Intramolecular Disulfide Bond of Tim22 Protein Maintains Integrity of the TIM22 Complex in the Mitochondrial Inner Membrane

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Eukaryotic cells contain highly developed membrane structures called organelles. Each organelle consists of a characteristic set of proteins and lipids to exert its specialized functions. Mitochondria are essential organelles consisting of four compartments, the outer membrane (OM), inner membrane (IM), intermembrane space (IMS), and matrix. Because nearly all the mitochondrial proteins are synthesized in the cytosol, they have to be imported into mitochondria, sorted to one of the four submitochondrial compartments, where they function, and attain their functional native conformation, which is often facilitated by assembly into the membrane or multiprotein complex. Previous studies have revealed that these processes are mediated by elaborate protein machineries called translocators in the outer and inner membranes for import into and sorting to their destination submitochondrial compartments. Among them, the TIM22 complex mediates insertion of polytopic membrane proteins into the inner membrane, and Tim22 constitutes its central insertion channel. Here we report that the conserved Cys residues of Tim22 form an intramolecular disulfide bond. By comparison of Tim22 Cys → Ser mutants with wild-type Tim22, we show that the disulfide bond of Tim22 stabilizes Tim22 especially at elevated temperature through interactions with Tim18, which are also important for the stability of the TIM22 complex. We also show that lack of the disulfide bond in Tim22 impairs the assembly of TIM22 pathway substrate proteins into the inner membrane especially when the TIM22 complex handles excess amounts of substrate proteins. Our findings provide a new insight into the mechanism of the maintenance of the structural and functional integrity of the TIM22 complex.

Mitochondrial proteins require protein machineries called translocators in the outer and inner membranes for import into and sorting to their destination submitochondrial compartments. Among them, the TIM22 complex mediates insertion of polytopic membrane proteins into the inner membrane, and Tim22 constitutes its central insertion channel. Here we report that the conserved Cys residues of Tim22 form an intramolecular disulfide bond. By comparison of Tim22 Cys → Ser mutants with wild-type Tim22, we show that the disulfide bond of Tim22 stabilizes Tim22 especially at elevated temperature through interactions with Tim18, which are also important for the stability of the TIM22 complex. We also show that lack of the disulfide bond in Tim22 impairs the assembly of TIM22 pathway substrate proteins into the inner membrane especially when the TIM22 complex handles excess amounts of substrate proteins. Our findings provide a new insight into the mechanism of the maintenance of the structural and functional integrity of the TIM22 complex.

Significance: Tim40(Mia40)/Erv1-independent disulfide bond formation contributes to protein stability in mitochondria.

Conclusion: The disulfide bond of Tim22 has a role in stabilization of the TIM22 complex, which is important for the TIM22 protein assembly pathway.

Results: Lack of the disulfide bond of Tim22 destabilizes Tim22 and impairs substrate protein assembly.

Background: Tim22 is a central component of the mitochondrial inner membrane protein insertion machinery TIM22 complex.
Disulfide Bond of Tim22 in Mitochondria

### TABLE 1
Yeast strains used in this study

| Strain               | Genotype*                      | Source         |
|----------------------|--------------------------------|----------------|
| Tim22WT              | MATa ade2-1 his3-11, 15 sra3-1 leu2-3, 112 trp1-1 can1-100 tim22Δ-CgHIS3 | This study     |
| tim22-C42S           | MATa ade2-1 his3-11, 15 sra3-1 leu2-3, 112 trp1-1 can1-100 tim22Δ-CgHIS3 | This study     |
| tim22-C141S          | MATa ade2-1 his3-11, 15 sra3-1 leu2-3, 112 trp1-1 can1-100 tim22Δ-CgHIS3 | This study     |
| tim22-C42/141S       | MATa ade2-1 his3-11, 15 sra3-1 leu2-3, 112 trp1-1 can1-100 tim22Δ-CgHIS3 | This study     |
| Tim18FLAG/Tim22WT    | MATa ade2-1 his3-11, 15 sra3-1 leu2-3, 112 trp1-1 can1-100 tim22Δ-CgHIS3 | This study     |
| Tim18FLAG/tim22-C42S | MATa ade2-1 his3-11, 15 sra3-1 leu2-3, 112 trp1-1 can1-100 tim22Δ-CgHIS3 | This study     |
| Tim18FLAG/tim22-C141S| MATa ade2-1 his3-11, 15 sra3-1 leu2-3, 112 trp1-1 can1-100 tim22Δ-CgHIS3 | This study     |
| Tim18FLAG/tim22-C42/141S| MATa ade2-1 his3-11, 15 sra3-1 leu2-3, 112 trp1-1 can1-100 tim22Δ-CgHIS3 | This study     |
| Tim40                 | MATa ade2-1 his3-11, 15 sra3-1 leu2-3, 112 trp1-1 can1-100 GAL7-TIM40-CgHIS3 | This study     |
| Erv1                 | MATa ade2-1 his3-11, 15 sra3-1 leu2-3, 112 trp1-1 can1-100 GAL7-ERV1-CgHIS3 | This study     |

