Thioredoxin-2 but not Thioredoxin-1 is a Substrate of Thioredoxin Peroxidase-1 from Drosophila melanogaster

Isolation and characterization of a second thioredoxin in D. melanogaster and evidence for distinct biological functions of Trx-1 and Trx-2

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Running title: Distinct functions of the thioredoxins in Drosophila

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Abbreviations

Ag, Anopheles gambiae; cumeneOOH, cumene hydroperoxide; Dm, Drosophila melanogaster; DTT, dithiothreitol; EST, expressed sequence tag; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulfide; IgG, immunoglobulin G; IPTG, isopropyl-β-D-thiogalactopyranoside; M, molecular mass; Trx, thioredoxin; TrxR, thioredoxin reductase; TPx, thioredoxin peroxidase; tBuOOH, t-butyl hydroperoxide;
SUMMARY

As Drosophila melanogaster does not contain glutathione reductase the thioredoxin system has a key function for glutathione disulfide reduction in insects (Kanzok S. M., Fechner A., Bauer H. et al. Science 291, 643-647). In view of these unique conditions, the protein systems participating in peroxide metabolism and in redox signaling are of special interest. The genes for a second thioredoxin (DmTrx-2) and a thioredoxin peroxidase (DmTPx-1) were cloned and expressed, and the proteins were characterized. In its disulfide form, the 13 kDa protein thioredoxin-2 is a substrate of thioredoxin reductase-1 ($K_m = 5.2 \mu M$, $k_{cat} = 14.5 s^{-1}$) and in its dithiol form an electron donor of TPx-1 ($K_m = 9 \mu M$, $k_{cat} = 5.4 s^{-1}$). DmTrx-2 is capable of reducing glutathione disulfide with a second order rate constant of 170 M$^{-1}$ s$^{-1}$ at pH 7.4 and 25 °C. Western blot analysis indicated that this thioredoxin represents up to 1% of the extractable protein of D. melanogaster Schneider cells or whole fruit flies. Recombinant thioredoxin peroxidase-1 (subunit $M_r = 23$ kDa) was found to be a decameric protein that can efficiently use Trx-2 but not Trx-1 as a reducing substrate. The new electron pathway found in D. melanogaster is also representative for insects that serve as vectors of disease. As a first step we have cloned and functionally expressed the gene that is the orthologue of DmTrx-2 in the malaria mosquito Anopheles gambiae.
INTRODUCTION

Aerobic life conditions that are based on the metabolization of molecular oxygen are inseparably linked to the occurrence of reactive oxygen species (ROS). These compounds are cytotoxic both for the organism but even more so for invaders such as microorganisms and tumor cells. This oxidative challenge is counteracted by a shield of enzymatic and nonenzymatic defense systems including superoxide dismutase ($2 \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$), catalase ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$), peroxidases ($\text{ROOH} + \text{AH}_2 \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{A}; \text{AH}_2 = \text{electron donor}$) and the tripeptide glutathione (1).

Glutathione is the most abundant intracellular non-protein thiol compound. Being present in millimolar concentrations, reduced glutathione (GSH) serves as a powerful antioxidant and effective radical scavenger. The oxidized or disulfide form of glutathione (GSSG) is recycled to the thiol form by the NADPH-dependent flavoenzyme glutathione reductase (GR):

$$\text{NADPH} + \text{H}^+ + \text{GSSG} \rightarrow \text{NADP}^+ + 2 \text{GSH}$$

(2). GR occurs in many eukaryotic and prokaryotic organisms but not in dipteric insects such as *Drosophila melanogaster* and *Anopheles gambiae*. Thus the redox metabolism of these species and probably also of further insects differs significantly from most other forms of life (3). Since glutathione is a key player for intracellular redox homeostasis also in *D. melanogaster* (4), high GSH levels must be maintained by an alternative pathway. A potent candidate to substitute for GR is the thioredoxin system.

Thioredoxins (Trx) are small thiol proteins with a molecular weight of about 12 kDa that are present in all organisms studied so far. Reduction of the protein disulfide (TrxS$_2$) to the dithiol form (Trx(SH)$_2$) is catalyzed by the homodimeric NADPH-dependent flavoenzyme thioredoxin reductase (TrxR: NADPH + H$^+$ + TrxS$_2 \rightarrow \text{NADP}^+ + \text{Trx(SH)}_2$). TrxR is both structurally and mechanistically closely related to GR (5).
The main difference between GR and TrxR is an additional redox center located in the C-terminal region of high Mₚ thioredoxin reductases as they are found in most eukaryotes (6). Mammal TrxRs have an adjacent redox active cysteine-selenocysteine pair (7,8) whereas in the thioredoxin reductase of the malaria parasite *Plasmodium falciparum* this redox center is represented by two cysteines separated by a spacer of 4 amino acids (9). Thioredoxin reductase is also present in the fruit fly *Drosophila melanogaster*. These insect thioredoxin reductases (DmTrxR-1 and DmTrxR-2) are the prototype of a third class of high Mₚ TrxRs: they possess two neighboring C-terminal cysteine residues that are essential for thioredoxin reduction (3).

