Cytosolic thioredoxin system facilitates the import of mitochondrial small Tim proteins

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Thiol-disulphide redox regulation has a key role during the biogenesis of mitochondrial intermembrane space (IMS) proteins. Only the Cys-reduced form of precursor proteins can be imported into mitochondria, which is followed by disulphide bond formation in the mitochondrial IMS. In contrast to the wealth of knowledge on the oxidation process inside mitochondria, little is known about how precursors are maintained in an import-competent form in the cytosol. Here we provide the first evidence that the cytosolic thioredoxin system is required to maintain the IMS small Tim proteins in reduced forms and facilitate their mitochondrial import during respiratory growth.

Keywords: redox regulation; mitochondrial import; thioredoxin; oxidoreductase; folding

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INTRODUCTION

Mitochondria has important roles in various regulatory processes ranging from ATP generation to cell growth and apoptosis. Not surprisingly, therefore, mitochondrial dysfunction leads to life-threatening diseases, including diabetes, stroke, Alzheimer, and cancer. Protein import is essential for the biogenesis of mitochondria, as the majority (99%) of mitochondrial proteins are synthesized in the cytosol on cytosolic ribosomes, and thus have to be imported into mitochondria for their function. How precursor proteins are imported into mitochondria is a subject of intensive study and at least four main import pathways have now been characterized [1,2]. In contrast, little is known about how mitochondrial precursors are maintained in an import-competent form in the cytosol.

The import of many essential mitochondrial intermembrane space (IMS) proteins is regulated by their thiol-disulphide redox state [3–6]. Although disulphide bond formation is crucial for the

function of these proteins inside mitochondria, oxidized precursor proteins cannot be imported into mitochondria and only Cys-reduced forms are import-competent [5,7,8]. Many IMS proteins, such as members of the ‘small Tim’ (e.g., Tim9, Tim10) and Cox17 (e.g., Cox17, Cox19) families, contain conserved Cys residues. Import of these proteins depends on the redox-sensitive mitochondrial import and assembly (MIA) pathway. Mia40 and Erv1 are the central components of the MIA pathway; they form a disulphide relay system in the IMS mediating the import and oxidative folding of these Cys-containing proteins [2,9].

Using the small Tim proteins as models, it has been shown that the oxidized proteins are thermodynamically stable under cellular glutathione redox conditions [7,10,11]. These proteins have a standard redox potential of -0.31 V to -0.33 V, which is more negative than that of the glutathione redox conditions in both the cytosol and mitochondrial IMS [7,10,12,13]. Such a redox stability of the small Tim proteins is consistent with their oxidized (disulphide bonded) state in the IMS, but not their reduced (thiol) states in the cytosol. Furthermore, studies showed that the precursors become oxidized during mitochondrial import and that this oxidation kinetically competes with their import [7]. Cytosolic factors are required to maintain these redox-sensitive precursors in a Cys-reduced, import-competent form in the cytosol before their import into mitochondria. Although zinc-binding can stabilize the small Tim proteins in their reduced forms *in vitro*, their relatively low-binding affinities (submicromolar to nanomolar) suggest that zinc-binding might not be the only or an important stabilizing factor during normal cell growth conditions [14,15]. How these redox-sensitive IMS precursors are maintained in an import-competent form in the cytosol is unknown.

The thioredoxin (Trx) and glutaredoxin (Grx) systems are ubiquitous oxidoreductases required for cellular thiol regulation and oxidative stress defence [16,17]. In the yeast *Saccharomyces cerevisiae*, there are two cytosolic Trx homologues (Trx1, 2) and two classical dithiol Grx homologues (Grx1, 2) located in the cytosol. A main function of these enzymes is to reduce disulphide bonds in their substrate proteins using electrons donated by nicotinamide adenine dinucleotide phosphate (NADPH). Oxidized Trx is reduced directly by NADPH and thioredoxin reductase (Trr), whereas Grx is reduced by glutathione using electrons donated by

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Fig 1 | The cytosolic Trx system is required for mitochondrial function. (A) Spot testing of cell growth with the wild-type (WT) and mutant yeast strains. The WT, *trx1 trx2* and *grx1 grx2* double or *trx1* and *trx2* single-deletion strains were spotted on YPD (left column) and YPEG (right column) plates at a series of 10-fold dilutions, followed by incubation at 30 °C for 2 days. grx, glutaredoxin; Trx, thioredoxin.

