The TIM10 complex, composed of the homologous proteins Tim10 and Tim9, chaperones hydrophobic proteins inserted at the mitochondrial inner membrane. A salient feature of the TIM10 complex subunits is their conserved “twin CX3C” motif. Systematic mutational analysis of all cysteines of Tim10 showed that their underlying molecular defect is impaired folding (demonstrated by circular dichroism, aberrant homo-oligomer formation, and thiol trapping assays). As a result of defective folding, clear functional consequences were manifested in (i) complex formation with Tim9, (ii) chaperone activity, and (iii) import into Tim9ts mitochondria lacking both endogenous Tim9 and Tim10. The organization of the four cysteines in intrachain disulfides was determined by trypsin digestion and mass spectrometry. The two distal CX3C motifs are juxtaposed in the folded structure and disulfide-bonded to each other rather than within each other, with an inner cysteine pair connecting Cys\textsuperscript{40} with Cys\textsuperscript{61} and an outer pair between Cys\textsuperscript{40} and Cys\textsuperscript{65}. These cysteine pairs are not equally important for folding and assembly; mutations of the inner Cys are severely affected and form wrong, non-native disulfides, in contrast to mutations of the outer Cys that can still maintain the native inner disulfide and display weaker functional defects. Taken together these data reveal this specific intramolecular disulfide bonding as the crucial mechanism for Tim10 folding and show that the inner cysteine pair has a more prominent role in this process.

Mitochondria import the vast majority of their proteins from the cytosol (1). To cope with the variety of these precursors, they have dedicated translocation machineries that facilitate the import through the outer (TOM translocase) and inner membrane (TIM translocase) (1–4). Most presequence-containing precursors undergo import pathways that are variants of (i) complex formation with Tim9 and Tim10 (the TIM10 complex) (16–20) or Tim8 and Tim13 (21, 22). The TIM10 complex appears to be a major player because it is in large excess over the Tim8-Tim13 complex, and both Tim9 and Tim10 genes are essential in yeast, whereas Tim8 and Tim13 are dispensable (18, 22–24).

The TIM10 complex binds at an early stage to the transmembrane segments of the carrier precursor, thus facilitating passage across the outer membrane (16, 19, 20, 25, 26). It then escorts the hydrophobic carrier across the intermembrane space, thus preventing its aggregation and acting as a chaperone (8, 17, 20, 27, 28). Subsequently, the precursor is inserted in the membrane by the 300-kDa TIM22 complex in a membrane potential-dependent manner (29, 30). Insertion of the polypeptide into the membrane occurs via a two-stage mechanism involving two distinct voltage-dependent steps (31). The insertion stage is probably the rate-determining step in this process, with subsequent dimerization of the carrier to its final functional dimer occurring very fast in the membrane (32).

The hetero-hexameric TIM10 complex (20, 25, 28) is made exclusively of Tim9 and Tim10 that are necessary and sufficient to form the complex (20). They bind to each other with 1:1 stoichiometry (three molecules of Tim9 with three molecules of Tim10) and an affinity of $5 \times 10^6 \text{ M}^{-1}$ (28). The TIM10 complex has been reconstituted in vitro either by co-expression of its two subunits in bacteria (20, 25) or by separately purifying them (28). In both cases, the complex reconstituted with the recombinant proteins is indistinguishable from the authentic mitochondrial complex (20, 25, 28).

A striking characteristic of all small Tim proteins is the presence of a strictly conserved “twin CX3C” motif separated by 11–16 residues. Initially, it had been proposed that the Cys in this zinc finger-like domain bind zinc (17), but a later report contradicted this and suggested that the Cys are connected in disulfide bonds (25). Recently, we have shown that the complex can only be formed by oxidized subunits and that zinc can bind only to the fully reduced state but without promoting complex formation.\textsuperscript{1} Whatever the case, it is clear that the twin CX3C is important for the structure and possibly the function of these proteins, but the specific roles of the Cys residues in complex formation, import, or substrate binding remains unresolved. An interesting observation along these lines is that mutation of the fourth cysteine residue in DDP1 (the human homologue of...
Circular Dichroism Analysis—CD spectra were acquired using a JASCO J-810 spectropolarimeter in 5 mM Tris, pH 7.4, at 25 °C using a 1-mm cuvette and protein samples of 0.1 mg/ml. For the α helix-containing domain of Tim10, denaturation was performed for 1 h at 25 °C prior to measurement on the spectropolarimeter. Each spectrum represents an average of four scans from 260 to 195 nm at 0.2-nm intervals. The base line was established by subtracting the spectrum of the buffer 0.1 mg/ml alone.