A large number of cellular functions are based on thioredoxins. They have an important role in DNA synthesis as electron donors for ribonucleotide reductase (10) and are involved in the cellular response to oxidative stress (11). Numerous disulfide bonds participating in protein folding or in redox control of transcription are effectively and selectively reduced by thioredoxins (12-14). Thioredoxins are also able to perform a dithiol-disulfide exchange reaction with glutathione disulfide (GSSG) thus producing GSH. In organisms possessing GR this reaction can serve as a backup system under conditions of GR insufficiency (15). In insects this normally supportive trait has evolved to a key pathway. Furthermore thioredoxins exert antioxidative functions as electron donors of thioredoxin peroxidases (TPx), protective enzymes that reduce hydrogen peroxide as well as organic peroxides. Thioredoxin peroxidases obviously have also regulatory functions in redox state-associated signaling, e.g. in pathways mediated by tumor necrosis factor-α (TNF-α) (16) or epidermal growth factor (EGF) (17).

In organisms containing different thioredoxins it is a key issue if these isoproteins differ not only in their intracellular localization but also in their function. A thioredoxin from *Drosophila* named deadhead gene product (dhd, DmTrx-1) has already been functionally characterized and recombinantly expressed (3,18). Here we introduce a second thioredoxin
(DmTrx-2) from *Drosophila melanogaster* and a closely related thioredoxin from the mosquito *Anopheles gambiae* (AgTrx-1). In order to specify the functions of DmTrx-2 we studied its reaction with DmTrxR-1 as well as the important dithiol-disulfide exchange with GSSG, and its role in peroxide detoxification. For this objective we recombinantly expressed and characterized thioredoxin peroxidase-1 from *D. melanogaster* (DmTPx-1). DmTrx-2 but not DmTrx-1 was found to be a substrate for this enzyme. This result indicates a clear separation of cellular functions between thioredoxins from the same organism.
EXPERIMENTAL PROCEDURES

Materials

Fruit flies, *Drosophila melanogaster* Schneider cell lines, *Anopheles gambiae* cDNA and cells were kindly provided by Dr. Hans-Michael Müller from the EMBL, Heidelberg, Germany. *E. coli* Trx and TrxR were gifts from Professor Charles H. Williams, Jr., University of Michigan, USA. HIS-tagged human thioredoxin mutant C73S (hTrx(C73S)) (19) and thioredoxin-1 from *Plasmodium falciparum* (PfTrx-1) (15) were recombinantly expressed in *E. coli*.

*D. melanogaster* thioredoxin reductase-1 (DmTrxR-1) and thioredoxin-1 (DmTrx-1) were expressed and purified as previously described (3). PCR chemicals and restriction enzymes were purchased from MBI Fermentas, precast polyacrylamide gels from Bio-Rad and molecular weight standards from Amersham Pharmacia Biotech. Antibiotics, substrates for enzymatic assays, and further chemicals were from BioMol, Roche Molecular Biochemicals, or Sigma. All compounds were of the highest available purity.

Cloning of *D. melanogaster* thioredoxin-2 (DmTrx-2)

*Drosophila* EST clone GH15403 containing the complete *dmtrx-2* gene was obtained from Research Genetics (Huntsville, AL, USA). For ligation into the expression vector pQE-30 (Qiagen), the *dmtrx-2* gene was PCR-cloned using the forward primer 

GCGC|GGATCC|GTG|TAC|CAG|GTG|AAA|GAT|AAG|G containing a *Bam*HI restriction site and CGC|AAGCTT|TTA|GAT|ATT|GGC|CTT|GAT|GAC|ATC as a reverse primer with a *Hind*III restriction site. The pET-30a vector (Novagen) was applied for expression of wild-type DmTrx-2. For this PCR-cloning we used the forward primer GCGC|CATATG|GTG|TAC|CAG|GTG|AAA|GAT|AAG|G which contains an *Nde*I restriction site. The reverse primer was the same as above. Hot start PCR was conducted using the following conditions: 3 min at 95 °C, 80 °C hold, and then 95 °C for 30 sec, 60 °C for 1
min, 72 °C for 30 sec (30 cycles), 3 min at 72 °C, 15 °C hold. The PCR fragments were isolated from an agarose gel using the QIAquick Gel Extraction Kit (Qiagen), digested with the restriction enzymes and ligated to the expression vectors. *E. coli* NovaBlue cells (Novagen) were then transformed with the plasmids and the inserts were verified by sequencing (MWG Biotech, Ebersberg, Germany).

**Protein expression of HIS-tagged DmTrx-2**

HIS-tagged DmTrx-2 (pQE-30/dmtrx-2) was overexpressed in *E. coli* NovaBlue. The cells were incubated overnight at 37 °C in 2xYT medium containing 50 µg carbenicillin/ml. Protein expression was induced with 0.5 mM IPTG for 3h at 37 °C. After centrifugation at 3000 g for 10 min (T = 4 °C), cells were resuspended in phosphate buffer (300 mM NaCl, 50 mM sodium phosphate, 10 mM imidazole, pH 7.4) and treated with lysozyme (0.2 mg/ml) and DNAse (0.02 mg/ml) for 20 min at room temperature. As protease inhibitors, 100 µM phenylmethylsulfonyl fluoride (PMSF), 3 µM pepstatin and 80 nM cystatin were added and cells were disintegrated by ultrasound. The homogenate was centrifuged at 38000 g for 30 min at 4 °C. The supernatant was purified over a Ni-NTA (Qiagen) column with a yield of 150 mg of HIS-tagged protein per liter culture. SDS-PAGE using a 15 % gel showed a single band at the expected size of 13 kDa, the purity being >95 %.