NADPH through glutathione reductase (Glr). In this present study we show that the cytosolic Trx system is required for yeast growth under respiratory condition and facilitates the import of mitochondrial MIA substrates by maintaining the precursors in a reduced form. An efficient disulphide bond transfer reaction was reconstituted using purified proteins, and we show that the Trx system preferentially catalyses the reduction of folding intermediates, rather than the fully oxidized protein.

RESULTS AND DISCUSSION

The Trx system is required for respiratory growth

To determine whether the cytosolic Trx and Grx systems are required for mitochondrial function, the wild-type (WT), *trx* and *grx* mutant strains were spotted onto fermentative and respiratory growth media (Fig 1). Although a double *TRX* deletion mutant (*trx1 trx2*) grows somewhat slower than the WT strain during fermentative growth (YPD), it is particularly inhibited for growth under respiratory conditions (YPEG). In contrast, the double *GRX* deletion mutant (*grx1 grx2*) grew comparably to the WT under both conditions. These results indicate that the Trx system might have an important role during the biogenesis of mitochondria. Growth of both the *trx1* and *trx2* single mutants was unaffected under respiratory conditions, consistent with an overlapping role for the two Trx proteins. This indicates that the respiratory growth defect of *TRX* deletion mutant (*trx1 trx2*) is not simply because of respiratory reactive oxygen species (ROS) production, as *trx2* mutants are hypersensitive to ROS, in contrast to *trx1* mutants that have WT levels of resistance [18]. Taken together, these results indicate that the cytosolic Trx system is required for maintaining mitochondrial function and probably facilitating the biogenesis of mitochondria. However, we cannot rule out that the defects seen in the *trx1 trx2* mutant strain is also contributed by other effects, as Trx proteins participate in various processes and have many client proteins.

To assess the function of Trx in biogenesis of mitochondrial proteins, protein expression was examined. While antibodies against Trx and Grx confirmed the deletion of these proteins, there were no obvious differences in the levels of the mitochondrial proteins (Mia 40 and MtHsp 70), although Tim9 and Tim10 seem to be slightly decreased in the *trx1 trx2* mutant (Fig 2A). To check this, mitochondria were isolated from the WT and mutant cells grown in YPEG, and protein concentrations determined. The steady-state levels of mitochondrial proteins from all four subcompartments are similar (not shown). This was not surprising, as the mutant was not unviable but grows slowly, and a small intensity difference will not be detectable by western blot. A similar result was shown for a *TOM5* (a non-essential component of the mitochondrial TOM translocase complex) deletion mutant [19]. Thus, the levels of the mitochondrial proteins under more stressful conditions were analysed. Cells were grown in fermentative YPD followed by a medium shift to respiratory YPEG for 6 h before mitochondria were isolated. The results clearly showed that the levels of the small Tim proteins (Tim9, Tim10 and Tim13) were decreased in the mitochondria of the *trx1 trx2* mutant strain, while no obvious decrease was observed for the other control proteins (Fig 2B). The same mitochondrial isolation and western blotting experiment were performed three times, and the levels of the small Tim proteins of the mutant strain were statistically lower than that of the WT strain ($P < 0.05$, Fig 2C). These results provide *in vivo* evidence that the cytosolic Trx system is involved in facilitating biogenesis of the mitochondrial small Tim proteins.

