58, 59). Based on these considerations, it is tempting to speculate that both kinds of Grx may be able to promote the activity of PtTrx4 and its orthologs. Moreover, there are many naturally occurring monothiol Grx in plants. It will be of considerable interest to compare the relative efficiencies of the corresponding proteins for PtTrx4 catalysis.

The physiological role of this particular kind of GSH/Grx reducible Trx remains to be elucidated. Although PtTrx4 is unable to reduce several isoforms of poplar glutathione peroxidases,4 the enzyme is capable of reducing a cytosolic MsrA and unable to reduce several isoforms of poplar glutathione peroxidase (4). More studies are required to identify other possible targets, keeping in mind that the E value of PtTrx4 is significantly more positive than that of most Trxs and thus thermodynamic considerations would limit the number of protein disulfides that PtTrx4 is capable of reducing.

Acknowledgments—We thank F. Favier for careful reading of the manuscript. We are very grateful to the FIP team in ESRF, Grenoble, France, and the DESY team in EMBL-Hamburg Outstation, Germany, for providing access to beamlines BM30A, X11, and BW7A.

REFERENCES

1. Meyer, Y., Vignols, F., and Reichheld, J. P. (2002) Methods Enzymol. 347, 394–402.
2. Schürmann, P. (2003) Antioxid. Redox Signal. 5, 69–78.
3. Gelhaye, E., Rouhier, N., Navrot, N., and Jacquot, J. P. (2004) Cell Mol. Life Sci. 61, 24–35.
4. Laloï, C., Rayapuram, N., Chartier, Y., Grienengerber, J. M., Bonnard, G., and Meyer, Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 14144–14149.
5. Gelhaye, E., Rouhier, N., Navrot, N., and Jacquot, J. P. (2004) Plant Physiol. Biochem. 42, 265–271.
6. Bréhélin, C., Laloï, C., Setterdahl, A. T., Knaff, D. B., and Meyer, Y. (2004) Photosynth. Res. 79, 295–304.
7. Gelhaye, E., Rouhier, N., Gerard, J., Jolivet, Y., Gualberto, J., Navrot, N., Ohlsson, P. I., Wingsle, G., Hirasaki, M., Knaff, D. B., Wang, H., Dizen-gemel, P., Meyer, Y., and Jacquot, J. P. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 14454–14450.
8. Gelhaye, E., Rouhier, N., and Jacquot, J. P. (2003) FEMS Lett. 555, 443–448.
9. Juttner, J., Olde, D., Langridge, P., and Baumann, U. (2000) Eur. J. Biochem. 267, 7109–7117.
10. Coudévelly, N., Thureau, A., Hemmerlin, C., Gelhaye, E., Jacquot, J. P., and Cung, M. T. (2005) Biochemistry 44, 2001–2008.
11. Peterson, F. C., Lyle, B. L., Sampath, S., Vinarov, D., Tyler, E., Shahan, M., Markley, J. L., and Volkman, B. F. (2005) Protein Sci. 14, 2195–2200.
12. Jacquot, J. P., Rivera-Madrid, R., Marinho, P., Kollarova, M., Le Marechal, P., Migniac-Maslow, M., and Meyer, Y. (1994) J. Mol. Biol. 235, 1357–1363.
13. Rouhier, N., Gelhaye, E., Gualberto, J. M., Jordy, M. N., De Fay, E., Hirasa-wa, M., Duplessis, L., Lemaire, S. D., Frey, P., Martin, F., Manieri, W., Knaff, D. B., and Jacquot, J. P. (2004) Plant Physiol. 134, 1027–1038.
14. Rouhier, N., Gelhaye, E., and Jacquot, J. P. (2002) J. Biol. Chem. 277, 13609–13614.
15. Rouhier, N., Gelhaye, E., Sautiere, P. E., and Jacquot, J. P. (2002) Protein Expression Purif. 24, 234–241.
16. Schenk, P. M., Baumann, S., Mattes, R., and Steinbiss, H. H. (1995) Bio-techniques. 19, 196–200.
17. Navrot, N., Collin, V., Gualberto, J., Gelhaye, E., Hirasaki, M., Ray, P., Knaff, D. B., Issakidis, E., Jacquot, J. P., and Rouhier, N. (2006) Plant Physiol. 142, 1364–1379.
18. Krimm, I., Lemaire, S., Ruelland, E., Migniac-Maslow, M., Jacquot, J. P., Hirasaki, M., Knaff, D. B., and Lancelin, J. M. (1998) Eur. J. Biochem. 255, 185–195.
19. Hirasaki, M., Ruelland, E., Schepens, I., Issakidis-Bourguet, E., Migniac-Maslow, M., and Knaff, D. B. (2000) Biochemistry 39, 3344–3350.
20. Masuda, S., Dong, C., Swem, D., Knaff, D. B., and Bauer, C. E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7078–7083.
21. Deiana, L., Carru, C., Pes, G., and Tadolini, B. (1999) Free Radic. Res. 31, 237–244.
22. Retwinowsky, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326.
23. Kabsch, W. (1993) J. Appl. Crystallogr. 26, 795–800.
24. CCP4 Collaborative Computational Project Number 4 (1994) Acta Crys-
 tallogr. Sect. D Biol. Crystallogr. 50, 760–763.
25. Sheldrick, G. M., and Schneider, T. R. (1997) Methods Enzymol. 277, 319–343.
26. de la Fortelle, E., and Bricogne, G. (1997) Methods Enzymol. 276, 472–494.
27. Abrahams, J. P., and Leslie, A. G. (1996) Acta Crystallogr. Sect. D Biol. Crystallogr. 52, 30–42.
28. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) Nat. Struct. Biol. 6, 458–463.
29. Roussel, A., and Cambillau, C. (1989) Silicon Graphics Geometry Partners Directory, pp. 77–78, Silicon Graphics, Mountain View, CA.
30. Vagin, A., and Teplyakov, A. (1997) J. Appl. Crystallogr. 30, 1022–1025.
31. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J., Scheline, J., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921.
32. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 2126–2132.
33. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291.
34. DeLano, W. L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific, Palo Alto, CA.
35. Kleywegt, G. J., and Jones, T. A. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 179–185.
36. Rouhier, N., Gelhaye, E., and Jacquot, J. P. (2002) FEMS Lett. 511, 145–149.
37. Holmgren, A., Söderberg, B. O., Eklund, H., and Brandén, C. I. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2305–2309.
38. Katti, S. K., LeMaster, D. M., and Eklund, H. (1990) J. Mol. Biol. 212, 167–184.
39. Wechsler, A., Gasdaska, J. R., Powis, G., and Monfort, W. R. (1996) Structure 4, 735–751.