* Plasmids are in square brackets.

CX₃C or CX₆C (24–28). Tim40 transiently binds to the incoming substrate proteins in the IMS to form a mixed disulfide intermediate. Subsequently, Erv1, an FAD-containing sulfhydryl oxidase, oxidizes Tim40 to release substrate proteins to the IMS with transfer of a disulfide bond into substrate proteins (24–28).

Although previous studies have revealed a number of IMS proteins or protein domains that contain disulfide bond(s), which are likely transferred from Tim40, there are still many proteins or protein domains in the IMS that contain Cys residues whose formation of disulfide bonds remains to be experimentally confirmed (38, 39). Here we find that the conserved Cys residues in Tim22 form an intramolecular disulfide bond. Genetic and biochemical analyses reveal that the disulfide bond of Tim22 stabilizes Tim22 through its interactions with Tim18, and this stabilization is important for the stability and function of the Tim22 complex. Our findings provide a new insight into the mechanism of the maintenance of the structural and functional integrity of the TIM22 complex.

### EXPERIMENTAL PROCEDURES

**Plasmids, Strains, and Growth Conditions**—pRS316-Tim22 or pRS314-Tim22, a CEN-URA3 or CEN-TRP1 plasmid expressing Tim22, was constructed as follows. The TIM22 gene was PCR-amplified from yeast genomic DNA using primers SacII-Tim22-F (5'-TCC CGG CGG AGA ATG TCT TGG AGA CAG ATT-3) and Xhol-Tim22-R (5'-CCG CTC GAG TTA TTT GAA AGT GTG ACT ATC ACT-3), digested with SacII and Xhol, and cloned into pRS316 or pRS314.

pRS314-Tim22-C42S, -C141S, and -C42/141S, CEN-TRP1 plasmids expressing Tim22-C42S, -C141S, or -C42/141S, were constructed as follows.4 The C42S or C141S mutation was introduced into the TIM22 gene on pRS314-Tim22 by QuikChange PCR using primers Tim22-C42S-F (5'-TCA TGA CTT CCT CTC CTG GAA AAT C-3') and Tim22-C42S-R (5'-GAT TTT CCA GGA GAG GAC ATG A-3') or Tim22-C141S-F (5'-CTG GGG TGG AGT CTC TGA TCT AGT C-3') and Tim22-C141S-R (5'-GAC TCT ATG ACA GAC TCC ACC CCA G-3'). To obtain pRS314-Tim22-C42/141S, pRS314-Tim22-C42S was mutated by QuikChange PCR using primers Tim22-C141S-F and Tim22-C141S-R.

For *in vitro* translation of Tim22 and its mutants, the TIM22, tim22-C42S, C141S, or C42/141S gene was amplified from plasmid pRS314-Tim22, Tim22-C42S, C141S, or C42/141S, using primers BamHI-Tim22-(30bp)-F (5'-CAG GAT CAT TCT CAC ACC CAA ACC TCA CTT GCT GCT) and BamHI-Tim22-(30bp)-R (5'-GTC TGC AGT CAT TCT TTA AAA TAC TTT-3') and PstI-Tim22-R (5'-GTC TGC AGT CAT TCT TTA AAA TAC TTT-3'), digested with BamHI and PstI, and cloned into pGEM-4Z (Promega).