The Trx system facilitates the import of IMS proteins *in vitro*

To verify whether the Trx system has an effect on the import of mitochondrial proteins, mitochondrial import was examined using radiolabelled Tim9 and Tim10, synthesized in rabbit reticulocyte lysates, in the presence or absence of purified Trx1, Trx2 and/or NADPH. To eliminate the effect of metal ions such as Zn^{2+} , all import experiments were carried out in the presence of 2 mM EDTA. After import for 30 min, reactions were treated with trypsin to remove un-imported materials, and mitochondrial import was analysed using SDS-PAGE (Fig 3A), revealing that in the presence of the Trx1 system (lane 2) the import level was increased compared with the control (lane 1; Fig 3A). A partial Trx system (without Trx1 or Trx2) slightly enhanced the import level, but not as efficiently as the full system. It should be noted that small amounts of the Trx and/or Grx system components might be present in reticulocyte lysates; however, the addition of the purified yeast system clearly increased import (lane 1 versus lane 2) confirming that the Trx system facilitates import of the small Tim proteins. The same effect was observed when Cox19, a CX₉C motif containing substrate of the MIA pathway (Fig 3A), was used as an import substrate. In contrast, no obvious effect was observed on the import of the matrix marker proteins F₁-ATPase subunit-β (F₁β) and mtHsp60, the inner membrane (IM) protein AAC, or the outer membrane porin. A time-course experiment confirmed that the Trx system increases the import of the small Tim proteins (Fig 3B). While the import seems to reach a stationary level after 5 min in the absence of Trx system, it was continuously increased with the presence of the Trx system over the whole time course. Furthermore, an 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) assay showed that the redox state of mitochondrial Mia 40 was not

Fig 2 | Effects of the Trx system on protein expression level. (A) Western blots of cellular extracts from the wild-type (WT) and mutant cells. Yeasts were grown to exponential phase in YPEG medium, then lysed and analysed using antibodies against the indicated proteins. Cytosolic protein G6PDH was used as a loading control. (B) Western blots with mitochondria isolated from the WT and mutant cells. The WT and *trx1 trx2* yeast cells were grown in YPD to OD₆₀₀ of 10 and shifted to YPEG for 6 h. Then mitochondria were isolated and analysed using antibodies against the indicated proteins. 25, 50 and 75 μg mitochondria were loaded. The IMS proteins (Tim9, Tim10, Tim13 and Cytb2); Mia40: IMS/IM anchored; the outer membrane Tom40; IM AAC; and matrix protein malate dehydrogenase (MDH), mtHsp70 and co-chaperone GrpE were analysed. (C) Quantification of proteins in the mitochondria isolated from the WT (black solid squares) and *trx1 trx2* (blue open squares) cells as described in B. The levels of the small Tim proteins were significantly different (by Student's *t*-test: Tim10 $P < 0.05$ at all points, Tim9 and Tim13 $P < 0.05$ at 50 and 75 μg), and not significantly different for the marker proteins. Error bars represent s.e. ($n = 3$). grx, glutaredoxin; IM, inner membrane; IMS, intermembrane space; Trx, thioredoxin.

affected by the addition of the components of Trx system in the reactions (Fig 3C), and Mia40 was mainly in the oxidized state as shown previously [20]. Taken together, these results indicate that (i) the Trx system selectively facilitates the import of the redox-sensitive precursors of mitochondrial IMS proteins, and (ii) the general mitochondrial import machineries are not affected by the presence of the Trx system.

The Trx system keeps small Tims in reduced forms

We anticipated that the mechanism of enhanced import of the small Tim proteins was that the Trx system can maintain

the precursors in a reduced form. To confirm this idea, following the import assay described above, the redox state of the remaining un-imported proteins was analysed using the AMS thiol-modification assay (Fig 4A). The results indicate that while all the un-imported proteins were oxidized in the control (lane 1), the Trx1 system can maintain a large fraction of the proteins in a reduced form (lane 2). A small fraction of reduced proteins also existed in the reaction containing Trx1 (lane 4), but not Trx1 alone (lane 3). The same result was obtained for both Tim9 and Tim10.