Isoelectric Focusing—Tim9 and Tim10 (wt and mutant) proteins were solubilized in 9 mM urea, 2% thiourea, 2% CHAPS, 65 mM DTT, 0.5% IPG buffer, pH 3–10, for 1 h at room temperature to give a concentration of 20 μg in 350 μl. This solution was then injected into an IPGphor isolectric focusing strip holder (Amersham Biosciences), and 50 μl was loaded onto top of the IPG strip. The gel was run down. 2 μl of paraffin oil was applied on top of the strip to minimize evaporation, and the cover was placed on the strip holder. The holder containing the strips was then placed onto the IPIphor platform. Focusing consisted of 12 h of rehydration at 2 °C and then focusing at (i) S1 500 V, 500-V h; (ii) S2 1000 V, 1000-V h; and (iii) S3 8000 V, 32000 V h.

Focusing was conducted using a current of 50 μA/strip and a Step-Hold gradient. Once completed the strips were placed in Petri dishes and stained. The strips were fixed for 10 min in 20% trichloroacetic acid and washed for 3 min in a 1:1 ratio of 0.2% (w/v) CuSO4, 20% acetic acid, and 80% (v/v) methanol. The strips were stained in staining buffer that consisted of 1.1 ratio of 0.2% (w/v) CuSO4, 20% acetic acid, 88% Meambo Blue R-250 (Merek), 40% distilled water, 60% methanol (100%) solution. All of the solutions were added to the strips and mixed gently while incubating.

Intramolecular Disulfide Bond Mapping by Trypsin Digestion MALDI-TOF-MS—Purified oxidized proteins were separated from their intermolecular disulfide species by SDS-PAGE using gel sample buffer without DTT. The monomer bands of the proteins were cut out from Coomassie-stained gels. Each protein sample from the gel was divided into two, washed with acetonitrile, dried by vacuum centrifugation, and dissolved in a small volume of 25 mM NH4HCO3. One sample was kept as the oxidized sample, and the other was reduced with 10 μM DTT at 55 °C for 1 h and alkylated with 55 mM iodoacetamide for 45 min. In-gel trypsin digestion was performed essentially as described by Shakevchenko et al. (35), and the digestion products were extracted with 20 μl of NH4HCO3, 5% formic acid in 50% acetonitrile and concentrated using a vacuum centrifuge. The samples were desalted using a C4 or C18 ZipTip, and 1 μl of the desalted mixture was mixed with an equal volume of 50 mM α-cyano-4-hydroxycinnamic acid. The mixture (1 μl) was applied to a TOF-Spec target and analyzed by MALDI-TOF-MS in the positive ion mode, using a Voyager-DE STR. Angiotensin I, adrenocorticotropic hormone, and oxidized insulin B chain were used as internal standards. MALDI-TOF-MS spectra for oxidized and reduced peptides were recorded.