**Protein expression of wild-type DmTrx-2**

Wild-type DmTrx-2 (pET-30/dmtrx-2) was expressed in *E. coli* BL21(DE3) (Novagen). Cells were grown overnight at 30 °C in 2xYT medium and 40 µg kanamycin/ml. Protein expression was induced with 0.5 mM IPTG for 3h at 37 °C. The raw protein extract with a content of approx. 130 mg thioredoxin-2 from one liter culture was isolated as described above and was then treated with a heat shock of 60 °C for 1 min. The denaturated *E. coli* proteins were separated by centrifugation at 38000 g for 30 min at 4 °C. Subsequently the supernatant was
subjected to ammonium sulfate fractionation at 4 °C (50% followed by 70% saturation). The 70% pellet, containing approx. 90 mg thioredoxin, was redissolved in 100 mM potassium phosphate and 1 mM EDTA, pH 7.0 and purified over a Sephadex G75® column. The overall yield was about 60 mg/l medium. SDS-PAGE using a 15% gel revealed a single band at the expected size of 11.7 kDa; purity was >90%.

**Cloning of Anopheles gambiae thioredoxin-1 (AgTrx-1)**

The *agtrx-1* gene was PCR-cloned from an *A. gambiae* cDNA using the forward primer GCGC|GGATCC|GTG|TAC|ATG|GTG|AAA|GAT|TCC containing a *Bam*HI restriction site and GCGC|AAGCTT|TTA|CGC|CGA|ATG|CTG|CTG|G as a reverse primer with a *Hind*III restriction site. Hot start PCR was conducted using the following conditions: 3 min at 95 °C, 80 °C hold, and then 95 °C for 30 sec, 57 °C for 1 min, 72 °C for 30 sec (30 cycles), 3 min at 72 °C, 15 °C hold. The cloning procedure was identical to that described for *dmtrx-2*. *E. coli* NovaBlue competent cells were transformed with the plasmid and the insert was verified by sequencing (MWG Biotech).

**Protein expression of HIS-tagged AgTrx-1**

*Agtrx-1* in pQE-30 vector was overexpressed in *E. coli* NovaBlue. The cells were incubated overnight at 30 °C in 2xYT medium containing 50 µg carbenicillin/ml. Protein expression was induced with 0.5 mM IPTG for 4h at 30 °C. Homogenisation and purification were identical to DmTrx-2 giving a yield of 40 mg/l of HIS-tagged protein. Purity was checked by SDS-PAGE with a 15 % gel showing a single band at the expected size of 13 kDa.

**Cloning of Drosophila melanogaster thioredoxin peroxidase-1 (DmTPx-1)**

DmTPx-1 was PCR-cloned from EST clone LD33645 (Research Genetics) using GCGC|GGATCC|CCC|CAG|CTA|CAG|AAG|CCC as a forward primer with *Bam*HI
restriction and TAGGTGACACTATAGAACTCGAG as a POT vector reverse primer. Conditions for the hot start PCR were as follows: 3 min at 95 °C, 80 °C hold, and then 95 °C for 30 sec, 60°C for 1 min, 72 °C for 45 sec (30 cycles), 3 min at 72 °C, 15 °C hold. The PCR product of approximately 930 bp was blunt end cloned into pT7blue vector (Novagen Perfectly Blunt Cloning Kit) and sequenced (MWG Biotech). The sequence was identical to the DmTPx-1 cDNA (GeneBank acc. AF167098). A PstI restriction site is contained in the fragment downstream of the stop codon. The fragment was therefore digested with BamHI and PstI giving a 789 bp product that was cloned into the pQE-30 expression vector.

**Protein expression of HIS-tagged DmTPx-1**

*E. coli* BL21 (Novagen) cells were transformed with the (pQE-30/dmtpx-1) plasmid and grown overnight at 37 °C in 2x YT medium containing 50 µg carbenicillin/ml. Protein expression was then induced with 0.5 mM IPTG for 3 h at 37 °C. Cells were disintegrated as above and the supernatant was purified over a Ni-NTA column. The yield was 20 mg/l culture medium and SDS-Page revealed a single band at approx. 20 kDa, which was lower than the expected 23 kDa.

**Gel filtration of DmTPx-1**

For determination of molecular mass, 1 mg of DmTPx-1, 0.5 mg glutathione reductase (GR) (M_r = 110 kDa), 1 mg aldolase (M_r = 160 kDa), 1 mg catalase (250 kDa) and 1 mg of ferritin (450 kDa), respectively, in 100 mM potassium phosphate, 1 mM EDTA, pH 7.0 were applied to a Sephadex G200® column (d = 1 cm; l = 100 cm). Proteins were separated at a flux of 13ml/h at T = 4 °C and collected in 1.3 ml fractions. A second gel filtration with 1 mg of glutathione reductase and 1 mg of DmTPx-1 was conducted as described above but in a buffer additionally containing 5 mM DTT.
**Thioredoxin Reductase Assays**

Assays were conducted at 25 °C using a reaction mixture volume of 1 ml consisting of buffer T (100 mM potassium phosphate, 2 mM EDTA, pH 7.4) and 100 µM NADPH. For determination of $K_m$ values, thioredoxin concentrations were varied from 3 to 50 µM (39 to 650 µg/assay). The assay was started with 10 mU of DmTrxR-1 (1 U = 1µmol NADPH consumption per min), and thioredoxin-dependent NADPH oxidation was measured spectrophotometrically at 340 nm. $K_m$ and $V_{max}$ values were obtained applying the Michaelis-Menten equation.