Our results indicate that the presence of the Trx system causes an increased level of reduced precursors, and improved import of

Fig 3 | The Trx system facilitates the import of MIA substrates. (A). Effects of Trx system on the mitochondrial import. *In vitro* translated radioactive precursor proteins as indicated were incubated with isolated mitochondria in the presence or absence of the purified Trx system at 25 °C for 30 min, followed by trypsin treatment to remove un-imported proteins. Then, all mitochondrial imports were analysed by SDS-PAGE and autoradiography. (B) Time course of Tim9 and AAC import in the presence (+ TS) or absence (- TS) of the Trx system, and the qualification for Tim9 (bottom, - TS, 30 min as 100%). Error bars represent s.e. ($n > 3$). (C) Western blot of 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid assay of Mia40 after incubating with components of Trx system at 25 °C for 20 min. Lanes 1–5 as in A, lanes 6 and 7 were oxidized (Ox) and reduced (Red) controls. MIA, mitochondrial import and assembly; NADPH, nicotinamide adenine dinucleotide phosphate; TS, Trx system; Trr, thioredoxin reductase; Trx, thioredoxin.

these precursors. However, as the mitochondrial import system contains many components, it remained possible that our results could be because of an indirect effect of the Trx system on some of these components. To address this, we asked whether the Trx system can directly reduce disulphide bonds of the small Tim proteins. Purified oxidized Tim10 was incubated with purified Trx1 in the presence and absence of purified Trr1 for 10 min, followed by the AMS assay. The results showed that a fraction of Tim10 was reduced by the presence of the Trx1 system (Fig 4B, lane 3), suggesting that Tim10 is a substrate of the Trx1 system. Consistently, the small Tim proteins cannot be reduced by Trx1 or Trr1 alone (lanes 2 and 4; note that in the absence of Trr1, Trx1 was in the oxidized inactive form). Thus the small Tim proteins can be reduced by the Trx system directly. However, only a fraction of the protein was reduced under these experimental conditions, indicating that the fully oxidized proteins might not be very good substrates of the Trx1 system.

Small Tims proteins are good substrates of Trx system

There are two intramolecular disulphide bonds in Tim9 and Tim10 formed between the Cys residues of the twin CX3C motif in juxtaposition (C1–C4, and C2–C3). Previous work showed that oxidative folding of the small Tim proteins occurs through formation of single disulphide-bonded intermediates and that these intermediates are also import-incompetent [7]. Thus, we asked whether the Trx system can reduce these folding intermediates more efficiently. For this, double Cys mutants with

each disulphide bond mutated to Ser (C1,4S and C2,3S) were used. As shown in Fig 4C, all the Cys mutants can be fully reduced by the Trx1 system, and a time-course analysis confirmed that reduction of the mutants was very rapid. While only about 10% of native Tim10 was reduced after 10 min, all of the single disulphide-bonded mutants were reduced within 1 min (Fig 4D). Thus, reduction of the single-disulphide intermediates by the Trx1 system is at least 100-fold faster than that of the fully oxidized proteins.

To obtain more quantitative results, the reactions were measured by following absorption changes at 340 nm because of NADPH oxidation (Fig 5A), and the results were analysed using the Michaelis–Menten equation (Fig 5B). The catalytic constant k_{cat} and Michaelis constant K_m were determined to be 23 min⁻¹ and 6.0 μM for Tim10C1,4S, and 29.0 min⁻¹ and 5.3 μM for Tim10C2,3S, respectively, and the substrate efficiency (k_{cat}/K_m) was 3.8×10^6 M⁻¹ min⁻¹ for Tim10C1,4S, and 5.5×10^6 M⁻¹ min⁻¹ for Tim10C2,3S. This substrate efficiency is similar to that of the well-characterized substrates of Trx, such as ribonucleotide reductase, arsenate reductase and insulin [21–23]. This result confirms that both single-disulphide intermediates are excellent substrates of the Trx system, supporting our conclusion that the Trx system maintains the small Tim proteins in a reduced form in the cytosol and thus facilitates their mitochondrial import.