For overexpression of ADP-ATP carrier (AAC), phosphate carrier (PIC), or dicarboxylate carrier (DIC), the AAC2, MIR1, or DIC1 gene was PCR-amplified from yeast genomic DNA using primers BamHI-AAC-F (5'-CGC GGA TCC ATG TAC TGG TCT TCC AAC GCC CAA GTC AAA ACC CCA CCT CCA G-3') and AAC-Xhol-R (5'-CCG CTC GAG TTA TTT GAA AGT GTG ACT ATC ACT-3'), digested with BamHI and Xhol, and cloned into pRS316 or pRS314.

Yeast strains used in this study are listed in Table 1. C-terminal FLAG tagging was accomplished by PCR-mediated gene replacement (30). The FLAG tag was amplified from pFA6a-FLAG-kanMX6 (31) using primers Tim18tag-F (5’-GAT CAA GAA ATT GTG ATT TTA) and Tim18tag-R (5’-TAT AAA TAC ACA TTT AAA ACA). Yeast strains used in this study are listed in Table 1. C-terminal FLAG tagging was accomplished by PCR-mediated gene replacement (30). The FLAG tag was amplified from pFA6a-FLAG-kanMX6 (31) using primers Tim18tag-F (5’-GAT CAA GAA ATT GTG ATT TTA) and Tim18tag-R (5’-TAT AAA TAC ACA TTT AAA ACA).

4 Throughout this study, C42/141S was used to indicate the double mutant C42S/C141S.
TTC ATC GTT CTG CGA AAT TGG CTA TCA CAG GAA ACA GCT ATG ACC-3′), and introduced into the TIM22 locus of a diploid strain, W303-AB (31). After introduction to the URA3-containing single-copy plasmid harboring the TIM22 gene, the diploid cells were subjected to sporulation and dissection. The resulting haploid strain that lacks the TIM22 gene in chromosomes with the complementing URA3-plasmid was transformed with a TRP1-containing single-copy plasmid harboring the TIM22, tim22-C42S, tim22-C141S, or tim22-C42/I41S gene. The resulting strains were cultured on SCD-Trp containing 5′-fluoroorotic acid to obtain tim22-C42S, tim22-C141S, tim22-C42/I41S, and the corresponding wild-type strains.

Cells were grown in lactate medium (0.3% yeast extract, 0.2% glucose, 0.05% CaCl2·2H2O, 0.05% NaCl, 0.06% MgCl2·6H2O, 0.1% KH2PO4, 0.1% NH4Cl, 2.0% lactic acid, pH 5.6), SCD (0.67% yeast nitrogen base without amino acids, 0.5% casamino acid, 2% glucose), 120 mM creatine phosphate, 120 mM creatine kinase, 2 mM methionine, 30 µg/ml each of l-leucine and l-lysine. Cells that have the KanMX6 gene were selected on YPD (1% yeast extract, 2% glucose), 200 µg/ml gentamicin, 2 mM mercaptoethanol, and 0.5 mM PMSF. Mitochondria were treated with 50 µg/ml valinomycin, mitochondria were treated with 50 µg/ml proteinase K for 10 min on ice to digest substrate proteins outside mitochondria.

**RESULTS**

**Tim22 Forms an Intramolecular Disulfide Bond**—Evidence has accumulated that a number of proteins form disulfide bond(s) in the mitochondrial IMS, usually with the aid of the Erv1-Tim40 disulfide bond relay system (23–27). Tim22 is a central channel-forming component of the TIM22 complex, the insertion machinery for polytopic IM proteins. Because Tim22 has two Cys residues that are well conserved among different organisms (Fig. 1A), we wondered whether Tim22 forms an intramolecular disulfide bond in the IMS. We thus compared the migration rates of Tim22 on SDS-PAGE gels between reducing (in the presence of β-mercaptoethanol (β-ME)) and nonreducing (in the absence of β-ME) conditions followed by immunoblotting with anti-Tim22 antibodies (Fig. 1B). Tim22 migrated faster under the nonreducing condition than the reducing condition, suggesting that Tim22 forms a disulfide bond. To confirm this further, we tested the presence of a free thiol group by incubating cell extracts with a thiol-trapping reagent, AMS and detected Tim22 by immunoblotting. AMS can be covalently attached to a free thiol group in a protein, which accompanies a 500-Da increase in the size of the modified protein. The size of Tim22 increased upon AMS treatment only when proteins were pretreated with DTT (Fig. 1C, DTT+, AMS+), but it did not increase without pretreatment with DTT (Fig. 1C, DTT−, AMS+). As a control, we analyzed AMS modification of another IM protein, Tim23, which is a Tim22 homolog and contains three Cys residues. Upon AMS treatment, Tim23 was modified even without DTT pretreatment, and the migration rates of AMS-modified Tim23 with or without DTT pretreatment were the same. These results show that Tim22, but not Tim23, forms a disulfide bond and does not contain free thiol group in mitochondria.

