**1H, 13C, and 15N assignment of the oxidized and reduced forms of T. brucei glutathione peroxidase-type tryparedoxin peroxidase**

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**Abstract** The cysteine-homologues of glutathione peroxidases in Trypanosoma brucei catalyze the trypanothenione/tryparedoxin-dependent reduction of hydroperoxides. We report the 1H, 13C, and 15N assignment of the oxidized and reduced form of the enzyme by NMR. Major changes between these two forms were only observed for residues close to the catalytic site.

**Keywords** T. brucei · Peroxidase · Tryparedoxin · Resonance assignment

**Biological context**

Trypanosoma brucei is the causative agent of African sleeping sickness. Trypanosomatids possess a unique thiol metabolism where the ubiquitous glutathione system is replaced by a trypanothione system (Fairlamb and Cerami 1992; Krauth-Siegel et al. 2005). Trypanothione (T(SH)2; N1,N8-bis(glutathionyl)spermidine), the main low molecular weight thiol of trypanosomatids, is kept in the reduced state by the flavoenzyme trypanothione reductase, which catalyzes the NADPH dependent reduction of trypanothione disulfide. In the mammalian host, trypanosomes are exposed to various reactive oxygen species such as hydrogen peroxide and superoxide anions, but their ability to cope with oxidative stress is surprisingly weak. Trypanosomatids lack catalases and classical selenocysteine-containing glutathione peroxidases but have 2-Cys peroxiredoxins (Nogoceke et al. 1997; Tetaud et al. 2001; Flohe et al. 2002) and cysteine-homologues of classical glutathione peroxidases (Wilkinson et al. 2000; Hillebrand et al. 2003; Schlecker et al. 2005). RNA interference studies revealed that both, 2-Cys peroxiredoxins and cysteine-homologues of classical glutathione peroxidases are essential for T. brucei. With NADPH as final electron donor, the reducing equivalents flow from trypanothione onto tryparedoxin (Gommel et al. 1997; Comini et al. 2005) and finally the peroxidase which then catalyzes the reduction of hydroperoxides (Nogoceke et al. 1997; Gommel et al. 1997).

**Methods and experiments**

**Cloning.** The T. brucei tryparedoxin peroxidase III gene was amplified by PCR from a pQE-30 plasmid (Quiagen) using a 5'-primer that lacked the mitochondrial targeting sequence starting at S13 (CAC83394) and a 3'-primer using the native C-terminus of the protein (residues 13–176 of the native sequence). The PCR product was cloned using the 3'-Acc65I and 5'-NcoI restriction sites into a modified pET-9d vector containing a TEV-protease-cleavable thioreredoxin- His6 fusion-vector which was kindly provided by Gunther Stier. The vector was transformed into E. coli BL21(DE3). The expression medium used for NMR
Experiments was LB or M9 minimal medium supplemented with $^{15}$NH$_4$Cl ± [U-$^{13}$C]-glucose. Deuterated Px III was expressed using Silantes OD$_2$ CDN medium. Selectively labeled protein was obtained expressing Px III in M9 medium containing unlabeled NH$_4$Cl and glucose. In addition, 100 mg of each amino acid were added to the medium except those which should be labeled. The cells were grown at 37°C to an OD$_{600}$ of 0.6. Expression was induced by adding 0.3 mM isopropyl-$\beta$-D-thiogalactopyranoside over night at 18°C. For selective labeling, the $^{15}$N or $^{15}$N/$^{13}$C labeled amino acids were added 10 min after induction.

The thioredoxin-His$_6$-tagged protein was purified over a Ni-NTA (Qiagen) column, subsequently digested with TEV protease and passed over a second Ni-NTA column to remove both the fusion tag and the His$_6$-tagged TEV-protease. Finally, the protein was applied on a PD10 column equilibrated with NMR buffer (50 mM potassium phosphate, 100 mM NaCl, pH 6.8). The monomeric protein was concentrated to 1.2 mM and for NMR experiments 10% D$_2$O was added. For experiments regarding the reduced form of the protein the intramolecular disulfide bond of Px III was reduced by adding DTT to a final concentration of 10 mM. Reoxidation was prevented by filling of the NMR tube with argon.

The $^1$H, $^{13}$C and $^{15}$N resonances of Px III with the intramolecular disulfide bond (oxidized form, Fig. 1) were assigned by standard triple resonance experiments (HNCACB, HNCA, CBCA(CO)NH, NH detected side chain TOCSY (H(CCO)NH, (H)C(CO)NH) and HCCH-TOCSY acquired at 34°C (Sattler et al. 1999). Experiments were recorded on Bruker DRX500, DRX600, DRX800, and DRX900 spectrometers equipped with cryoprobes. Since 40 NH resonances were missing, HNCA and CBCA(CO)NH were repeated at 22°C yielding 10 new resonances. Unfortunately, the HNCACB only showed the strongest 63 signals at this temperature whereas the majority of resonances were missing.

The reduced form of PxIII (free thiol groups, Fig. 2) precipitated at high concentrations (1 mM) at 34°C, so that the backbone assignment (HNCACB, HNCA, HN(CO)CA and CBCA(CO)NH) had to be performed at 22°C which required deuteration of the protein.
Assignments and data deposition

Complete chemical shift assignment was achieved for all secondary structure elements regions whereas in the large loops several resonances were exchange broadened. In the reduced form more residues were exchange broadened than in the oxidized protein. In the oxidized form (Fig. 1) resonances of residues 1, 37, 39–42, 68–70, 121, 124, and 126–130 were missing in the 1H–15N-HSQC (Fig. 1). The chemical shifts of the residues 37, 40, 41, 69, 70, 121, 126, 127, 129, and 130 could be assigned in a combination of 13C-edited TOCSY, 13C-edited NOESY and 15N-edited NOESY. In addition to the non-detectable resonances of the oxidized form, the signals of residues 38, 43, 44, 83, 85, and 86 were exchange broadened in the reduced protein. Residues 87 and 41, however, could be detected at 500 MHz. For residues 43 and 44 chemicals shift assignment was achieved in the 13C-edited NOESY. Selective labeling with 15N- and/or 15N–13C- labeled Lys, Ala, Thr, Phe, Tyr or Cys proved that the HN15N resonances of the majority of these residues were really line broadened and not hidden by spectral overlap.

Comparison of the reduced and oxidized protein shifts revealed that in the reduced protein numerous resonances showed significant chemical shift changes or disappeared due to exchange broadening. The majority of these resonances were located close to the redox active cysteines.

The chemical shifts have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under accession number 15597 (oxidized form) and 15598 (reduced form).

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