Crystal structures of oxidized and reduced forms of human mitochondrial thioredoxin 2

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Abstract

Mammalian thioredoxin 2 is a mitochondrial isoform of highly evolutionary conserved thioredoxins. Thioredoxins are small ubiquitous protein–disulfide oxidoreductases implicated in a large variety of biological functions. In mammals, thioredoxin 2 is encoded by a nuclear gene and is targeted to mitochondria by a N-terminal mitochondrial presequence. Recently, mitochondrial thioredoxin 2 was shown to interact with components of the mitochondrial respiratory chain and to play a role in the control of mitochondrial membrane potential, regulating mitochondrial apoptosis signaling pathway. Here we report the first crystal structures of a mammalian mitochondrial thioredoxin 2. Crystal forms of reduced and oxidized human thioredoxin 2 are described at 2.0 and 1.8 Å resolution. Though the folding is rather similar to that of human cytosolic/nuclear thioredoxin 1, important differences are observed during the transition between the oxidized and the reduced states of human thioredoxin 2, compared with human thioredoxin 1. In spite of the absence of the Cys residue implicated in dimer formation in human thioredoxin 1, dimerization still occurs in the crystal structure of human thioredoxin 2, mainly mediated by hydrophobic contacts, and the dimers are associated to form two-dimensional polymers. Interestingly, the structure of human thioredoxin 2 reveals possible interaction domains with human peroxiredoxin 5, a substrate protein of human thioredoxin 2 in mitochondria.

Keywords: human thioredoxin; X-ray crystal structure; mitochondria; oxidized state; reduced state

Thioredoxins (TXNs) are small ubiquitous protein–disulfide oxidoreductase enzymes conserved throughout evolution in all organisms from lower prokaryotes to higher eukaryotes (Holmgren 1985; Arner and Holmgren 2000; Powis and Montfort 2001). TXNs are redox proteins

characterized by a conserved active site sequence Cys-Gly-Pro-Cys. The catalytic cysteines of TXNs maintain thiols of protein substrates in a reduced state and thereby become oxidized themselves (Powis and Montfort 2001). These reductases have been implicated in a very large variety of biological functions such as cell proliferation, protection against oxidative stress, DNA synthesis, regulation of gene expression, and control of apoptosis (Arner and Holmgren 2000; Powis and Montfort 2001).

In mammals, cytosolic/nuclear TXN1 and mitochondrial TXN2 are two TXN isoforms encoded by two distinct genes (Arner and Holmgren 2000). Recently, based on protein sequence organization, a second group of TXNs has been distinguished composed of fusion proteins of TXN-like and additional domains (Sadek et al. 2003; Jimenez et al. 2004). TXN1 and TXN2 are part of the so-called cytosolic and mitochondrial TXN systems

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Abbreviations: ASK1, apoptosis signal-regulating kinase 1; TXN, thioredoxin; hTXN1, human cytosolic/nuclear thioredoxin 1; hTXN2, human mitochondrial thioredoxin 2; hPRDX5, human peroxiredoxin 5. Gene symbols in this article follow standard nomenclature defined by the Human Genome Organization Nomenclature Committee (<http://www.gene.ucl.ac.uk/nomenclature/>). For this reason TXN is used instead of the commonly used Trx for designating thioredoxin in the literature.

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including cytosolic and mitochondrial NADPH-dependent TXN reductases (TXNRDs) (Miranda-Vizuete et al. 2000). TXN systems are considered to act as antioxidant systems providing thiol-reducing equivalents to numerous substrate proteins, among which are found most of the mammalian thiol-dependent peroxidases named peroxiredoxins (PRDXs) (Wood et al. 2003).

Mammalian TXN1 has been shown to act as disulfide oxidoreductase for ribonucleotide reductase, methionine sulfoxide reductase; it modulates DNA binding of several transcription factors and facilitates refolding of disulfide-containing proteins (for review, see Arner and Holmgren 2000; Powis and Montfort 2001). Interestingly, TXN1 can be also secreted by a leaderless pathway and thereby stimulates the proliferation of a variety of mammalian cells including human tumor cell lines (Powis et al. 2000). Mammalian mitochondrial TXN2 was first cloned and characterized in rat (Spyrou et al. 1997) and more recently in human (Chen et al. 2002; Damdimopoulos et al. 2002). Overexpression of mitochondrial TXN2 in human cells shows that TXN2 interacts with components of the mitochondrial respiratory chain and plays a role in the regulation of the mitochondrial membrane potential (Damdimopoulos et al. 2002) as well as in the protection against peroxide-induced apoptosis (Chen et al. 2002). Also, TXN2 was involved in the inhibition of apoptosis signal-regulating kinase 1 (ASK1)-mediated apoptosis (Zhang et al. 2004). Finally, TXN2 inactivation in the chicken cell line and in the mouse revealed that mitochondrial TXN2 plays a crucial role in the regulation of the mitochondrial apoptosis signaling pathway, and is essential for normal development of mice, the embryonic lethality coinciding with maturation of mitochondria (Tanaka et al. 2002; Nonn et al. 2003).

In the present study we report, for the first time, crystal structures of human mitochondrial TXN2 (hTXN2) in reduced and oxidized forms. As expected from homologies between amino acid sequences of hTXN1 and hTXN2 (Fig. 1), the crystal structures reveal that hTXN2 presents

a typical thioredoxin-fold. The differences between hTXN1 and hTXN2 are discussed, laying emphasis on the transition between the oxidized and the reduced forms and on the formation of dimers. A possible interaction with hPRDX5, a substrate protein of hTXN2 in mitochondria, is also proposed.