We next constructed yeast strains containing Tim22 mutants with one or two Cys → Ser replacement(s) (C42S, C141S, or C42/I41S). The single Cys → Ser mutants, Tim22-
C42S and Tim22-C141S, and the double Cys → Ser mutant, Tim22-C42/141S, all exhibited similar migration rates, under both nonreducing and reducing conditions, eliminating the possibility that two Cys residues independently contribute to a disulfide bond formation or that Tim22 forms a disulfide-linked dimer (Fig. 1D). To rule out the trivial possibility that Tim22 forms a disulfide bond only after solubilization of mitochondria, we preincubated mitochondria with different concentrations of DTT on ice or at 30 °C, washed off DTT, and then solubilized the mitochondria under the nonreducing condition. The proteins were analyzed by SDS-PAGE in the absence of β-ME followed by immunoblotting with antibodies against Tim22. Tim22 was detected as a reduced form after pretreatment with 30 mM (30 °C) or 90 mM (0 °C) DTT (Fig. 1E). Taken together, we conclude that Tim22 forms an intramolecular disulfide bond in mitochondria.

**Effects of Cys → Ser Mutations on Assembly of Tim22 into the TIM22 Complex**—We next asked what the roles of the disulfide bond of Tim22 are. We first examined whether lack of the disulfide bond affects the assembly of Tim22 into the TIM22 complex. We synthesized radiolabeled wild-type Tim22 and its Cys → Ser mutants (C42S, C141S, and C42/141S) in vitro and imported them into mitochondria isolated from wild-type and tim22 Cys → Ser mutant cells were analyzed by SDS-PAGE under reducing (+ β-ME) or nonreducing (− β-ME) condition followed by immunoblotting with anti-Tim22 antibodies. Mitochondria isolated from wild-type yeast cells were incubated with the indicated concentrations of DTT on ice or at 30 °C for 30 min. After washing with SEM buffer, proteins were analyzed by SDS-PAGE under nonreducing conditions and immunoblotting with anti-Tim22 antibodies.

As reported previously, imported wild-type Tim22 formed a Tim22 dimer and was subsequently assembled into the 300-kDa TIM22 complex in a manner dependent on the membrane potential across the IM (ΔΨ) (23) (Fig. 2A). In contrast, the Tim22 mutants, Tim22-C42S, Tim22-C141S, and Tim22-C42/141S, hardly formed a dimer and were not assembled into the 300-kDa TIM22 complex efficiently (Fig. 2A). The amounts of Tim22 assembled into the TIM22 complex were normalized by those of imported Tim22 (SDS-PAGE) and plotted against time (Fig. 2C). These results indicate that both Cys-42 and Cys-141 are important for proper Tim22 assembly into the TIM22 complex through formation of the dimer.

We then compared the in vitro assembly of radiolabeled wild-type Tim22 into the TIM22 complex in mitochondria isolated from the wild-type, tim22-C42S, tim22-C141S, and tim22-C42/141S strains. Interestingly, assembly of wild-type Tim22 into the TIM22 complex was accelerated in tim22-C42S, tim22-C141S, and tim22-C42/141S mitochondria as compared with that in wild-type mitochondria (Fig. 2, B and D). We also noticed that the Tim22 dimer was hardly detected during the Tim22 assembly in tim22-C42S, tim22-C141S, and tim22-C42/
Disulfide bond formation of Tim22 affects assembly of Tim22 into the TIM22 complex in vitro. A, $^{35}$S-labeled Tim22 and Tim22 Cys $\rightarrow$ Ser mutants were synthesized in vitro and incubated with wild-type mitochondria for the indicated times in the presence (ΔΨ+ or absence (ΔΨ−) of valinomycin. Mitochondria were then treated with proteinase K, and proteins were analyzed by SDS-PAGE and radioimaging. For analysis of the assembly of Tim22 into the TIM22 complex, mitochondria were lysed in digitonin buffer, and proteins were analyzed by BN-PAGE and radioimaging. C lane indicates control. B, $^{35}$S-labeled wild-type Tim22 was synthesized and imported to mitochondria isolated from wild-type or tim22 Cys $\rightarrow$ Ser mutant cells. Imported and assembled Tim22 was analyzed as in A. Control (C) indicates 10% of protein used for the in vitro import assay. C and D, quantification of assembly of Tim22 to the TIM22 complex. The amounts of 300-kDa TIM22 complex were normalized by those of imported Tim22 at each time point. The amounts of wild-type Tim22 in A and Tim22-C42S in B at 30-min incubation are set to 100%, respectively. Values are mean ± S.E. (error bars) (n = 6 for wild-type, n = 3 for the others).
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141S mitochondria (Fig. 2B), probably due to rapid consumption of the Tim22 dimer intermediate for uptake by the TIM22 complex (23). These results suggest that the lack of the disulfide bond in Tim22 alters the quaternary structures of the TIM22 complex in such a way that newly imported wild-type Tim22 can exchange with pre-existing mutant Tim22 much faster than in wild-type mitochondria.