4 N. Navrot, unpublished results.
5 N. Rouhier, unpublished results.
Glutaredoxin-dependent Thioredoxin

40. Menchise, V., Corbier, C., Didierjean, C., Saviano, M., Benedetti, E., Jacquot, J. P., and Aubry, A. (2001) Biochem. J. 359, 65–75
41. Menchise, V., Corbier, C., Didierjean, C., Jacquot, J. P., Benedetti, E., Saviano, M., and Aubry, A. (2000–2001) Biopolymers. 56, 1–7
42. Krause, G., and Holmgren, A. (1991) J. Biol. Chem. 266, 4056–4066
43. Maeda, K., Hägglund, P., Finnie, C., Svensson, B., and Henriksen, A. (2006) Structure 14, 1701–1710
44. Reichheld, J. P., Khaif, M., Riondet, C., Droux, M., Bonnard, G., and Meyer, Y. (2007) Plant Cell 19, 1851–1865
45. Dyson, H. J., Jeng, M.-F., Tennant, L. L., Slaby, I., Lindell, M., Cui, D.-S., Kuprin, S., and Holmgren, A. (1997) Biochemistry 36, 2622–2636
46. Saarinen, M., Gleason, F. K., and Eklund, H. (1995) Structure 3, 1097–1108
47. Friemann, R., Schmidt, H., Ramaswamy, S., Forstner, M., Krauth-Siegel, R. L., and Eklund, H. (2003) FEBS Lett. 554, 301–305
48. Boschi-Muller, S., Arza, S., Sanguier-Ciaperani, S., Talfournier, F., Van Dorsselear, A., and Branlant, G. (2000) J. Biol. Chem. 275, 35908–35913
49. Wangensteen, O. S., Chueca, A., Hirasawa, M., Sahrawy, M., Knaff, D. B., and Lopez Gorge, J. (2001) Biochim. Biophys. Acta 1547, 156–166
50. Lennon, B. W., Williams, C. H., Jr., and Ludwig, M. L. (2000) Science 289, 1190–1194
51. Koh, C. S., Didierjean, C., Navrot, N., Panjikar, S., Mulliert, G., Rouhier, N., Jacquot, J. P., Aubry, A., Shawkataly, O., and Corbier, C. (2007) J. Mol. Biol. 370, 512–529
52. Crawford, N. A., Droux, M., Kosower, N. S., and Buchanan, B. B. (1989) Arch. Biochem. Biophys. 271, 223–239
53. Schmidt, H., and Krauth-Siegel, R. L. (2003) J. Biol. Chem. 278, 46329–46336
54. Cheng, Z., Arscott, L. D., Ballou, D. P., and Williams, C. H., Jr. (2007) Biochemistry 46, 7875–7885
55. Rouhier, N., Gelhaye, E., and Jacquot, J. P. (2004) Cell Mol. Life Sci. 61, 1266–1277
56. Aslund, F., Berndt, K. D., and Holmgren, A. (1997) J. Biol. Chem. 272, 30780–30786
57. Rouhier, N., Unno, H., Bandyopadhyay, S., Masip, L., Kim, S. K., Hirasawa, M., Gualberto, J. M., Lattard, V., Kusunoki, M., Knaff, D. B., Georgiou, G., Hase, T., Johnson, M. K., and Jacquot, J. P. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 7379–7384
58. Fernandes, A. P., and Holmgren, A. (2004) Antioxid. Redox Signal. 6, 63–74
59. Bushweller, J. H., Åslund, F., Wüthrich, K., and Holmgren, A. (1992) Biochemistry 31, 9288–9293