Results and Discussion

Quality of the structure and overall description

We have solved and refined three isomorphous structures of hTXN2. Each of them contains six independent molecules in the asymmetric unit, labeled A to F. In the first structure, despite the crystallization in the presence of DTT, we observe, respectively, the following distances between the S γ atoms of Cys31 and Cys34 (amino acid numbering refers to mature protein without its mitochondrial presequence; see Fig. 1), the two catalytic residues: 3.01, 2.92, 3.36, 3.38, 3.18, and 3.13 Å. These values are to be compared to the typical distance observed in a disulfide bridge, 2.05 Å, and to the sum of the van der Waals radii of two sulfur atoms, 3.6 Å, which should correspond to the minimum distance when the disulfide bridge is not present. With the exception of molecules C and D, which are on the whole reduced, all of the other molecules show distances intermediate between the values expected in the reduced form and the oxidized form. It must be concluded that in the crystal, some molecules are reduced while the other ones are oxidized and that the sulfur atoms appear in some average electron density. A S γ –S γ distance of 3.1 Å, similar to those of our initial structure of hTXN1 in the reduced state (Qin et al. 1994). However, this surprisingly short distance of hTXN1 was interpreted as an artifact allowed by the potential function used for structure refinement (Jeng et al. 1994). Since in the reduced form of hTXN2 the two sulfur atoms are

Figure 1. Sequence alignment of hTXN1 and mature hTXN2 without its mitochondrial presequence is presented according to the structural alignments in both cases. For hTXN2, amino acid numbering refers to proposed mature protein (Damdimopoulos et al. 2002). The secondary structural elements are underlined (double underline for helices; single underline for β -strands) according to the limits given in the PDB files (1eru for hTXN1; 1w4v for hTXN2). Identical residues are indicated in gray.

not too far away from each other, we supposed that oxidation and reduction could take place in already grown crystals. We have indeed succeeded to prepare the completely oxidized and the completely reduced crystal forms by soaking crystals in solutions containing hydrogen peroxide (1 mM) and tris(hydroxymethyl)phosphine (10 mM), respectively. The resulting S^{γ} - S^{γ} distances are 2.07, 2.12, 2.08, 2.08, 2.08, and 2.11 Å in the oxidized form, and 3.63, 3.52, 3.63, 3.73, 3.71, and 3.58 Å in the reduced form. These unambiguous results clearly demonstrate that the initial structure is in an intermediate redox state. In all 18 chains, the electron density is well-defined along the main chains and for most of the side chains, with the exception of the beginning of the C-terminal α -helix ($\alpha 4$) in reduced molecule D (residues D94–D96) and the region around D60 in the same molecule, where the electron density is somewhat more ambiguous. In molecules A, B, and D, we observe in the N-terminal part the last His residue of the 6 $\times$ His-tag and the short linker (Gln–Ser); in molecule D, the two residues of the short linker; and in molecule F, only one residue of the linker. The analysis of the Ramachandran plots (data not shown) computed with the program PROCHECK (Laskowski et al. 1993) shows that 91.3%, 93.5%, and 93.2% of the nonglycine residues are in the most favored regions, and that there are no residues in disallowed regions, in the initial, oxidized, and reduced structures, respectively. There is a *cis* proline at position 75 in all of the chains. In each molecule, the overall structure is characterized by the presence of a thioredoxin fold consisting of a four-stranded β -sheet and three flanking α -helices (Martin 1995). In the N-terminal part, each chain comprises an additional β -strand associated with the thioredoxin β -sheet to form a fifth strand and also an additional α -helix. In both the oxidized and the reduced structures, the “central molecule” was searched by means of the program LSQMAN (Kleywegt and Jones 1997). The central molecule is the molecule showing the lowest root-mean-square (RMS) C^{α} - C^{α} distances toward all of the other ones after superposition, and is thus the most representative among the molecules related by noncrystallographic symmetry. In both cases, molecule B was elected as the central molecule and will be used for various comparisons. The oxidized molecule B is illustrated in Figure 2A, with indication of the limits of the secondary structural elements.

Comparison between hTXN2 and hTXN1 molecules

The central molecules of the oxidized and reduced forms of hTXN2 have been superposed at the level of the C^{α} atoms on the crystal structures of the corresponding oxidized and reduced molecules of hTXN1 (PDB codes 1eru and 1ert,

respectively; Weichsel et al. 1996). The superposition was performed by the program LSQMAN (Kleywegt and Jones 1997). The results are very similar, and in each case, the same 103 C^{α} atoms (out of 107 in hTXN2) can be superposed with a maximum distance of 3.5 Å. The RMS distances are 1.36 Å and 1.25 Å, respectively. The sequence alignment is presented in Figure 1 according to the structural alignments in both cases. A stereo view of this superposition of the two oxidized molecules is given in Figure 2B. A very similar view (data not shown) could be obtained with the reduced molecules. The region of the active site (30–35 in hTXN2) is very well-conserved. The five strands of the central β -sheet are also well-aligned. The largest differences are observed at the level of the orientation of some α -helices and in some loops. The first discrepancy appears in the C-terminal part of helix $\alpha 1$ and continues in the loop between $\alpha 1$ and $\beta 2$. As shown on the alignment in Figure 1, it is caused by the insertion of an additional residue (Val16) at the end of $\alpha 1$ and the deletion of two residues in the loop. Then the $\beta 2$, $\alpha 2$ region remains well-aligned, including the zone of the active site, at the N-terminal part of $\alpha 2$. The loop between $\alpha 2$ and $\beta 3$ comprises in hTXN2 the insertion of residue Lys51 responsible for the deviation but the $\beta 3$, $\alpha 3$ region that follows is again well-