**Conserved Cys Residues of Tim22 Are Important for Stabilization of the TIM22 Complex**—It was reported previously that destabilization of the TIM22 complex by mutations in each of the subunits facilitates integration of newly imported, corresponding subunits into the TIM22 complex (23). The above results suggest that the lack of the disulfide bond in Tim22 alters the quaternary structures of the TIM22 complex in such a way that newly imported wild-type Tim22 can exchange with pre-existing mutant Tim22 much faster than in wild-type mitochondria.

We next analyzed the levels of the 300-kDa TIM22 complex by blue-native PAGE (BN-PAGE) after solubilization of mitochondria with 1% digitonin. The apparent sizes of the TIM22 complex detected with anti-Tim22 antibodies were slightly smaller in tim22 Cys → Ser mutant mitochondria at 30 °C (Fig. 3A). However, the steady-state levels of the Tim22 mutants (Tim22-C42S, Tim22-C141S, and Tim22-C42/141S) decreased when yeast cells were cultured at elevated temperature (37 °C) for 4 h before the isolation of mitochondria (Fig. 3B).

When anti-Tim54 antibodies were used for detection of the TIM22 complex, additional smaller complexes were observed for Tim22 Cys → Ser mutant mitochondria (Fig. 3C, asterisks). As negative controls, the amounts...
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FIGURE 4. Loss of the disulfide bond in Tim22 weakens Tim22-Tim18 interactions. Left panels, A, mitochondria isolated from wild-type and tim22 Cys → Ser mutant cells expressing Tim18-FLAG were lysed with digitonin and subjected to immunoprecipitation using anti-FLAG agarose. Bound proteins were eluted with boiled SDS-PAGE sample buffer. The mitochondrial lysate (Total; 20%) and bound proteins (Elute; 100%) are shown. B, mitochondria were pretreated at 37 °C for 10 min and subjected to immunoprecipitation as in A. C and D, mitochondria isolated from cells overexpressing wild-type Tim22 or Tim22 Cys → Ser mutants under their own promoter from a multicopy plasmid pYO324 were subjected to the same immunoprecipitation as in A and B, respectively. Right panels (A–D), protein amounts were quantified. The amounts for wild-type mitochondria are set to 100%. Values are mean ± S.E. (error bars) (n = 3). SDS-PAGE was performed under reducing conditions in A and B or nonreducing conditions in C and D.

of the TOM40 complex in the OM and the TIM23 complex in the IM were comparable between wild-type and tim22 Cys → Ser mutant mitochondria. The amounts of the TIM22 complex detected with antibodies against Tim22, Tim18, and Tim54 also decreased after treatment at 37 °C (Fig. 3D). These results indicate that C42S, C141S, and C42/141S mutations in Tim22 destabilize the TIM22 complex at 30 °C, and lead to decreased levels of the 300-kDa TIM22 complex after treatment at 37 °C. This is consistent with the idea that Cys → Ser mutations or most likely the lack of the conserved disulfide bond in Tim22 cause(s) destabilization of the TIM22 complex, which instead facilitates exchange of pre-existing Tim22 with newly imported Tim22.