**Thioredoxin Peroxidase Assays**

To the reaction mixture (1 ml, 25 °C) in buffer T containing 100 µM NADPH and 1 U DmTrxR-1, different amounts (3 - 50 µM; 39 - 650 µg) of the thioredoxins (DmTrx-1, DmTrx-2, AgTrx-1, *Plasmodium falciparum* thioredoxin (PfTrx-1) and human thioredoxin mutant C73S (hTrx(C73S)) were added in order to determine $K_m$ and $V_{max}$ values. NADPH consumption was followed at 340 nm. When thioredoxin reduction had reached an end point, 70 µM $t$BuOOH was added and the assay was started with 10 mU DmTPx-1. $K_m$ values for $H_2O_2$, $t$BuOOH and cumeneOOH were estimated by a similar assay at constant thioredoxin concentration. To the 1 ml mixture containing 50 µM DmTrx-2 (> 5 $K_m$), 100 µM NADPH and 1 U DmTrxR in buffer T, peroxides were added with resulting end concentrations of 3 to 50 µM. The reaction was started with 10 mU of thioredoxin peroxidase.

**Thioredoxin-dependent GSSG Reduction Assay (GHOST assay)**

The GHOST assay was conducted as described previously (15,19). The mixture contained 100 µM NADPH and DmTrx-2 concentrations from 5 - 50 µM. The assay was started by addition of 1 U DmTrxR-1 and NADPH oxidation was measured spectrophotometrically at
340 nm. After thioredoxin reduction was complete, 1 mM GSSG was added and GSSG reduction was followed by further NADPH consumption. The composition of the assay mixture guarantees that >98% of thioredoxin is present in reduced form. Under steady state conditions the overall reaction rate $v$ depends on the concentrations of $\text{Trx(SH)}_2$ and GSSG:

$$
\begin{align*}
  v &= -\frac{d[\text{Trx(SH)}_2]}{dt} = k_2[\text{Trx(SH)}_2][\text{GSSG}] \\
  &= \frac{d[\text{Trx(SH)}_2]}{dt} = V_{\text{max}}[\text{TrxS}_2]/(K_m + [\text{TrxS}_2]) \\
  &= V_{\text{max}}([\text{Trx}_{\text{tot}}] - [\text{Trx(SH)}_2])/(K_m + [\text{Trx}_{\text{tot}}] - [\text{Trx(SH)}_2])
\end{align*}
$$

This allows to determine $k_2$, the second order rate constant for the reaction $\text{Trx(SH)}_2 + \text{GSSG} \rightarrow \text{TrxS}_2 + 2 \text{GSH}$.

**Antibody production and immunoblots**

One milligram of recombinant DmTrx-2 and AgTrx-1, respectively, was injected as an antigen into rabbits (Bioscience, Göttingen, Germany). Insect cell extracts (10 $\mu$g and 50 $\mu$g total protein) as well as 500 ng purified DmTrx-1 and DmTrx-2 were applied to a 15% SDS-PAGE. After completion of the electrophoresis the proteins were blotted overnight onto a nitrocellulose membrane (20). After incubation with the anti(Trx) IgGs the second antibody (swine anti(rabbit IgG) antibody-horseradish peroxidase conjugate) was added. For staining, the nitrocellulose paper was exposed to a solution of 1-chloronaphthol and hydrogen peroxide in sodium citrate buffer.
RESULTS

When we attempted to characterize *D. melanogaster* thioredoxin peroxidase-1 (DmTPx-1) (21,22) in detail, it became obvious that the known thioredoxin isoprotein DmTrx-1 (3,23) is not the genuine substrate of this enzyme. Consequently we cloned and functionally expressed the gene of a second thioredoxin from *Drosophila* (DmTrx-2) and its putative orthologue from *Anopheles gambiae* (AgTrx-1). *A. gambiae* is the predominant mosquito vector of malarial parasites.

The dmtrx-2 and agtrx-1 genes and their gene products. Genome analysis shows that the dmtrx-2 gene coding for a protein of 106 amino acids contains three exons and two introns (see GeneBank acc. AE003625). According to the available EST sequences, dmtrx-2 is transcribed in all developmental stages of *Drosophila*. Western blot analyses revealed that DmTrx-2 is expressed in larvae and in imagines of the the fruit fly as well as in Schneider cell lines. The corresponding band could be clearly detected with 10 µg total protein and represents 0.3 - 1% of extracted protein (Fig. 1). The gene product from the malarial mosquito *A. gambiae* that is putatively isofunctional to DmTrx-2 was also studied. Like thioredoxin-2 from *Drosophila*, AgTrx-1 was found to be a highly expressed protein in vivo. The concentrations are in the same order of magnitude as estimated for DmTrx-2. As shown in Fig. 2, DmTrx-2 shares 56% sequence identity with its relative from *Anopheles gambiae* (AgTrx-1) but also considerable 50% with cytosolic human thioredoxin. In contrast, the similarity to DmTrx-1 is significantly lower showing only 37% identical residues. Thus DmTrx-1 differs from DmTrx-2 as much as *P. falciparum* thioredoxin does. We expressed HIS-tagged DmTrx-2 and AgTrx-1 as well as wild-type DmTrx-2 in *E. coli*. Purification of the authentic *Drosophila* thioredoxin was based on procedures established for thioredoxins from other sources (24) (see Experimental Procedures). *E. coli* thioredoxin is an inevitable contaminant of purified DmTrx-2 but it was found to be only <0.05 % when using *E. coli*
TrxR for detection. Furthermore *E. coli* thioredoxin is a poor substrate for *D. melanogaster* TrxR-1 ($K_m > 700 \mu M$) so that even several percent *E. coli* thioredoxin would not interfere with the kinetic characterization of DmTrx-2.