To understand why the Cys mutants, but not the WT proteins, are excellent substrates of the Trx system, overall folding of

Fig 4 | Effects of Trx system on the redox state of Tim9 and Tim10. (A) Import and the redox state of un-imported small Tim proteins. The proteins were imported in the presence or absence of Trx system (TS: 1.5 μ M Trx1, 0.5 μ M Trr1 and 0.6 mM NADPH) at 25 °C for 30 min, and then each reaction was divided into two. One treated with trypsin for import analysis (top panels), the other was centrifuged and the supernatant was treated with AMS (bottom panels). (B) The Trx system reduces disulphide bonds of purified Tim10. Tim10 (10 μ M) was incubated with 0.6 mM NADPH in the presence and absence of Trx1 (1.5 μ M) and/or Trr1 (0.5 μ M) at 25 °C for 10 min. Then the redox state was analysed by AMS assay. (C) AMS assay of the WT and double Cys mutant of Tim9 and Tim10 as described in B. (D) Time course of the reduction of WT and mutant Tim10 by the Trx system. (B–D), SDS–PAGE with Coomassie staining. The reduced (R) and oxidized (O) states are indicated. AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; NADPH, nicotinamide adenine dinucleotide phosphate; Trr, thioredoxin reductase; Trx, thioredoxin; WT, wild-type.

these proteins was studied using far-ultraviolet circular dichroism (CD; Fig 5C). As shown previously, the WT proteins are folded with α -helical structure, which becomes unfolded on addition of a reducing agent, Tris(2-carboxyethyl)phosphine (TCEP). Clearly, both double Cys mutants of Tim10 display significantly lower CD signals than the oxidized WT protein (Fig 5C curves a, b and c). Oxidized Tim10C1,4S seems to be as unfolded as the reduced protein, and there is no obvious spectrum change on addition of TCEP (curves b and e). In comparison to the WT protein, Tim10C2,3S is partially folded and becomes unfolded on addition of TCEP (curves c and f). Thus, together with the above enzyme kinetic study, our results revealed that the Trx enzymes preferably react with Tim10 in an unfolded or partially folded state. In other words, the Trx enzymes react with the folding intermediates preferably and effectively rather than the folded small Tim proteins, indicating a stereoselective control mechanism. This result is consistent with the fact that all the well-known substrate proteins of the Trx enzymes have a solvent-exposed disulphide bond. Such a stereoselective mechanism will not only allow the enzyme to recognize a wide range of substrates, but also to reduce disulphide bonds effectively at an early stage of protein folding. We presume that apart from the small Tim proteins, many more redox-sensitive proteins can be maintained in a reduced state through the redoxin system by preventing the formation of early folding intermediates.

CONCLUSION

In summary, whereas the MIA pathway is used for import and oxidative folding of many Cys-containing IMS proteins inside mitochondria, here we provide the first evidence that the cytosolic Trx system is required to keep the precursors in a reduced form in the cytosol and thus to facilitate their mitochondrial import. The Trx system specifically facilitates the import of redox-sensitive IMS proteins without affecting matrix and membrane proteins, through an efficient disulphide bond transfer reaction that could be reconstituted using purified proteins. Further, we show that single-disulphide folding intermediates of the small Tim proteins are excellent substrates of the Trx system. The Trx enzyme preferentially recognizes unfolded or partially folded Tim10, and thus catalyses the reduction of the folding intermediates rather than the fully oxidized small Tim proteins. Our findings provide important insight into the initial steps of mitochondrial protein biogenesis, specifically how mitochondrial precursors are maintained in an import-competent form in the cytosol.

METHODS

Materials. 4-Acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid were obtained from Invitrogen Molecular Probes. EDTA was from BDH Co, and all other chemicals were obtained from the Sigma at the highest grade. The yeast strains used in this study were the isogenic derivatives of W303 as described previously [18].