Figure 2. (A) Ribbon diagram of molecule B of hTXN2 (the “central” molecule) in the oxidized state. The secondary structural elements are labeled as well as the beginning and the end of each of them. The three helices and the four β -strands belonging to the thioredoxin fold are colored green and red, respectively, while the remaining elements are colored yellow. (B) Stereo view of the superposition of the C^{α} traces of the oxidized molecules. hTXN1 is colored green, and the central molecule (B) of hTXN2 is colored red. The orientation is the same as in A.

aligned. The orientation of the loop between $\alpha 3$ and $\beta 4$ is very different and the largest divergence occurs at the level of residue 73 (Cys in hTXN1, Ala in hTXN2). Interestingly, in the case of hTXN1, this residue Cys73 is implicated in the dimerization through the formation of a disulfide bond with the same Cys residue of the second monomer (see below). Moreover, in mammalian TXN1, oxidation of Cys73 residues and formation of an intermolecular disulfide bond was demonstrated to lead to a loss of enzymatic activity (Holmgren and Bjornstedt 1995). This inactivation has been proposed as a regulatory mechanism for TXN1 functions. The absence of corresponding Cys73 in hTXN2 and the absence of any disulfide bond involving catalytic Cys residues between two oxidized hTXN2 monomers as revealed by this study, but also as noted by Spyrou and collaborators (Spyrou et al. 1997; Dandimopoulos et al. 2002), suggest that such TXN1 inactivation mechanism would not occur in mitochondrial hTXN2. The loop between $\beta 4$ and $\beta 5$ (81–84) is also misaligned in spite of the absence of any insertion or deletion. Finally, the last large discrepancy occurs in the N-terminal part of helix $\alpha 4$

and is once more caused by the insertion of residue Glu95 in hTXN2. Most of the differences are thus caused by insertions or deletions except in the two loops $\alpha 3$ – $\beta 4$ and $\beta 4$ – $\beta 5$.

Flexibility of hTXN2 molecules

Considering together the three structures, a total of 18 hTXN2 molecules is available. The superposition of these molecules allows for an estimation of the mobility along the chain, but such an estimation would include the movements due to the transition between the oxidized and the reduced forms. For a pure description of the flexibility, we have superposed separately the six oxidized (overall RMS between the C^α atoms: 0.582 Å) and the six reduced molecules (overall RMS between the C^α atoms: 0.551 Å). In each case and for each of the 107 residues, 15 distances between the C^α atoms can thus be computed, as well as their RMS value (program LSQMAN, Kleywegt and Jones 1997), and finally, the RMS values between the two sets which will be called “RMS flexibility.” The results are presented in Figure 3,

Figure 3. (A) RMS flexibility (thick line) compared to RMS redox (thin line) as function of residue number. The RMS flexibility is defined as the RMS of the RMS distance between the C^α atoms of the six superposed oxidized molecules of hTXN2 and the RMS distance between the C^α atoms of the six superposed reduced molecules. This represents the flexibility of the 12 molecules independently of the movements due to the transition between the oxidized and the reduced states. The RMS redox is defined as the RMS distance between the C^α atoms in pairs of superposed equivalent molecules, respectively, in the oxidized and in the reduced states. Thus, chain A_{ox} is superposed to chain A_{red} , chain B_{ox} to chain B_{red} , etc. (B) Superposition of the 18 molecules of hTXN2 (six from each crystal form) colored according to the RMS flexibility defined above, from blue for the least flexible parts to red for the most flexible ones. The side chains of the two Cys residues of the oxidized and the reduced “central” molecules are represented.

A and B. The largest mobilities occur in the loop between $\alpha 2$ and $\beta 3$ (residues 46–50) and in the loop between $\beta 4$ and $\beta 5$ (residues 82–83). Surprisingly, the region containing the active site (residues 30–35) in which occurs the transition between the oxidized and the reduced form is not particularly agitated. The availability of this RMS flexibility will be very useful to assess the significance of movements observed during the oxidation or reduction.

Comparison of the oxidized and reduced molecules of hTXN2

Since there are six independent molecules of hTXN2 in the asymmetric unit, it is possible that some local differences between the molecules are the result of different packings. It would thus be dangerous to compare, for example, the oxidized molecule A with the reduced molecule F if the aim is to obtain information about the movements related to the oxidation and the reduction. For this reason, we have decided to compare only the molecules by superimposed pairs (A_{ox} and A_{red} , B_{ox} and B_{red} , etc.) in such a way that the packing environment of each pair of molecules remains essentially the same in the oxidized and in the reduced crystal forms. The overall RMS distance between the C^α atoms of the oxidized and the reduced molecules computed in this way is only 0.297 Å. This value is very small and is much lower than the RMS distance of the C^α atoms of the superimposed six molecules in the oxidized crystal (0.582 Å) or in the reduced crystal (0.551 Å). This indicates that the fold is unaffected by the oxidized or reduced state of the molecule. However, it does not mean that some significant local differences are not present. In order to examine possible local differences, for each residue in the sequence, the RMS distance (RMS redox) between the six available pairs of C^α atoms was computed and compared to the RMS flexibility defined in the previous paragraph. The results are shown in Figure 3, A and B. For a significant movement due to the oxidized–reduced transition, one would expect a value larger than the RMS flexibility and it can be seen that it never occurs, except very slightly at the level of Cys31. In agreement with Figure 4A showing the superposition of the central oxidized and reduced molecules, the conclusion could thus be that, concerning the disposition of the C^α atoms, there are no significant differences between the oxidized and the reduced states of the molecules, except if the direction of all of the movements is not random for example, if all of the atoms of the six molecules are moving in the same direction, even if the movement is very small. This is exactly what is observed in the Cys31–Cys34 region, and only in that region. As illustrated in Figure 4B, the group of Cys31 C^α atoms