**DmTrx-2 and AgTrx-1 as substrates of Drosophila thioredoxin reductase.** Wild-type as well as HIS-tagged DmTrx-2 are equally good substrates of *D. melanogaster* thioredoxin reductase-1 (Table I). The measured kinetic parameters, notably the $k_{cat}/K_m$ ratio of $3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$, are in the same order of magnitude as for the DmTrx-1/DmTrxR-1 system and for other homologous thioredoxin systems. Catalysis most likely depends on a dithiol-disulfide exchange between the C-terminal redox center of TrxR and its substrate Trx (6). The rate $k$ of this reaction is equal to $k_{cat}/K_m$ if a dithiol-disulfide exchange is the rate limiting chemical reaction during catalysis; otherwise $k$ is even greater, that is $> 3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$. AgTrx-1 was found to be an excellent heterologous substrate of DmTrxR-1, with the $k_{cat}/K_m$ ratio being $1.2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$.

**DmTrx-2 as a glutathione disulfide-reducing protein.** Since the *Drosophila* genome does not encode a genuine glutathione reductase (3) the maintenance of redox homeostasis and antioxidative defense by the thioredoxin system is a key issue (Fig. 3). Thioredoxin reductase is likely to substitute for GR in an indirect way because the broad TrxR substrate spectrum does not include GSSG. Glutathione is most probably kept in reduced state by a nonenzymatic dithiol-disulfide exchange reaction with Trx(SH)$_2$. Using the GHOST assay (see Experimental Procedures), $k_2$ was determined to be $170 \text{ M}^{-1} \text{s}^{-1}$ ($= 0.01 \mu \text{M}^{-1} \text{min}^{-1}$) at 25 °C. This is basically identical with the published value for DmTrx-1 as a GSSG-reducing protein (3). Under assumed *in situ* conditions - 10 μM Trx-1, 50 μM Trx-2, 200 μM GSSG and 1 U/ml TrxR - the thioredoxin system would allow GSSG fluxes of more than 100 μM min$^{-1}$.

**Thioredoxin peroxidase-1 (DmTPx-1).** While DmTrx-1 and DmTrx-2 reduce glutathione disulfide equally well this is not the case for the reduction of DmTPx-1. The occurrence of this enzyme had already been shown in different developmental stages of *Drosophila* (21). As
described by Orr and collaborators (22), DmTPx-1 is a cytosolic protein and overexpression of the gene in Schneider cells results in higher survival rate under peroxide treatment. DmTPx-1, a protein of 194 amino acids and a molecular mass of 21.7 kDa per subunit, shows sequence elements that are typical for type I TPx, notably in the environment of the two conserved cysteines Cys\textsuperscript{47} and Cys\textsuperscript{168} (Fig. 4).

The enzyme was recombinantly expressed in \textit{E.coli} as a HIS-tagged protein. Gel filtration under reducing (5 mM DTT) and non-reducing conditions revealed that DmTPx-1 has an apparent M\textsubscript{r} of 250 kDa which would correspond to a homodecamer (230 kDa) or a homododecamer (approx. 276 kDa). This result is in good agreement with the known decameric crystal structure of TPx-B (= hTPx-1) from human erythrocytes (25); as shown in Fig. 4, hTPx-1 is a 2-Cys peroxiredoxin exhibiting over 70% sequence identity with DmTPx-1. Notably, most residues representing the intersubunit contacts are conserved when comparing the human with the \textit{Drosophila} TPx-1.

\textbf{Thioredoxins as substrates of DmTPx-1}. We tested the peroxidase activity with the two thioredoxins of \textit{Drosophila} (DmTrx-1 and DmTrx-2) as electron donors and with H\textsubscript{2}O\textsubscript{2}, tBuOOH and cumeneOOH as peroxide compounds. Furthermore transspecies activities of the enzyme were examined using AgTrx-1, PfTrx-1 and hTrx(C73S) as reductants. DmTrx-2, in tagged or wild-type form, turned out to be the preferred reducing substrate. The apparent K\textsubscript{m} and V\textsubscript{max} values were found to be 9 µM and 14 U mg\textsuperscript{-1}, respectively, when the assays were conducted in the presence of 70 µM tBuOOH (Table II). The thioredoxin from \textit{A. gambiae} (AgTrx-1) exhibits only slightly higher K\textsubscript{m} and lower V\textsubscript{max} values than DmTrx-2. It was surprising to find that DmTrx-1 was an even poorer substrate for DmTPx-1 than PfTrx-1. With a K\textsubscript{m} of 52 µM and a k\textsubscript{cat}/K\textsubscript{m} ratio 60fold lower than for the system DmTrx-2/DmTPx-1, thioredoxin-1 is probably not of physiological relevance as a peroxidase-1 substrate.