Fig 5 | Kinetic and structural analysis of the WT and mutant Tim10. (A) Time course of absorption change at 340 nm for 10 μM Tim10 WT (a), Tim10C1,4S (b) or Tim10C2,3S (c) in the presence of 0.1 μM Trx, 0.1 μM Trr1 and 140 μM NADPH, after subtraction of blank reaction in the absence of Tim10. (B) Michaelis-Menten plots of the WT (a), Tim10C1,4S (b) and Tim10C2,3S (c) as studied in A. The k_{cat} and K_m were determined to be 23 min⁻¹, 6.0 μM for Tim10C1,4S, and 29.0 min⁻¹, 5.3 μM for Tim10C2,3S, respectively. (C) Far-ultraviolet circular dichroism spectra of the WT Tim10 (a, d), Tim10C1,4S (b, e) and Tim10C2,3S (c, f), in the absence (a-c) or presence (d-f) of 1 mM TCEP at 25 °C for 1 h. NADPH, nicotinamide adenine dinucleotide phosphate; Trr, thioredoxin reductase; Trx, thioredoxin; WT, wild-type.

Protein preparations. Oxidized WT and mutant Tim10 were purified as described previously in buffer AE (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA) [10,24]. Trx1 and Trr1 were purified as described previously [25], and followed by gel filtration (Superdex × 200 column). Flavin adenine dinucleotide was added to Trr1 before gel filtration. Trx1 concentration was determined using extinction coefficient of 10,095 M⁻¹ cm⁻¹ at 280 nm, and Trr1 concentration was determined based on extinction coefficient of 11,300 M⁻¹ cm⁻¹ for flavin adenine dinucleotide at 450 nm.

Miscellaneous. All experiments were carried out at 25 °C in buffer AE unless stated. AMS assay was performed as described in [7]. CD spectra were recorded as described in [14]. Mitochondria isolation and protein import analysis were performed as described previously [26,27]. All import reactions were performed in the presence of 2 mM EDTA. For steady-state levels of mitochondrial protein analyses, the WT and *trx1 trx2* yeast cells were first grown in YPD until OD₆₀₀ of 10 and then, cells were isolated by centrifugation, washed twice with autoclaved milli-Q water, then shifted to grow in YPEG for 6 h before the mitochondria were isolated. For the Trx enzyme kinetic analysis, WT or mutant Tim10 (1–50 μM) was mixed with 0.1 μM Trx and 140 μM NADPH, and the reaction was initiated by adding 0.1 μM Trr1. The oxidation of NADPH was followed at 340 nm using a Cary300 spectrophotometer (Varian Ltd).

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REFERENCES

- Schmidt O, Pfanner N, Meisinger C (2010) Mitochondrial protein import: from proteomics to functional mechanisms. *Nat Rev Mol Cell Biol* **11**: 655–667
- Chacinska A, Koehler CM, Milenkovic D, Lithgow T, Pfanner N (2009) Importing mitochondrial proteins: machineries and mechanisms. *Cell* **138**: 628–644
- Herrmann JM, Hell K (2005) Chopped, trapped or tacked—protein translocation into the IMS of mitochondria. *Trends Biochem Sci* **30**: 205–211
- Herrmann JM, Kauff F, Neuhaus HE (2009) Thiol oxidation in bacteria, mitochondria and chloroplasts: common principles but three unrelated machineries? *Biochim Biophys Acta* **1793**: 71–77
- Lu H, Allen S, Wardleworth L, Savory P, Tokatlidis K (2004) Functional TIM10 chaperone assembly is redox-regulated *in vivo*. *J Biol Chem* **279**: 18952–18958
- Mesecke N, Terziyska N, Kozany C, Baumann F, Neupert W, Hell K, Herrmann JM (2005) A disulfide relay system in the intermembrane space of mitochondria that mediates protein import. *Cell* **121**: 1059–1069
- Morgan B, Lu H (2008) Oxidative folding competes with mitochondrial import of the small Tim proteins. *Biochem J* **411**: 115–122
- Tokatlidis K (2005) A disulfide relay system in mitochondria. *Cell* **121**: 965–967
- Hell K (2008) The Erv1-Mia40 disulfide relay system in the intermembrane space of mitochondria. *Biochim Biophys Acta* **1783**: 601–609
- Lu H, Woodburn J (2005) Zinc binding stabilizes mitochondrial Tim10 in a reduced and import-competent state kinetically. *J Mol Biol* **353**: 897–910