Figure 4. (A) Superposition in the same orientation as Figure 3B of the “central” oxidized (red) and reduced (blue) molecules of hTXN2. The side chains of the Cys residues are also shown. (B) The C^α traces of residues 27–38 colored red (oxidized) and green (reduced) after superposition of the 12 molecules. The side chains of Trp30, Cys31, and Cys 34 are also presented. (C,D) Electron density of a sigma map ($2wF_o - DF_c$) contoured at the level of 1.0 σ in the region Trp30–Lys35 of the central oxidized (C) and reduced (D) molecules. Trp30 is in the upper right part and Lys35 in the lower left part of the figure.

comes closer to the group of Cys34 C^α atoms in the oxidized molecules compared to the reduced molecules. Surprisingly, Cys34 C^α atoms remain practically unaffected but seem to show a slight movement in the opposite direction, increasing the distance between them and Cys31 C^α . In the oxidized molecules, the distance between the two C^α atoms is comprised between 5.32 Å and 5.54 Å, with a mean value of 5.40 Å and an RMS deviation of 0.22 Å. In the reduced molecules, the limits are 5.47 Å and 5.73 Å, the mean value is 5.64 Å, and the RMS deviation is 0.23 Å. Again, the difference of 0.24 Å is hardly significant except if an increase of the distance is observed in all six molecules, and this is the case. Furthermore, this difference of 0.24 Å is larger than the difference of 0.1 Å observed in the NMR structure of hTXN1 (Qin et al. 1994). The main difference allowing to bring together the two sulfur atoms must thus concern the side chains of the Cys residues. As can be seen in Figure 4, A and B, during the transition between the reduced and the oxidized states, the side chains of Cys34 residues remain practically unaffected while the side chains of Cys31 move in the direction of Cys34 to form the disulfide bridge between the two sulfur atoms; this movement is already sensible at the level of the C^β atom of Cys31. The situation is rather different in the

crystal structure of human hTXN1 (Weichsel et al. 1996): Even if the principal motion also concerns the side chain of Cys32, the authors noticed a movement of Cys32 toward Cys35 through shifts in Ala29–Cys35 (equivalent to Ala28–Cys34 in hTXN2) and a slight movement of Cys35 toward Cys32, with the most pronounced movement (~ 1 Å) at the level of the side chain of Trp31. In hTXN1, this residue is partially disordered in the reduced form and well-ordered in the oxidized state. As illustrated in Figure 4B, in hTXN2, we do not observe such a movement of the equivalent residue, Trp30. In the central molecule (B), the largest movement at the level of individual pairs of atoms of the indole ring is < 0.15 Å and if we consider the ensemble of the six independent molecules, no particular tendency is detectable between this side chain in the oxidized or the reduced form. Furthermore, in hTXN2, the Trp30 residue is well-defined in the electron density in both the oxidized and the reduced states, as shown in Figure 4, C and D. In the NMR structure of hTXN1 (Qin et al. 1994), the transition between the oxidized and the reduced states is mainly accomplished by a change in the χ_1 angle of Cys35, while in the crystal structure of hTXN2, the equivalent residue, Cys34, is practically unaffected and the main change occurs at the level of Cys31. A situation similar to that observed in the crystal structure of hTXN1 is also present in the transition between the oxidized and the reduced states of TXN from *Drosophila melanogaster* (Wahl et al. 2005), where a concerted motion of the Cys32 sulfhydryl group and the Trp31 indole ring is envisaged to support withdrawal of Cys32 from Cys35 upon reduction. In spinach chloroplast TXN m (Capitani et al. 2000) a rotation around the χ_1 torsion of Cys37 moves its sulfhydryl group away from Cys40 but no particular movement of Trp36 is observed, and this behavior is more similar to our observations in hTXN2. As is also the case in the crystal structure of human hTXN1 (Weichsel et al. 1996), the position of Cys31 with respect to Cys34 is stabilized in the oxidized state by a hydrogen bond between S^γ of Cys31 and the amide nitrogen of Cys34 (mean distance 3.25 Å) and this hydrogen bond disappears in the reduced state (mean distance 4.01 Å). In the solution structure of human hTXN1 (Qin et al. 1994), this hydrogen bond is described in both the oxidized and the reduced states, and appears to be involved in the stabilization of the anionic form of Cys32 by reducing Cys32 pK_a value. We also observe in the reduced state an alignment of the Cys31 and Cys34 sulfhydryls favorable for the formation of an hydrogen bond between Cys34 S^γ acting as a proton donor and Cys31 S^γ as a proton acceptor, as described in the crystal structure of hTXN1 (Weichsel et al. 1996). In the present case, the mean S^γ – S^γ distance is 3.63 Å and the mean $C31C^\beta$ – $C31S^\gamma$ – $C34S^\gamma$