\textbf{Peroxides as substrates of DmTPx-1}. DmTPx-1 is inactivated by peroxides, an effect that has been previously described for other peroxidases (26). Under the used coupled assay
conditions with 1 U/ml DmTrxR-1, this phenomenon is visible as a constantly decreasing rate of NADPH consumption that can be reversed by addition of fresh peroxidase aliquots (Fig. 5). Reaction is usually started by peroxidase addition to the standard assay mixture but preincubation of the assay mixtures containing the peroxidase and t-butyl hydroperoxide results in the same kinetic behavior. This indicates that DmTPx-1 in oxidized form is not affected by peroxides; obviously the enzyme undergoes substrate inactivation only during catalysis. Since the syncatalytic loss of activity depends on the peroxide concentration, an accurate determination of the $V_{\text{max}}$ value for the tested peroxide substrates is impossible. With the high and saturating peroxide levels used for $K_m$ determination of thioredoxins, the apparent $V_{\text{max}}$ values are considerably lower than those determined with low peroxide concentrations under thioredoxin saturation. DmTPx-1 has no detectable glutathione peroxidase (GPx) activity. This was shown using a coupled assay containing 1 mM GSH and 1 U/ml human glutathione reductase instead of Trx and TrxR.
DISCUSSION

The characterisation of a second thioredoxin (DmTrx-2) and a thioredoxin peroxidase (DmTPx-1) contributes to the elucidation of redox homeostasis and redox signaling in the fruit fly.

*Antioxidative defense networks in dipteric insects. Drosophila* is a model organism representative for insects such as the malaria vector *Anopheles gambiae* and numerous other disease-transmitting dipters. These insects have specific morphologic risk factors, for instance a tracheal respiratory system and translucent surfaces where reactive oxygen species (ROS) are generated in UV radiation-catalysed reactions. The dithiol protein-based redox buffers of these insects differ significantly from most other organisms. Reduced glutathione (GSH) occurring in millimolar concentrations is also in *Drosophila* a key player of intracellular redox homeostasis. A high GSH/GSSG ratio is maintained (4) although dipteric insects do not possess the enzyme glutathione reductase (3). As proposed before, the function of GSSG reduction in insects is most likely performed by the thioredoxin system which consists of NADPH and the dithiol proteins thioredoxin reductase and thioredoxin (Fig. 3). The crucial role of thioredoxin reductase *in vivo* is supported by the fact that DmTrxR null mutations are lethal in larval stage and that reduced TrxR activity severely affects life span of the insects (27,28). The rate constant for the dithiol-disulfide exchange reaction between GSSG and reduced thioredoxin was determined to be 170 M⁻¹ s⁻¹ (= 0.01 µM⁻¹ min⁻¹) at 25 °C (Table I). This value is almost identical when comparing the two cytosolic thioredoxins, DmTrx-1 and DmTrx-2. We could demonstrate that DmTrx-2 is indeed a major protein *in situ*. As suggested by Western blot analysis it represents approximately 0.3 to 1% of the protein extracted from Schneider cells or whole insects (Fig. 1). Assuming a protein concentration of 100 mg/ml cytosol and 50% cytosol in the cell, the cytosolic Trx-2 concentration is estimated to be above 50 µM. At physiological concentrations of GSSG (200 µM) and thioredoxin reductase
(1 U/ml), the resulting GSSG flux $v = k_2[GSSG][Trx(SH)_2]$ would therefore be in the range of physiological demand, that is approx. 100 µM min$^{-1}$ (29), and may be further adapted to oxidative challenge by increasing the thioredoxin concentration.

*Thioredoxin peroxidase and its reducing substrate DmTrx-2.* The two cytosolic thioredoxins do not exhibit immunochemical cross-reactivity (Fig. 1). Another important difference is their behavior as electron donors for thioredoxin peroxidase (DmTPx-1). To study this crucial peroxide reduction pathway in more detail we cloned and characterized the enzyme. TPx-1 was found to be a well conserved homodecameric protein. For the structurally known enzyme of human erythrocytes it has been discussed that the decameric state may occur only in an oxidative environment (25). For DmTPx-1, however, we found that this aggregate is also the major molecular form in the presence of 5 mM dithiothreitol. TPx is not only involved in direct detoxification of reactive peroxides. In yeast it was found that at least one TPx-1 orthologue is essential for launching off the cell’s response to oxidative stress (30), a process which is based on up and down regulation of numerous gene activities. DmTPx-1 belongs to a group of proteins in *Drosophila* that are differentially expressed in the developmental stages of *Drosophila* (21). This is consistent with observations on other organisms where TPx is involved in redox signaling pathways (16,17) and repression of apoptosis (31).