Import into tim9ts Mitochondria—Pure proteins were used for import studies (20), and Western blot analysis was performed to ascertain whether import was successful. For each import, 4 μg of Tim10 was precipitated in 2 volumes of saturated ammonium sulfate in 50 mM Hepes, pH 7.4, for 30 min on ice. This solution was centrifuged at 25,000 × g for 30 min. The resulting pellet was resuspended in 8 mM urea, 10 mM EDTA, 10 mM β-mercaptoethanol, 50 mM Hepes-KOH, pH 7.4, and incubated for 1 h at room temperature. Meanwhile 200 μg of mitochondria for each import was washed with breaking buffer (20 mM Hepes-KOH, pH 7.4, 0.6 M sorbitol), centrifuged at 12,000 × g for 5 min at 4 °C, and then resuspended in 20 μl of breaking buffer. The mitochondria were then incubated in import buffer consisting of 100 mM Hepes-KOH, pH 7.1, 1.2 M sorbitol, 4 mM KHPO4, 100 mM KCl, 20 mM MgCl2, 5 mM Na2EDTA, 10 mM t-methionine, 2 mg/ml fatty acid-free bovine serum albumin in the presence of 2.0 mM ATP and 2.5 mM NADH. Tim10 was then added to this import mix and vortexed briefly, and import was carried out for 15 min at 30 °C. The mix was centrifuged at 12,000 × g for 4 min, the supernatant was removed, and the pellet was resuspended in 200 μl of 0.1 M guanidine hydrochloride containing 0.1 M NaAc on ice. 2 μl of 0.2% phenylmethylsulfonyl fluoride was added to all samples and centrifuged at 12,000 × g for 4 min, the supernatant was removed, and the pellet was resuspended in 20 μl of 2% sample buffer containing 2 mM phenylmethylsulfonyl fluoride. All of the samples were analyzed by SDS-PAGE and Western analysis using Tim10 antibodies. For restoration assays, 8 μg of Tim10 wt protein was imported first, and mitochondria were then centrifuged at 12,000 × g for 5 min at 4 °C and resuspended in 400 μl of the import mix for subsequent import of the Tim10 protein.

Thiol Trapping Assays—Tim10 at 30 μM was either incubated with 1
Probes) (36) for 10 min at 4°C left untreated (oxidized sample). The protein was then incubated in 2 nonreducing sample buffer with or without 20 mM AMS (Molecular Tim10. wild type Tim10 (native and denatured) and single cysteine mutants of isoelectric points are also indicated. and the four cysteine Tim10 mutants. The experimentally measured Tim10. A mM Tris-(2-carboxyethyl) phosphine hydrochloride (Molecular Probes) plex formation were performed as previously described (20, 28).

RESULTS

Cysteine Connectivities and Mutational Tim10 Folding Defects

mm Tris-(2-carboxyethyl) phosphine hydrochloride (Molecular Probes) overnight at 4°C to completely reduce the protein (reduced sample) or left untreated (oxidized sample). The protein was then incubated in 2× nonreducing sample buffer with or without 20 mM AMS (Molecular Probes) (36) for 10 min at 4°C. 20 mM N-ethylmaleimide was added to the samples, which were then boiled at 95°C for 3 min. 5 μg of protein was analyzed by SDS-PAGE on a 16% Tris-Tricine gel, followed by Coomassie Blue staining.

Miscellaneous Methods—Western blotting, gel filtration, in vitro chaperone activity using Luciferase refolding assays, and TIM10 complex formation were performed as previously described (20, 28).

Fig. 1. Mutation of the cysteine residues affects the folding of Tim10. A, Coomassie stained SDS-PAGE of purified wt Tim9, Tim10, and the four cysteine Tim10 mutants. The experimentally measured isoelectric points are also indicated. B, circular dichroism analysis of wild type Tim10 (native and denatured) and single cysteine mutants of Tim10.

All of the mutants were less soluble than the wt (mainly partitioning in inclusion bodies), indicating that their structure is affected. This was tested directly by CD. wt Tim10 and the four cysteine mutants were analyzed by far-UV CD as shown in Fig. 1B. The wt protein displays two minima at 209 and 222 nm, indicative of predominantly α-helical structure (28). The four cysteine mutants maintained these two minima, but notably, their molar ellipticity was significantly lower than the wild type protein, indicating that the proteins are less folded than the wt. As a control, the spectrum for the guanidinium unfolded wt protein is also presented. These data are in agreement with previous observations that reduction of Tim10 by DTT or Tris-(2-carboxyethyl) phosphine hydrochloride results in unfolding of the protein1 and directly show that the primary molecular defect of the cysteine mutants is impaired folding.