11. Tienson HL, Dabir DV, Neal SE, Loo R, Hasson SA, Boontheung P, Kim SK, Loo JA, Koehler CM (2009) Reconstitution of the mia40-erv1 oxidative folding pathway for the small tim proteins. *Mol Biol Cell* **20**: 3481–3490
12. Hu J, Dong L, Outten CE (2008) The redox environment in the mitochondrial intermembrane space is maintained separately from the cytosol and matrix. *J Biol Chem* **283**: 29126–29134
13. Ostergaard H, Tachibana C, Winther JR (2004) Monitoring disulfide bond formation in the eukaryotic cytosol. *J Cell Biol* **166**: 337–345
14. Ivanova E, Ball M, Lu H (2008) Zinc binding of Tim10: Evidence for existence of an unstructured binding intermediate for a zinc finger protein. *PROTEINS: Struct Func Bioinf* **71**: 467–475
15. Lu H, Golovanov AP, Alcock F, Grossmann JG, Allen S, Lian LY, Tokatlidis K (2004) The structural basis of the TIM10 chaperone assembly. *J Biol Chem* **279**: 18959–18966
16. Holmgren A (1989) Thioredoxin and glutaredoxin systems. *J Biol Chem* **264**: 13963–13966
17. Meyer Y, Buchanan BB, Vignols F, Reichheld JP (2009) Thioredoxins and glutaredoxins: unifying elements in redox biology. *Annu Rev Genet* **43**: 335–367
18. Garrido EO, Grant CM (2002) Role of thioredoxins in the response of *Saccharomyces cerevisiae* to oxidative stress induced by hydroperoxides. *Mol Microbiol* **43**: 993–1003
19. Dietmeier K, Honlinger A, Bomer U, Dekker PJ, Eckerskorn C, Lottspeich F, Kubrich M, Pfanner N (1997) Tom5 functionally links mitochondrial preprotein receptors to the general import pore. *Nature* **388**: 195–200
20. Terziyska N, Grumbt B, Kozany C, Hell K (2009) Structural and functional roles of the conserved cysteine residues of the redox-regulated import receptor Mia40 in the intermembrane space of mitochondria. *J Biol Chem* **284**: 1353–1363
21. Zahedi Avval F, Holmgren A (2009) Molecular mechanisms of thioredoxin and glutaredoxin as hydrogen donors for Mammalian s phase ribonucleotide reductase. *J Biol Chem* **284**: 8233–8240
22. Messens J, Van Molle I, Vanhaesebrouck P, Limbourg M, Van Belle K, Wahni K, Martins JC, Loris R, Wyns L (2004) How thioredoxin can reduce a buried disulphide bond. *J Mol Biol* **339**: 527–537
23. Holmgren A (1979) Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. *J Biol Chem* **254**: 9627–9632
24. Ivanova E, Jowitt TA, Lu H (2008) Assembly of the mitochondrial Tim9-Tim10 complex: a multi-step reaction with novel intermediates. *J Mol Biol* **375**: 229–239
25. Mukhopadhyay R, Shi J, Rosen BP (2000) Purification and characterization of ACR2p, the *Saccharomyces cerevisiae* arsenate reductase. *J Biol Chem* **275**: 21149–21157
26. Meisinger C, Pfanner N, Truscott KN (2006) Isolation of yeast mitochondria. *Methods Mol Biol* **313**: 33–39
27. Morgan B, Ang SK, Yan G, Lu H (2009) Zinc can play chaperone-like and inhibitor roles during import of mitochondrial small Tim proteins. *J Biol Chem* **284**: 6818–6825