Disulfide Bond of Tim22 Is Important for Tim22-Tim18 Interactions—We further asked which interactions among the subunits of the TIM22 complex were destabilized by the Tim22 Cys → Ser mutations. We isolated mitochondria from wild-type and tim22 Cys → Ser mutant cells expressing C-terminally FLAG-tagged Tim18 instead of authentic Tim18. The mitochondria were solubilized with digitonin and subjected to co-immunoprecipitation using anti-FLAG agarose. The levels of Tim22 and Tim54 co-immunoprecipitated with Tim18-FLAG were comparable between wild-type and tim22 Cys → Ser mutant mitochondria (Fig. 4A). Tom70 was used as a negative control (Fig. 4A). However, when mitochondria were preincubated for 10 min at 37 °C before solubilization, the amounts of Tim22 and Tim54 co-immunoprecipitated with Tim18-FLAG were significantly reduced (Fig. 4B). The reduction of the co-immunoprecipitated proteins may simply reflect the decreased amounts of total Tim22 Cys → Ser mutants after heat treatment (Fig. 4B, Total). To distinguish the effects of the absence of the disulfide bond on possible decreased intersubunit inter-
actions from the decreased amounts of Tim22, we overexpressed wild-type or mutant Tim22 proteins from multicopy plasmids and performed similar co-immunoprecipitation experiments with or without preincubation at 37 °C. Now due to overexpression, total amounts of wild-type and mutant Tim22 are comparable between wild-type and \textit{tim22} \textit{Cys} \textit{3} \textit{Ser} mitochondria even after heat treatment at 37 °C (Fig. 4, \textit{C} and \textit{D}). Nevertheless, the amount of Tim22 co-immunoprecipitated with Tim18-FLAG was still significantly reduced after heat treatment for \textit{tim22} \textit{Cys} \textit{3} \textit{Ser} mutant mitochondria, although reduction of co-immunoprecipitated Tim54 is marginal (Fig. 4D). These results indicate that the disulfide bond of Tim22 is important for Tim22-Tim18 interactions in the TIM22 complex at elevated temperature.

\textbf{Tim22 Is Susceptible to Degradation due to the Loss of the Disulfide Bond}—When wild-type Tim22 was overexpressed, the reduced form of Tim22 was observed, yet it was gone after heat treatment at 37 °C (Fig. 4, \textit{C} and \textit{D}). Therefore, the reduced form of Tim22 is likely unstable and degraded efficiently at elevated temperature. We next performed cycloheximide chase experiments to compare the stability or degradation of wild-type and mutant Tim22 as well as Tim18 and Tim54 in the TIM22 complex. Briefly, we stopped protein synthesis in wild-type and \textit{tim22} \textit{Cys} \textit{3} \textit{Ser} mutant cells by the addition of a ribosomal inhibitor, cycloheximide. Then, whole cell extracts were prepared from the cells cultivated for different time periods (1–3 h) at 30 °C or 37 °C after translation inhibition to follow the fate of each protein. Immunoblotting of Tim22 showed that Tim22 Cys \textit{3} Ser mutants were rapidly degraded as compared with wild-type Tim22, and rapid degradation was more prominent at 37 °C than at 30 °C (Fig. 5). We also observed accelerated degradation of Tim18 at 37 °C in \textit{tim22} \textit{Cys} \textit{3} \textit{Ser} mutant cells. However, degradation rates of another subunit of the TIM22 complex, Tim54, and a control protein, Tom70, did...
not significantly differ between wild-type and \textit{tim22} Cys → Ser mutant cells (Fig. 5). These results clearly indicate that the disulfide bond plays an important role in stabilizing Tim22 especially at elevated temperature.

**Protein Assembly via the TIM22 Complex Is Impaired in \textit{tim22} Cys → Ser Mutant Mitochondria**—We asked whether the disulfide bond of Tim22 plays important roles in protein import and assembly via the TIM22 complex. We thus performed \textit{in vitro} import assays using an IM protein, Tim23, as a substrate for insertion into the IM via the TIM22 complex.