Tim10 Cysteine Mutants Have an Increased Tendency to Form Aberrant, DTT-dependent Higher Oligomers—Gel filtration under nonreducing conditions was used to determine the oligomeric state of the proteins. Wild type pure Tim9 and Tim10 in solution exist as monomer/dimer species in equilibrium (28) that elute primarily in fraction 12 when injected onto a Superdex 75 column (Fig. 2A). Interestingly, when the purified cysteine mutants were analyzed under the same conditions, they migrated differently from the wt with the majority of the protein eluting in fractions 9–11 (Fig. 2A), suggesting an increased tendency for oligomer formation. These higher oligomers were DTT-dependent, as when fraction 10 was treated with DTT and re-run over the column, a shift back to fraction 12 was observed (data not shown). Additionally, a shift to the higher fractions was observed after incubation of fraction 12 at 4°C for 1–3 days. These higher oligomers could arise from intermolecular disulfides between free cysteines in the mutants. In the wt Tim10, all four cysteines are involved in intramolecular disulfides (25); therefore, in the case of the single cysteine mutants only one intramolecular disulfide pair can form per molecule, leaving the third cysteine free to form intermolecular S–S bridges. This was tested directly by analyzing the different gel filtration fractions on SDS-PAGE under reducing or nonreducing conditions (Fig. 2B). For the wt Tim10, fraction 12 is predominantly monomeric (lanes 1 and 2). In contrast, in the case of the C40S mutant, the protein is almost exclusively found in a DTT-sensitive covalent dimer (lanes 3 and 4). The other three single cysteine mutants gave similar results. These data hence indicate that loss of one of the cysteine residues dramatically affects the oligomeric state of the protein and increases its propensity to form intermolecular, covalent S–S paired dimers.

Thiol Trapping Assays Reveal the Status of the Thiol Groups in the Tim10 Cysteine Mutants—To ascertain the redox state of the Cys residues in the mutants compared with the wt protein, we used a thiol trapping assay by AMS. This reagent binds to free thiols with the addition of 0.5 kDa for every thiol trapped, which can then be visualized by a band shift in high resolution SDS-PAGE. wt Tim10 and the cysteine mutants were analyzed for their binding to AMS in both the oxidized and reduced forms. The principle of the assay for this case is shown in Fig. 3A, and the results obtained are shown in Fig. 3B. In the reduced form, with all cysteines free, four AMS molecules bind to wt Tim10, increasing its molecular mass by 2 kDa (indicated by an asterisk, lane 4). In contrast, only three AMS molecules can bind to the cysteine mutants, increasing their molecular masses by 1.5 kDa (indicated by a caret, lane 8 for C40S; lane 12 for C44S; lane 16 for C61S; and lane 20 for C65S). In the oxidized form, wt Tim10 has all four cysteines in a disulfide-bonded state (intramolecularly); hence no AMS can bind and
there is no increase in SDS-PAGE mobility (lanes 1 and 2). On the other hand, the single cysteine mutants in their oxidized state should have only one disulfide pair because the other is broken due to the presence of the serine residue. In this case AMS binds to one free thiol group, thus increasing the molecular mass of the mutants by 0.5 kDa (indicated by a small open circle, lane 6 for C40S; lane 10 for C44S; lane 14 for C61S; and lane 18 for C65S). An additional interesting point that became obvious in this experiment is the significantly enhanced propensity of the C44S mutant to give higher oligomeric species (compare lanes 9 and 10 for C44S with lanes 5 and 6 for C40S, or lanes 13 and 14 for C61S, or lanes 17 and 18 for C65S). For C44S, trimeric and tetrameric species are much more abundant, indicating that mutation of Cys44 causes a very severe structural defect.

Mapping of the Intramolecular Disulfide Pairs Present in Tim10 in Vitro and in Vivo by MALDI-TOF-MS—It has been postulated that formation of intramolecular disulfides is important to maintain the structure of the individual subunits Tim9 and Tim10 in the TIM10 complex (25). In light of the behavior of the cysteine mutants described here, it was of great importance to understand the arrangement and connectivities of the cysteines in the intramolecular disulfides in the wt Tim10 and particularly how they change, if at all, in the cysteine mutants. To address this point, we assigned the intramolecular disulfide bonds in the wild type Tim10 as well as the four Cys mutants combining trypsin digestion of oxidized or reduced and alkylated proteins with subsequent peptide mapping by mass spectrometry. For the wild type Tim10, a peak corresponding to fragments containing Cys44 and Cys61 in a disulfide-bonded form was present in the oxidized protein and largely disappeared in the reduced and alkylated protein. Instead, a new peak corresponding to fragments containing alkylated Cys44 and Cys61 appeared (Fig. 4B). This shows that Cys44 and Cys61 are disulfide-bonded in the wt Tim10. Because it has been previously shown that all four Cys residues are disulfide-bonded in the oxidized state, it is evident that the remaining two Cys40 and Cys65 must also be connected by a disulfide bond. We therefore conclude that the four cysteines are connected via two disulfide pairs between two distal CX3C motifs rather than within each one of them. An inner pair connects Cys44 of the first CX3C motif to Cys61 of the second CX3C motif, and an outer pair connects Cys40 of the first CX3C motif to Cys65 of the second CX3C motif. By this mechanism, Tim10 folds so that the two distal CX3C are juxtaposed by covalent bonding of the distal Cys (Fig. 4).