Our kinetic data show that only one of the two cytosolic thioredoxins in *D. melanogaster*, namely DmTrx-2, serves as a substrate of DmTPx-1. DmTrx-1 is unlikely to be a substrate of physiological importance because its $K_m$ of 52 µM is too high and its $k_{cat}$ of $10^4$ M$^{-1}$ s$^{-1}$ (Table II) is extremely low. In contrast, the kinetic parameters for DmTrx-2 ($K_m = 9$ µM; $k_{cat}/K_m = 6 \times 10^5$ M$^{-1}$ s$^{-1}$) are most favorable for serving as a TPx-1 substrate in situ. Thus Trx-2 has a function which is not shared by the isoprotein Trx-1 probably operating in the same cell compartment. To our knowledge this is the first clear example of functional differentiation of thioredoxin isoproteins. It will be interesting to see if Trx-2 can substitute for Trx-1 as a morphogenetic factor since Trx-1 is the deadhead-preventing protein of *D.*
melanogaster larvae (18,23). Obviously Trx-1 and Trx-2 share a number of functions. In their oxidized form TrxS2, both isoproteins are oxidizing substrates of thioredoxin reductase, and in their reduced forms they are equally efficient in GSSG reduction and thus in redox buffering. It should be noted that the functions of TPx-1 delineated above are influenced by the concentration and the redox-state of Trx-2 but not of Trx-1. Thus Trx-2 is expected to have additional indirect effects which are not shared by Trx-1.

The thioredoxin system of disease transmitting insects. The experimental results on the thioredoxin system of D. melanogaster probably apply also for disease-transmitting insects. As a case in point, Anopheles gambiae thioredoxin-1 was found to be closely related to DmTrx-2 both in structure and function. The A. gambiae protein serves as a substrate of DmTrxR-1 and of DmTPx-1, and it reduces GSSG. Thus the redox metabolism on the basis of thioredoxins and thioredoxin reductases - instead of GR which is not present in Drosophila nor in Anopheles - offers a new target for the combat against insects and insect borne parasitic diseases like malaria (32,33). The causative agent of tropical malaria, Plasmodium falciparum, is particularly sensitive to oxidative stress. Severe disturbance of the redox buffering capacity in the human host erythrocyte is caused, for instance, by glucose-6-phosphate dehydrogenase deficiency or inhibition of erythrocytic GR. These conditions in the host compromise the development of parasitic blood stages (34,35). Not only in the human host, but also in the insect vector, oxidative compounds like peroxynitrite are produced and launched against the parasite (36,37). In analogy to GR inhibitors as antiparasitic drugs in man (38), TrxR inhibitors would be promising agents against the insect stages of the parasite but also as insecticides. This perspective is supported by the fact that there are great structural and functional differences among the thioredoxin-reducing centers of the TrxRs from insects, malarial parasites and mammals (15). Only the mammalian enzymes contain a selenocysteine as a functional group so that the difference between disulfide and selenyl sulfide biochemistry can be used for the design of specific enzyme inhibitors. Ideally, such an inhibitor would
inactivate both parasite TrxR and insect TrxR and thus serve as a prototype of parasiticide/insecticide that is taken up by the insect when feeding on blood of treated patients.

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FIGURE LEGENDS

**Fig. 1.** Western blot of recombinant and wild-type DmTrx-2. In lane 1, Schneider cell extract containing 10 µg protein was applied, and in lanes 2 and 3, 1 µg each of recombinant DmTrx-1 and DmTrx-2. The blot was incubated with rabbit anti(DmTrx-2) serum and stained with swine anti(rabbit) immunoglobulins-HRP (horseradish peroxidase). As expected the band in lane 1 appears at a slightly lower M_r (approx. 11.7 kDa) than the recombinant DmTrx-2 (13 kDa). As shown in lane 2, DmTrx-1 does not cross-react with anti(DmTrx-2) serum. The additional weak bands in lane 2 and 3 are *E. coli* proteins.

**Fig. 2.** Multiple sequence (Clustal W) alignment of thioredoxins. This was conducted with DmTrx-2 (NCB acc. AAF52794), DmTrx-1 (NCB acc. P47938), thioredoxin from man (hTrx, NCB acc. JH0568), *Anopheles gambiae* (AgTrx-1, NCB acc. AAF68382), and with a *P. falciparum* thioredoxin (PfTrx-1, NCB acc. AAF34541). Only few amino acid sequences like the active site motif WCGPC with its two cysteines (marked by plus signs) are conserved among all species.

**Fig. 3** Interactions of the thioredoxin system in *Drosophila melanogaster*. Due to the absence of a GR the thioredoxin system has a key function in the redox metabolism of the fruit fly and other dipteric insects. GSSG reduction is based on dithiol-disulfide exchange with reduced thioredoxin. This function is shared by Trx-1 and Trx-2, but only Trx-2 serves as a substrate of TPx-1.
Fig. 4. 2-Cys peroxiredoxins from eukaryotes. The alignment includes enzymes from *Drosophila* (DmTPx-1, NCB acc. AAF42985), man (hTPx-1, NCB acc. P32119), *Schizosaccharomyces pombe* (SpTPx, NCB acc. T41413) and *Plasmodium falciparum* (PfTPx-1, NCB acc. AAF67110). The two redox active cysteine residues are marked with plus signs. The F-motif starting with phenylalanine and including the cysteine homologous to Cys47 of DmTPx-1 is highly conserved.

