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The conserved Cys76 plays a crucial role for the conformation of reduced glutathione peroxidase-type tryparedoxin peroxidase

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ABSTRACT

The crystal structure of reduced tryparedoxin peroxidase shows Cys47 close to Gln82 and Trp137 and helix formation of residues 87 to 97 whereas the NMR structure of the reduced C76S mutant adopts a different conformation similar to the oxidized protein. Circular dichroism (CD), fluorescence and NMR spectroscopy reveal that the fully active C76S mutant differs from the wildtype (WT) enzyme mainly in its reduced form both in secondary structure content and Trp137 environment. This implies that Cys76 plays a critical role for the reduced enzyme assuming different conformational states and that the catalytic triad may only be necessary as short-lived intermediate during catalysis.
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1. Introduction

Trypanosoma brucei, the causative agent of African sleeping sickness, encodes three nearly identical genes for two cytosolic (Px I and Px II) and a mitochondrial (Px III) form of glutathione peroxidase-type enzymes, which functionally act as tryparedoxin peroxidases (for recent reviews see [1,2]). In the first half reaction of catalysis, the enzyme with Cys47 and Cys95 in the dithiol state reacts with the hydroperoxide substrate generating the oxidized peroxidase with an intramolecular disulfide. The second half reaction describes the interaction with tryparedoxin and thus regeneration of the reduced enzyme [3]. In addition to the redox active dithiol/disulfide couple, *T. brucei* Px III contains Cys76. A cysteine at this position is highly conserved throughout the glutathione peroxidase protein family independent of the nature of the active site residue being selenocysteine or cysteine. However, the functional role of the residue is not clear. In enzymes from Chinese cabbage and *Drosophila melanogaster*, replacement of the cysteine by a serine [4,5] or alanine [5] residue, lowers the peroxidase activity by 30–70%. Substitution of Cys76 by a serine residue in *T. brucei* Px III results in a mutant with wildtype (WT) activity [3]. Similarly, replacement of the cysteine by Gly or Ser did not significantly perturb the catalytic activity of *T. cruzi* Px I [6]. Comparison of more than 400 sequences revealed that nearly 10% of the glutathione peroxidases possess a genuine serine or alanine at this position [7].

Recently we have reported on the crystal structure of the oxidized C76S mutant of *T. brucei* Px III as well as on the NMR structures of the oxidized and reduced protein species [8]. In parallel, the crystal structure of reduced WT Px II has been published [9]. The NMR structure of reduced C76S Px III showed a high overall similarity to that of the oxidized protein. No major conformational rearrangements were observed that would allow formation of a catalytic triad between Cys47, Gln82 and Trp137. In contrast, the structure of the reduced WT Px II revealed the presence of the catalytic triad as well as a newly formed helix from Glu87 to Lys97 [9]. In order to unravel these discrepancies we followed the reduction of WT and C76S Px III by circular dichroism (CD), tryptophan fluorescence, and NMR spectroscopy.

2. Materials and methods

2.1. Expression and purification of the Px III species

Recombinant WT and C76S Px III (without mitochondrial signal sequence) were prepared as previously described for the mutant [8]. The concentration of the stock solution was determined by

Abbreviations: CD, circular dichroism; Px, glutathione peroxidase-type tryparedoxin peroxidase; RmsD, root mean square deviation; WT, wildtype

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absorption spectroscopy using the theoretical extinction coefficient of 17 545 $M^{-1}cm^{-1}$ which perfectly agrees with the experimental data showing that a 1 mg/ml solution of Px III corresponds to a $\Delta A_{280\ nm}$ of 1.0 [10]. For selective labeling with ^{15}N-tryptophan, the M9 minimal medium was supplemented with all unlabeled amino acids apart from tryptophan. To avoid scrambling, a two-fold additional excess of unlabeled amino acids was added at the moment of induction, and the ^{15}N-tryptophan was added 10 min later [11]. The bacterial cell pellet was harvested 1.5 h after induction.

2.2. CD spectroscopy

CD spectra were recorded at 20 °C on a Jasco J-810 spectropolarimeter (Jasco Co., Tokyo, Japan) in a cuvette with 1 mm path length and a water-thermostated cell holder. The protein (15 μM) was dissolved in 20 mM potassium phosphate, pH 6.8, 100 mM NaF. After recording the spectrum of the oxidized proteins, DTT was added to a final concentration of 1 mM and spectra were recorded at the time points indicated. The spectra are an average of three scans at a scan rate of 10 nm min^{-1}, 8 s response time and 1 nm band width and smoothened by the adaptive smoothing method, which is part of the Jasco Spectra Analysis software. The buffer spectrum was subtracted. Reproducibility was confirmed by using three different protein batches for each Px III variant and acquiring at least two spectra series for both Px III variants.