and $C31S^\gamma$ – $C34S^\gamma$ – $C34C^\beta$ angles are 79.2° and 104.4° , respectively. The corresponding values in hTXN1 are 3.9 Å, 70° , and 105° and in the neutron structure of L-cysteine (Kerr and Ashmore 1975) 3.85 Å, 69.8° , and 95.9° . This hydrogen bond is supposed to be implicated in the depressed Cys31 pK_a (Weichsel et al. 1996). According to the proposed mechanism (Kallis and Holmgren 1980), the redox reaction would require a thiolate on Cys31 of hTXN2. At the acidic pH used for hTXN2 crystallization, both active site SH-groups are certainly protonated and the hydrogen bond is thus between thiol–thiol forms; this is also the case in the neutron structure of L-cysteine. Another possible reason for the depressed Cys31 pK_a value is the stabilization through interaction with the dipole of $\alpha 2$ helix (Kortemme and Creighton 1995). As in the crystal structure of hTXN1 (Weichsel et al. 1996), the dipole is pointed in the correct direction for stabilization of Cys31 thiolate anion but Cys34 is also close to the N terminus of the helix and has a normal pK_a value (Jeng et al. 1994). Thus, this interaction does not seem to be the main explanation of the lowered pK_a of Cys31. During the transition between the two states, rigid body motions of the helices relative to the underlying sheet are described in the NMR structure of hTXN1 (Qin et al. 1994), and as can be seen in Figure 4A, this effect is not observed in the central molecule of hTXN2 or in the other molecules (data not shown).

Dimerization of hTXN2 in the crystal

In the crystal structure of hTXN1 (Weichsel et al. 1996), the formation of regulatory homodimers is described, in which two monomers are related through a crystallographic twofold axis. These dimers exist in the oxidized and in the reduced states and are stabilized by a disulfide bond between residues Cys73 of two monomers. The dimer is also observed in the C73S mutant where the two Ser residues are connected by a hydrogen bond. Similar dimers are also present in hTXN2 (oxidized and reduced states) in spite of the impossibility to form the disulfide bond (or the hydrogen bond as in the C73S mutant of hTXN1) since Cys73 is not present in the sequence of hTXN2, and the corresponding residue in the structural alignment is an alanine (Ala73). Three dimers are formed between molecules A and B, C and D, and E and F, respectively, and as shown in Figure 5, A and B, in each case, the two monomers are related through a noncrystallographic twofold axis. The contacts involve 15 residues belonging to the three loops presented in the upper part of Figure 2A ($\beta 2$ – $\alpha 2$, $\beta 3$ – $\alpha 3$, $\alpha 3$ – $\beta 4$), to the N-terminal part of $\alpha 2$ and to $\alpha 3$. Four of these residues (30–33) belong to the active site. About 490 Å² per monomer are buried in this dimerization. The

Figure 5. (A) Dimer formed by molecules A (blue) and B (red) of hTXN2 in the oxidized state. The view is perpendicular to the noncrystallographic twofold axis. The side chains of some important residues are shown: Trp30, Cys31, Cys34, and Asp60. (B) Disposition of the three dimers in the unit cell. (C) Schematic representation of the contacts producing two dimensional polymers in the x,y plane. Molecules C', D', E', and F' are symmetry-related (lattice translation) molecules. The thick lines correspond to the dimer formation, the thin lines are the principal bonds between adjacent dimers, and the dotted lines are less systematic contacts. (D) Fragment of one slice of the two dimensional polymer formed in the crystallographic a,b plane. Each molecule is represented as a C α trace with the same coloring as in Figure 5B. The crystallographic *a* axis is horizontal.

contacts are mainly hydrophobic, Trp30 is the most concerned residue, and Ile59, Ala66, Ile67, and Val74 are also involved. In hTXN1, an important movement of Trp31 (equivalent to Trp30 in hTXN2) toward Trp31' of the other monomer was noticed upon oxidation: The distance between the C ζ^2 side-chain atoms from each monomer was reduced from 5.6 Å in the reduced state to 4.0 Å in the oxidized state. In hTXN2, this movement is completely absent since the mean distance between Trp30 C ζ^2 atoms remains 4.1 Å in the oxidized or the reduced dimers. An important hydrogen bond is also observed at the dimer interface between the side chains of Asp60 belonging to the two monomers. This hydrogen bond is only possible if Asp60 is protonated. It is possible that the proximity of Asp58 affects the protonation state of Asp60 by increasing the Asp60 pK_a value, as in the crystal structure of hTXN1 (Weichsel et al. 1996). The bond is very strong in the oxidized state, with a mean distance of 2.46 Å between the two O δ^1 oxygen atoms and slightly looser in the reduced state with a mean distance of 2.92 Å (in the reduced state, the C–D dimer was not taken into account, since the side

chain of residue 60 of molecule D is not well-defined, as explained in Results). Again, a rather different situation occurs in hTXN1: This hydrogen bond is well-observed in the reduced state but disappears in the oxidized state and is replaced by a hydrogen bond between Asp60 and the indole nitrogen of Trp31 in the same monomer. In hTXN2, an intramolecular hydrogen bond between Asp60 and Trp30 N ϵ^1 remains present in all cases. Surprisingly, this intramolecular hydrogen bond introduces some dissymmetry between the two monomers. In molecules A, C, and E, Trp30 N ϵ^1 is bonded to Asp60 O δ^1 , the oxygen atom that also takes part to the intermolecular bond with Asp60', while in molecules B, D, and F, Trp30 N ϵ^1 is bonded to Asp60 O δ^2 . Furthermore, in molecules A and E, an additional intermolecular hydrogen bond occurs between Asp60 O δ^2 and Thr63 O γ of the other monomer (B and F, respectively). The mean length of this hydrogen bond remains unaltered in the oxidized or the reduced state, at ~2.65 Å. There is thus some complicity between the dissymmetry of the hydrogen bonds involving Asp60, Trp30, and Thr63, which could explain why only approximate (noncrystallographic)