Fig. 5. Characteristics of a typical coupled thioredoxin peroxidase assay. 100 µM NADPH, 1 U DmTrxR-1 and 10 µM DmTrx-2 as a reductant cascade were used. NADPH consumption was measured at 340 nm. The typical inactivation of DmTPx-1 by 70 µM tBuOOH is easily recognized.
Table I. Basic biological properties of *D. melanogaster* thioredoxin-2 (DmTrx-2, NCB acc. AAF52794) and *Anopheles gambiae* thioredoxin-1 (AgTrx-1, NCB acc. AAF68382) and their kinetic characteristics as a substrate of DmTrxR-1. A dash between two numbers indicates that these refer to wild-type and HIS-tagged protein, respectively.

|                     | DmTrx-2       | AgTrx-1       |
|---------------------|---------------|---------------|
| No. of amino acids  | 106 / 117     | 107 / 118     |
| Mr [kDa]            | 11.7 / 13.0   | 12.1 / 13.3   |
| K_m [µM]            | 5.9 ± 0.8 / 5.2 ± 0.9 | n.d. / 9 ± 0.9 |
| V_max [U mg⁻¹]      | 16.0 ± 1.4    | 11.0 ± 1.3    |
| k_cat [s⁻¹]         | 14.5 ± 1.2    | 10.0 ± 1.2    |
| k_cat/K_m [M⁻¹ s⁻¹] | 2.8 x 10⁶     | 1.2 x 10⁶     |
| k₂ of the dithiol-disulfide exchange reaction | 170 ± 17 | 140 ± 17 |
Table II. Kinetic parameters of *D. melanogaster* thioredoxin peroxidase-1 (DmTPx-1, NCB acc. AAF42985). As reducing substrates the thioredoxins from *Drosophila* (DmTrx-1 and DmTrx-2), from *A. gambiae*, *P. falciparum*, and humans were used. The three peroxide substrates exhibited similar $k_{cat}/K_m$ values.

| Substrate            | $K_m$ [µM] | $V_{max}$ [U mg$^{-1}$] | $k_{cat}$ [s$^{-1}$] | $k_{cat}/K_m$ [M$^{-1}$ s$^{-1}$] |
|----------------------|------------|-------------------------|----------------------|-----------------------------------|
| DmTrx-2 (wild-type)  | 8.2 ± 1.2  | 13 ± 1.7                | 5.0 ± 0.6            | 6.1 x 10$^5$                      |
| DmTrx-2 (HIS-tagged)| 9.0 ± 1.4  | 14 ± 1.6                | 5.4 ± 0.6            | 6.0 x 10$^5$                      |
| DmTrx-1              | 52 ± 17    | 1.6 ± 0.6               | 0.6 ± 0.2            | 0.1 x 10$^5$                      |
| hTrx(C73S)           | 19 ± 2     | 7.4 ± 0.7               | 2.9 ± 0.2            | 1.5 x 10$^5$                      |
| AgTrx-1              | 11 ± 1.5   | 7.0 ± 0.8               | 2.7 ± 0.3            | 2.5 x 10$^5$                      |
| PfTrx-1              | 25 ± 3     | 7.2 ± 0.6               | 2.8 ± 0.2            | 1.1 x 10$^5$                      |
| $H_2O_2$*            | 15 ± 5     | 40 ± 15                 | 15.5 ± 5.8           | 10.3 x 10$^5$                     |
| tBuOOH               | 6.0 ± 1.5  | 44 ± 6                  | 17.0 ± 2.3           | 28.3 x 10$^5$                     |
| cumeneOOH            | 7.3 ± 0.5  | 30 ± 4                  | 11.6 ± 1.5           | 15.9 x 10$^5$                     |

*Hydrogen peroxide is reduced in significant amounts by the system DmTrx-2/DmTrxR. This activity makes it difficult to determine more exact values.*
Fig. 1
| Protein     | Sequence                                      | Length |
|-------------|-----------------------------------------------|--------|
| DmTrx-2     | KKYQVKKDNADLDGQLTKASGKLVVLDFFATWCGPCKMISPKLVELSTQFADNVVVLKVDV 60 |        |
| AgTrx-1     | KKYQVKKDNADLDGQLTKASGKLVVLDFFATWCGPCKMISPKLVELSTQFADNVVVLKVDV 60 |        |
| PfTrx-1     | KKVIVTSQSSSIISQ--NEIVVDFDATWCGPCKMISPKLVELSTQFADNVVVLKVDV 60 |        |
| hTrx        | KKVIVTSQSSSIISQ--NEIVVDFDATWCGPCKMISPKLVELSTQFADNVVVLKVDV 60 |        |
| DmTrx-1     | -MASVRTMNDYHKRIEAADKLIVLDFYATWCGPCKEMESTVKSLARKYSSKAVVLKIDV 59 |        |

*Fig. 2*
NADPH

TrxR

Trx(SH)₂

GR

2 GSH

GSSG

TrxS₂

Trx-1

Trx-2

TPx-1

• powerful and abundant antioxidant
• detoxification of electrophiles and radicals
• glutaredoxin reduction
• enzyme regulation

• ribonucleotide reduction
• transcriptional control
• enzyme regulation

• peroxide detoxification
• regulatory functions in e.g. cell differentiation

Fig. 3
Fig. 4
Fig. 5
Thioredoxin-2 but not thioredoxin-1 is a substrate of thioredoxin peroxidase-1 from Drosophila melanogaster - Isolation and characterization of a second thioredoxin in D. melanogaster and evidence for distinct biological functions of Trx-1 and Trx-2

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