2.3. Fluorescence spectroscopy

Tryptophan fluorescence spectra were acquired at room temperature in a 1 cm cuvette on a Perkin Elmer LS 50B spectrometer. The proteins were dissolved at a concentration of 13 μM in the buffer used for the CD experiments, but NaF was exchanged against 100 mM NaCl. Fluorescence was excited at 290 nm and the emission was recorded from 300 to 450 nm. After the addition of 1 mM DTT (final concentration) spectra were recorded at different time intervals. The buffer spectrum was subtracted. Reproducibility was confirmed by using two different protein batches for each Px III variant and acquiring at least two spectra series for each.

2.4. NMR spectroscopy

^{1}H^{15}N-HSQC spectra were acquired at 25 °C on a Bruker Avance II 600 spectrometer equipped with a broadband triple resonance probe head with 128 scans per increment and a total of 128 increments in the indirect dimension. Both proteins were measured in 50 mM potassium phosphate, 100 mM NaCl, pH 6.8 at a concentration of 230 μM. To analyze the putative two conformations of the reduced enzyme species, the NMR samples were treated with 50 mM DTT. The spectra of the proteins with selectively labeled ^{15}N-tryptophan were acquired at a protein concentration of 130 μM. Data were processed and analysed with TOPSPIN software (Bruker Biospin, Karlsruhe).

3. Results

3.1. Comparison of the NMR structure of reduced C76S Px III with the crystal structure of reduced WT Px II

Superposition of the structures of reduced C76S Px III (2RM6) [8] and WT enzyme (2VUP) [9] yielded a root mean square deviation (RmsD) of 4.3 Å over the backbone atoms. If the regions Ser45(Ser37) to Cys47(Cys39), Asn77(Asn69) to Phe102(Phe94), and Thr132(Thr124) to Phe139(Phe131) were excluded [the numbering in brackets corresponds to that in Px II (2VUP)] the RmsD reduced to 1.0 Å, which revealed that the differences are located in the area around the catalytic centre. Whereas in the C76S mutant the long loop extending from Ser76(Cys68) to His116(His108) keeps its extended conformation observed in the oxidized structure (2RM5, 3DWV [8]), it moves to a new position with the concomitant formation of a long α-helix from Glu87(Glu79) to Lys97(Lys89) in WT Px II [9] (Fig. 1). Since α-helix 1 starts three residues earlier in the WT enzyme, the loop Ser45(Ser37) to Gly52(Gly44) also changes its position and places the peroxidatic

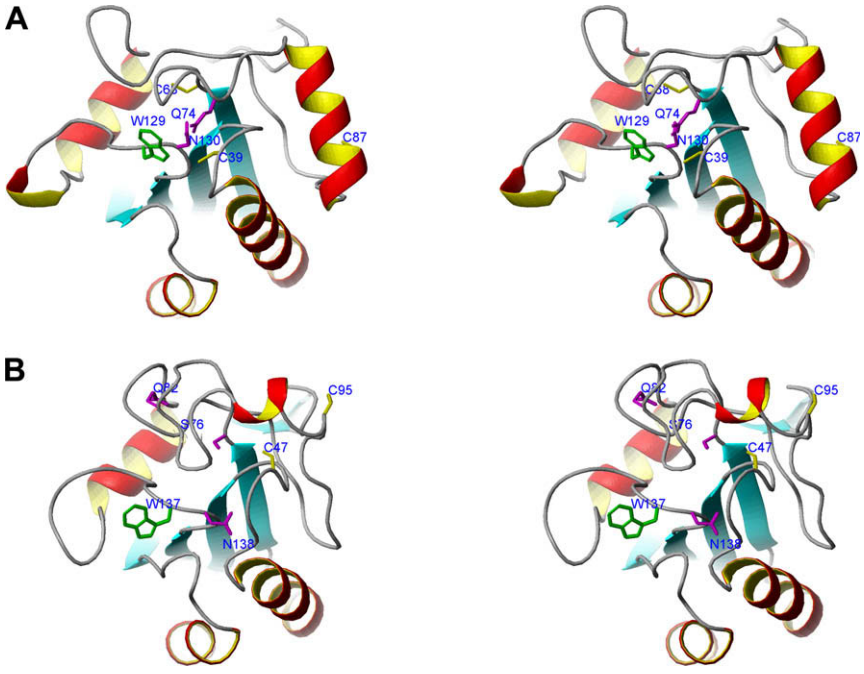

Fig. 1. Stereo view of partial structures of (A) reduced WT Px II (2VUP) [9] and (B) reduced C76S Px III (2RM6) [8] highlighting details of the catalytic site. The side chains of Cys39/Cys47, Cys87/Cys95, Cys68/Ser76, Gln74/Gln82, Trp129/Trp137 and Asn130/Asn138 in the WT/C76S protein, respectively, are depicted in colour. α-Helices are coloured in red and β-sheets in blue. The additional α-helix 87–97 harbouring Cys95 is seen on the right side of the WT structure.

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