**FIGURE 6. Import and assembly of Tim23 are impaired in \textit{tim22} Cys → Ser mutant mitochondria.** A. 35S-labeled Tim23 was imported into wild-type and \textit{tim22} Cys → Ser mutant mitochondria at 37 °C for the indicated times. Imported and assembled Tim23 were analyzed by SDS-PAGE (uppermost panels) and BN-PAGE (central panel) followed by radioimaging. The amounts of the longest time point for wild-type mitochondria are set to 100%. B, C-terminally FLAG-tagged Tim23 was synthesized with wheat germ extract and imported into wild-type and \textit{tim22} Cys → Ser mutant mitochondria at 25 °C for the indicated times. To assess the amounts of imported Tim23, the mitochondria were treated with proteinase K. Imported Tim23-FLAG was analyzed by SDS-PAGE followed by immunoblotting with the anti-FLAG antibody. 10% cont. indicates 10% of protein used for the \textit{in vitro} import assay. Lower panel, imported Tim23-FLAG was quantified. The amount of the longest time point for wild-type mitochondria is set to 100%.
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chondria and analyzed by SDS-PAGE to assess the amounts of Tim23 imported into mitochondria; the other half was solubilized with digitonin and analyzed by BN-PAGE to monitor its assembly into the TIM23 complex. When analyzed by BN-PAGE, imported Tim23 was assembled into the 100-kDa TIM23 core complex consisting of Tim23 and Tim17 efficiently as well as the TIM23 holo-complex containing Tim21 and/or Tim50 with higher molecular weights (Fig. 6A). Assembly of imported Tim23 into the TIM23 complex was less efficient in tim22 Cys → Ser mutant mitochondria. Although import of radiolabeled Tim23 into mitochondria was only slightly affected by tim22 Cys → Ser mutation (Fig. 6A), this defect became enhanced when we used excess amounts of C-terminally FLAG-tagged Tim23, which was synthesized in vitro with wheat germ extracts, for in vitro import assays (Fig. 6B). Therefore, the disulfide bond of Tim22 is required for efficient import and assembly of multispansing IM proteins such as Tim23 via the TIM22 complex.

Overexpression of Carrier Proteins Compromises Growths of tim22 Cys → Ser Mutant Cells—Our in vitro experiments suggest that the disulfide bond of Tim22 is important for protein import and assembly through the TIM22 pathway. Because the TIM22 protein import pathway is essential for mitochondrial biogenesis, hence yeast cell growth, we wondered whether Cys → Ser mutations of Tim22 affect cell growth. Because defects of the TIM22 pathway caused by the Cys → Ser mutations of Tim22 are more prominent when excess amounts of substrate proteins are imported (Fig. 6B), we tested the effects of overexpression of metabolite carrier proteins such as AAC, PIC, and DIC, which are inserted into the IM via the TIM22 complex, on the growths of tim22 Cys → Ser mutant cells. Although tim22 Cys → Ser mutant cells grew normally on fermentable and nonfermentable media at all temperatures we tested as compared with wild-type cells (Fig. 7A), overexpression of AAC, PIC, or DIC exacerbated the growths of tim22 Cys → Ser mutant cells more significantly than that of wild-type cells (Fig. 7B). In contrast, overexpression of an OM protein, Tom22, or a matrix-targeted protein, pb2(167)Δ19-DHFR, a fusion protein consisting of the matrix-targeting signal derived from cytochrome b$_2$ followed by mouse dihydrofolate reductase (DHFR), did not affect the growths of tim22 Cys → Ser mutant cells at all (Fig. 7B). These results support our idea that the disulfide bond of Tim22 is functionally important especially when the TIM22 complex handles excess amounts of its substrate proteins.

DISCUSSION

In this study, we found that two conserved Cys residues of Tim22 form an intramolecular disulfide bond, which is important for stability and functions of the TIM22 complex. To assess the roles of the disulfide bond in Tim22, we constructed three tim22 Cys → Ser mutant cells, in which one or both conserved Cys residues are replaced with Ser. Lack of the disulfide bond in Tim22 renders interactions between Tim22 and Tim18 weak in the TIM22 complex especially at elevated temperature. These weakened Tim22-Tim18 interactions apparently lead to rapid exchange of Tim22 assembled in the TIM22 complex with a pool of free Tim22 (e.g. newly imported Tim22), to rapid deg-

FIGURE 7. Overexpression of carrier proteins leads to growth defects of tim22 Cys → Ser mutant cells. A, 10-fold serial dilutions of wild-type and tim22 Cys → Ser mutant cells were spotted on SCD (~ Trp) (Glucose) and SC-Lac (~ Trp) plates (Lactic acid) and cultivated at the indicated temperatures for 2 and 4 days, respectively. B, IM carrier proteins, AAC, PIC, and DIC, an OM protein Tom22, and a matrix protein, pb2(167)Δ19-DHFR, were overexpressed under the GAL1 promoter in wild-type and tim22 Cys → Ser mutant cells. 10-fold serial dilutions of these cells were spotted onto SCGal (~ Trp, ~ Ura) plates and cultivated at 30 °C for 2 days.