twofold symmetry exists between the monomers of hTXN2 while a strict crystallographic symmetry is present in hTXN1. The remaining intermolecular hydrogen bonds described in hTXN1 are not directly observed in hTXN2. The bonds involving Ser67 O^γ are not possible because the structural alignment of the sequences shows that this residue is replaced by Ile67 in hTXN2. Finally, the short intermolecular β-sheet of hTXN1 composed of two short strands (72–74) is now separated in hTXN2 by two water molecules. These solvent molecules are hydrogen bonded to each other (mean distance 2.84 Å), and each of them is also bonded to the amide nitrogen atom of residue 74 in one monomer and the carbonyl oxygen atom of residue 72 in the other monomer. Again, the situation is exactly the same in the oxidized or in the reduced state, except that in the reduced EF dimer, only one of the two water molecules is observed, but there remains some weak unexploited electron density corresponding to the second one. As a conclusion concerning the dimer formation, the hydrophobic contacts in hTXN2 are very similar to those observed in hTXN1 while the disulfide bond is absent and the hydrogen bonds are rather different. There are nearly no movements in the dimer interface of hTXN2 when changing from the oxidized to the reduced states while important modifications are observed in hTXN1. It was also tempting to postulate that the regulatory function of this dimerization could exist in hTXN2 at least at low pH (pH 4.6) since one of the catalytic residues (Cys31) remains buried in the dimer formation, as is also the case with Cys30 in hTXN1. However, analyses of hTXN2 on gel filtration columns in Tris-HCl buffer at pH 7.5 and pH 4.6 yielded a peak at 13 kDa, consistent with the existence of a monomer in solution at neutral as well as at low pH (data not shown).

Polymerization of hTXN2 in the crystal

As can be seen in Figure 5, B, C, and D, there are close contacts between adjacent dimers, A–B being in contact with D–C and F–E in the same unit cell, but also with D'–C' (equivalent position $x, y - 1, z$) and with F'–E' (equivalent position: $x + 1, y, z$). The result is thus a two-dimensional polymer in the crystallographic a, b plane, and this particular feature is completely new since it was not observed in any TXN crystal structure up to now. The contacts A–D, F–A, C–B, and B–E are very similar, and the mean buried surface is 390 Å² per monomer, slightly less than for the dimer formation. They involve in the first molecule of the quoted pairs (A, F, C, B) residues from the α2 helix, the β5–α4 loop, and the N-terminal part of α4, and in the second molecule (D, A, B, E) residues from the β5 strand and the C-terminal part of α4. The principal hydrophobic contacts involve Ile36, Ile92 in the first

molecule and Val86, Leu105 in the second one. A hydrogen bond is also observed between Arg40 N^{η2} (first molecule) and the carbonyl oxygen atom of Lys104 (second molecule). Since in the first molecule of the pairs (A, F, C, B) the N-terminal part of the α2 helix takes part to the contacts, these contacts contribute to burying the active site. Surprisingly, this feature does not concern molecules D and E. The four principal contacts with lattice translated molecules, A–D', F'–A, C'–B, and B–E' are also very similar and bury a mean surface of 480 Å² per monomer in the first molecule (A, F', C', B) and of 530 Å² per monomer in the second one (D', A, B, E'), values comparable to those involved in the dimer formation. The secondary regions concerned in these contacts are α1 and α3 in the first molecule and β1, α2 and the α2–β3 loop in the second molecule. There are mainly three hydrogen bonds to be quoted: Glu68 O^{ε1}–His49 N^{ε2}, Asp64 O^{δ1}–Thr1 N, and Asp64 N–Ser0 O^γ. The buried surface involved in the remaining contacts (A–E', B–D', and D–E') are ~380 Å² per monomer in the first two cases and 490 Å² in the last one. Since these contacts are less systematic and only concern some of the molecules, they will not be described in detail. It is, however, worth noting that in the first two cases, the N-terminal part of the α2 helix of the second molecule (E', D') is involved and that this also contributes to burying their active site. These are the two molecules (D and E) that were surprisingly missing in the previous list of additional burying. As shown on Figure 5D, the two-dimensional polymerization results in the formation of juxtaposed octamers of dimers. Each octamer has the shape of a lozenge: The corners of the lozenges are occupied by A–B dimers, while the other dimers (C–D and E–F) are located along the edges of the lozenges. The diameter of the central cavity is ~20 Å. There are only a few contacts between two polymeric slices. They involve molecule C of one polymer and two F molecules belonging to the adjacent one, and vice versa. The aggregation of TXN molecules was already observed for reduced *Escherichia coli* TXN at low pH (Laurent et al. 1964). In order to determine if intermolecular contacts similar to those observed in hTXN2 polymerization are also possible in *E. coli* TXN, a structural alignment was performed between the X-ray structure of *E. coli* TXN (Katti et al. 1990) and hTXN2 (data not shown). The principal residues implicated in the hydrophobic contacts and in the hydrogen bonds described above were superposed upon residues incompatible with similar contacts. The modes of aggregation are thus not equivalent.