radation of the Tim22 mutants in the IM, and to destabilization of the TIM22 complex. Besides, the TIM22 complex with destabilized Tim22 mutants is defective in its function in assembly of polytopic IM proteins in the IM, especially when it has to handle excess amounts of substrate proteins, which is reflected in the impaired cell growth upon overexpression of carrier proteins.

During preparation of this manuscript, Chacinska and colleagues (34) reported that Tim22 forms a disulfide bond and that Tim22 Cys → Ser mutants fail in assembly into the TIM22 complex in vitro, which is essentially the same as we observed here. They also showed that Tim40/Mia40 drives import of Tim22 into the IMS through formation of the mixed disulfide intermediates between Tim22 and Tim40, although Tim22 does not contain a typical Cys motif for the Tim40 pathway such as CX$_2$C or CX$_3$C. Interestingly, they reported that the redox state of Tim22 is not affected in tim40-F311E and erv1-2 cells, suggesting that the Tim40-Erv1 system is not required for oxidation of Tim22 (34). Consistently, we observed normal disulfide bond formation of Tim22 even when Tim40 or Erv1 was depleted by GAL7 promoter shut-off (35, 36), although the steady-state level of Tim22 somehow decreased after depletion of Tim40 (data not shown). We also confirmed by in vitro import assays that newly imported Tim22 is mainly present as an oxidized form even in Tim40-depleted or Erv1-depleted mitochondria (data not shown).

Then what facilitates disulfide formation of newly imported Tim22 in mitochondria? Chacinska and colleagues (34) showed...
that ΔΨ is essential for the disulfide bond formation of Tim22 and that the oxidized form of newly imported Tim22 preferentially interacts with Tim18. These results suggest that the disulfide bond formation occurs after assembly of Tim22 into the TIM22 complex. Interestingly, when we overexpressed wild-type Tim22, not only oxidized Tim22 but also reduced Tim22 was detected in isolated mitochondria (Fig. 4C). This may suggest that excess Tim22 molecules that overflowed from the TIM22 complex may not form a disulfide bond. Supporting this idea, our co-immunoprecipitation experiments revealed stronger interactions of oxidized Tim22 with Tim18 than reduced Tim22 (Fig. 4C). In addition, the reduced form of Tim22 was unstable because it is rapidly degraded after heat treatment of mitochondria overexpressing Tim22 (Fig. 4D). On the basis of these observations, we propose that Tim22 spontaneously forms a disulfide bond between the conserved Cys residues after proper folding, which is achieved by correct assembly into the TIM22 complex and brings the Cys residues in close proximity (Fig. 8). In other words, even wild-type Tim22 cannot fold properly into its native conformation until it interacts with its proper binding partner, Tim18, in the TIM22 complex, and such incompletely folded Tim22, which cannot form a disulfide bond, becomes prominent when Tim22 is overexpressed.

Although Tim22 has been often assumed to have four hydrophobic transmembrane (TM) segments, precise membrane topology of Tim22 has not been experimentally determined so far. Possible TM segments are predicted by the TMpred program to be residues 50–69, 81–99, 129–146, and 174–191, although the second predicted TM segment (residues 81–99) is less hydrophobic (37). We can assume, on the basis of the sequence similarity between Tim22 and Tim23, that the N terminus of Tim22 faces the IMS (12). If Tim22 contains four TM segments, Cys-41, which is likely present in the IMS, needs to be inserted into the IM to form a disulfide bond with Cys-141, which is localized near the matrix side in the third TM helix. Alternatively, if Tim22 contains only three TM segments, leaving the second predicted TM segment uninserted into the IM, the disulfide bond may be formed more easily because Cys-141 should be close to the IMS side in the third predicted TM helix, which could be more consistent with our finding of the disulfide bond between Cys-42 and Cys-141. Clearly, biochemical and structural analyses to determine the membrane topology of Tim22 are essential for further understanding of the functional details of the TIM22 complex in mitochondrial protein import.

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