Possible interactions between hTXN2 and hPRDX5

In order to understand the recognition mechanism between hTXN2 and one of its target protein in mitochondria,

peroxiredoxin 5 (PRDX5) (Knoops et al. 1999; Declercq et al. 2001), profit was reaped from the availability of the crystal structure of *Haemophilus influenza* hybrid Prx5 (Kim et al. 2003). This hybrid Prx5 structure reveals the presence of two domains: a Prx5 domain homologous to hPRDX5 and a glutaredoxin (Grx) domain. Like TXN, Grx is an electron donor protein, capable of reducing an oxidized target protein. Interestingly, in this crystal structure, Prx5 and Grx domains belonging to different monomers interact with each other and are both in the reduced form. Furthermore, it was shown that this interaction exists independently on redox conditions (Kim et al. 2003). It can thus be postulated that this kind of interaction between the electron donor protein and the electron acceptor target protein can exist before the establishment of the disulfide bond to be reduced in the target protein. In the A(Prx5)–D(Grx) interaction, the peroxidatic cysteine residue of the Prx5 domain is located in the N-terminal part of the $\alpha 2$ helix as in the available structure of the reduced form of hPRDX5 (PDB code 1hd2; Declercq et al. 2001). In the A(Prx5)–D(Grx) contact, the major interaction force is derived from two charge interactions: negative on the Prx5 domain and positive on the Grx domain. For simulating a similar interaction between reduced hTXN2 and reduced hPRDX5, the reduced molecule B of hTXN2 was superposed on D(Grx) and human PRDX5 on A(Prx5) of the hybrid Prx structure, using “brute force” and “improve” procedures of the program LSQMAN (Kleywegt and Jones 1997). In both cases, this structural alignment resulted in a good spatial coincidence of the cysteine residues of the active sites. In the formation of this complex, surfaces of 155 Å² and 127 Å² are buried, respectively, in the hPRDX5 and hTXN2 molecules, values to be compared to 255 Å² and 222 Å² in hybrid Prx5. In hPRDX5, these contacts involve Pro45 (11 Å²), Gly46 (10 Å²), Lys49 (100 Å²), Thr50 (7 Å²), and Leu149 (27 Å²), and in hTXN2, Trp30 (34 Å²), Asp58 (1 Å²), Asp60 (66 Å²), Asp61 (9 Å²), and Thr63 (17 Å²). As shown in Figure 6, the three Asp residues (60, 61, 58) of hTXN2 involved in this contact form a negatively charged cavity with the most exposed residue (Asp60) located in the bottom part. Simultaneously, Lys49 of hPRDX5 appears as a positively charged protuberance, which could fit the cavity of hTXN2. At the same time, Trp30 of hTXN2 forms a hydrophobic contact with Leu149 and, eventually, Thr50 of hPRDX5. This also brings Cys31 of hTXN2 in close vicinity to the pocket containing the peroxidatic residue Cys47 of hPRDX5. These two Cys residues (hTXN2–Cys31 and hPRDX5–Cys47) are too far away (17 Å) for a direct interaction but it must be kept in mind that both molecules are in the reduced state and that the oxidation of hPRDX5 probably involves an unwinding of the N-terminal part of the $\alpha 2$ helix containing Cys47 (Choi et al. 2003; Evrard et al. 2004). Such an unwinding could bring the two cysteine

Figure 6. Molecular surfaces colored according to the local electrostatic potential, ranging from blue (the most positive region) to red (the most negative). Two orientations of hPRDX5 appear in *A* and *C*, and two orientations of hTXN2, in *B* and *D*. The residues involved in the proposed intermolecular contacts between hPRDX5 and hTXN2 are labeled and contoured by a yellow line. The orientation of *C* and *D* allows for seeing the protruding Lys49 in hPRDX5 and the cavity of hTXN2 at the bottom of which lies Asp60.

residues much closer to each other to allow for the formation of a transient intermolecular disulfide bridge supposed to be formed in the mechanism of reduction of PRDXs by TXNs (Hofmann et al. 2002). It must be pointed out that some residues of hTXN2, Trp30, Asp60, and Thr63, putatively involved in hTXN2–hPRDX5 contacts, take part in the hTXN2 dimer formation too. Thus, the interaction between hTXN2 and hPRDX5 is impossible with the dimeric form of hTXN2 observed in the crystal structures but is compatible with the existence of a monomeric hTXN2 in solution, detected by gel filtration analyses.

Materials and methods

Expression and purification of hTXN2

Human *TXN2* cDNA (GenBank accession number NM_012473; Damdimopoulos et al. 2002) cloned into pCR2.1 (Invitrogen) was amplified by PCR using forward primer 5'-AGGATCGGATCCACAACCTTTAATATCCAGGATG-3' (BamHI site underlined) and reverse primer 5'-ATCCCTAAGCTTCAGCCAATCAGCTTCTTCAG-3' (HindIII site underlined). The PCR product coding for mature hTXN2 without its predicted mito-

chondrial presequence (Damdimopoulos et al. 2002) was digested with BamHI and HindIII, and ligated into pQE-30 expression vector (Qiagen). The insert was sequenced and the N-terminal fusion with the hexahistidine (6×His) tag was confirmed. The resulting vector was used to transform *E. coli* strain M15 (pRep4). *E. coli* were grown at 37°C in LB medium containing 1 mM IPTG. Pelleted cells were lysed in 10 mM imidazole, 50 mM phosphate, 300 mM NaCl (pH 8) by sonication and clarified by centrifugation. The supernatant containing 6×His-tagged hTXN2 was loaded onto Ni²⁺-NTA column (Qiagen). The column was washed and the protein was eluted with 50 mM phosphate, 300 mM NaCl, 250 mM imidazole (pH 8). Eluted protein was then dialysed against PBS (pH 7.2) and stored at −20°C before use for crystallization.

Crystallization

The initial hTXN2 protein crystals were grown in 0.2 M ammonium sulfate, 0.1 M sodium acetate buffer (pH 4.6), 22% (w/v) PEG 3350, 1 mM 1,4-dithio-dl-threitol (DTT) as antioxidant, and 0.02% (w/v) sodium azide by the hanging drop vapor diffusion method at 18°C, using a protein concentration of 6 mg mL^{−1}. The volume of the crystallization solution was 500 µL, and the crystallization drop was formed by mixing equal amounts (2 µL) of the protein solution and of the crystallization solution. Crystals appear after 3 d. They look like thin plates and grow to a size of 0.4×0.4×0.05 mm³. All of the attempts to grow crystals in the absence of DTT were unsuccessful. The oxidized form of hTXN2 was obtained by soaking already grown crystals in 1 mM H₂O₂ for 90 sec. Many unsuccessful trials were made to get the reduced form of hTXN2 by soaking in DTT or β-mercaptoethanol at different concentrations and for different durations, and finally it was obtained by soaking crystals for 90 sec in 10 mM Tris(hydroxymethyl)phosphine.

Data collection

Before data collection, the crystals were soaked for a few seconds in a cryosolution similar to the mother liquor but containing 20% glycerol as cryoprotectant, and flash-cooled at 100 K. Some statistics of data collection and processing of the three crystals are presented in Table 1.

Data processing, structure solution, and refinement

The diffraction images of the different crystals were processed and merged with the XDS program package (Kabsch 1993). The crystals are triclinic, space group P1, with six molecules in the asymmetric unit. A self-rotation function allowed for identifying the presence of three more or less coplanar noncrystallographic twofold axes and one fourfold axis perpendicular to this plane. The initial hTXN2 structure solution was attempted by molecular replacement, using human hTXN1 (Weichsel 1996) as model (PDB code 1ert). The sequence identity of the model is 35.2% and probably due to the high number of molecules in the asymmetric unit and the lack of symmetry of the space group; all of the attempts using classical molecular replacement programs (Amore, Molrep, CNS, X-plor, EP-MR) were unsuccessful. Finally, the structure was solved by the program BEAST (Read 2001) based on the maximum

Table 1. Statistics of data collection and refinement

	Initial hTXN2	Oxidized hTXN2	Reduced hTXN2
Data collection			
Space group	P1	P1	P1
Cell dimension			
a (Å)	49.038	49.060	49.580
b (Å)	49.087	49.140	49.610
c (Å)	78.977	78.710	80.020
α (°)	87.96	87.75	87.77
β (°)	83.19	82.77	82.30
γ (°)	79.08	79.20	79.31
Synchrotron source	DESY- EMBL, Hamburg	ESRF, Grenoble	DESY- EMBL, Hamburg
Beamline	BW7B	BM30A	BW7A
Detector type	MAR 345	MAR CCD	MAR CCD
Wavelength (Å)	0.8441	0.9799	0.9686
Resolution (Å)			
Overall (ov)	23.5–2.0	19.3–1.8	19.4–2.0
Highest shell (hs)	2.2–2.0	1.9–1.8	2.1–2.0
Measured reflections	114,426	165,308	130,833
Unique reflections	47,020	65,984	50,114
Completeness (%)			
ov/hs	96.9/90.4	99.3/99.9	97.1/97.8
R _{sym}	0.060/0.22	0.055/0.27	0.071/0.24
I/σ(I) ov/hs	9.72/4.22	11.67/3.4	11.42/4.29
Refinement			
R factor (R _{free})	0.1997	0.1692	0.2170
Overall	(0.2652)	(0.2283)	(0.2841)
Highest shell	0.354	0.218	0.269
	(0.367)	(0.294)	(0.357)
Estimated overall coordinate error (Å)			
based on			
R _{free}	0.193	0.128	0.203
Maximum likelihood	0.153	0.098	0.171
R.M.S deviations from ideality			
Bonds (Å)	0.023	0.012	0.022
Angles (°)	2.017	2.947	2.324
Mean B value (Å ²)			
before TLS refinement			
Main chain	32.37	24.21	28.57
Side chain	36.04	27.36	31.58
Solvent	40.63	35.88	35.39

likelihood principle. The knowledge of the noncrystallographic symmetry (NCS) was essential for selecting the correct rotation values. Electron density maps were interpreted using O (Jones et al. 1991). After applying the NCS-phased refinement procedure available in the CCP4 suite (Collaborative Computational Project, Number 4 1994), the refinement was pursued with REFMAC5 (Murshudov et al. 1997). Since the oxidized hTXN2 and reduced hTXN2 structures were isomorphous with the initial hTXN2 structure, only some manual adjustments with O (Jones et al. 1991) were necessary and followed by refinement using REFMAC5 (Murshudov et al. 1997). In all cases, during the final steps, the hydrogen atoms were incorporated in riding positions and the mean-square displacements of rigid bodies were refined, each polypeptide chain

being defined as a different TLS group. Some refinement statistics are given in Table 1.

Graphics

The figures were prepared using MOLSCRIPT (Kraulis 1991) and RASTER3D (Merritt and Bacon 1997) or using O (Jones et al. 1991) and Molray (Harris and Jones 2001). Figure 6 was prepared using GRASP (Nicholls et al. 1993).

Protein Data Bank accession numbers

Atomic coordinates and structure factors have been deposited in the Protein Data Bank. Accession codes are 1uvz (initial hTXN2), 1w4v (oxidized hTXN2), and 1w89 (reduced hTXN2).

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