The same disulfide pairs were found to be present in (i) the recombinant Tim10 in complex with Tim9 and (ii) the authentic Tim10 present in the TIM10 complex isolated from mitocho-
Cysteine Connectivities and Mutational Tim10 Folding Defects

Fig. 3. AMS free thiol trapping of Tim10. **A**, model showing the thiol groups of Tim10 (wt and C40S) in the oxidized and reduced form and their susceptibility to covalent interactions with AMS. Similar considerations apply to the other Cys mutants. **B**, Coomassie-stained SDS-PAGE showing the AMS assay. The four AMS molecules bound are shown by an asterisk; the three AMS molecules bound are shown by carets; the one AMS molecule bound is shown by a small open circle. Red, reduced sample; Ox, oxidized sample.

The same method was applied to all four Cys mutants, and as shown in Table I the native disulfide Cys44–Cys61 was identified in both C40S and C66S mutants. However, a peak corresponding to fragments containing Cys61 and Cys65 in a disulfide-bonded form was present in the oxidized state of C44S mutant, and it disappeared completely in the reduced and alkylated protein with a concomitant appearance of a new peak corresponding to fragments containing alkylated Cys61 and Cys65. This suggests that a non-native disulfide Cys61–Cys65 was formed in the C44S mutant. For the C61S mutant, no disulfide pair could be identified because of the position of the remaining Cys in the sequence. It is therefore clear that the intramolecular disulfide pairs in Tim10 that are important for the folding of the protein connect the distal outer cysteines at positions 40 and 65 and that the inner ones connect at positions 44 and 61.

Complex Formation of Tim10 with Tim9 Is Severely Affected but Not Completely Abolished by the Tim10 Cysteine Mutants—wt Tim9 and Tim10 can form a complex that can be analyzed and isolated by gel filtration analysis. Here we applied this methodology to check the effects of the Tim10 mutants on complex formation with wt Tim9. The top panel of Fig. 5A shows complex formation between wt Tim9 and the first two cysteine mutants C40S and C44S is shown. The shift to fraction 10 (indicative of complex formation) in both cases is significantly reduced compared with the wt protein (top panel), but it is not completely abolished. Immunoblotting of the fractions confirmed that the large majority of Tim10 mutants and Tim9wt are still in fraction 12, i.e. in an unassembled form. Similar results were observed with C61S and C65S (Fig. 5A). Although all four cysteine mutants are clearly affected in their ability to form a complex with wt Tim9, C44S is mostly impaired: (i) it has the lowest yield of all in complex formation, and (ii) it is smaller than wt complex or the complex made by the other mutants.

In the middle panel of Fig. 5A, complex formation between wt Tim9 and the first two cysteine mutants C40S and C44S is shown. The shift to fraction 10 (indicative of complex formation) in both cases is significantly reduced compared with the wt protein (top panel), but it is not completely abolished. Immunoblotting of the fractions confirmed that the large majority of Tim10 mutants and Tim9wt are still in fraction 12, i.e. in an unassembled form. Similar results were observed with C61S and C65S (Fig. 5A). Although all four cysteine mutants are clearly affected in their ability to form a complex with wt Tim9, C44S is mostly impaired: (i) it has the lowest yield of all in complex formation, and (ii) it is smaller than wt complex or the complex made by the other mutants.

The Inner Cysteine Pair Cys44–Cys61 Is More Important for the TIM10 Complex Chaperone Activity than the Outer Pair Cys40–Cys65—Because complex formation is affected but not completely blocked, it was of interest to test the activity of the complexes made from Tim10 Cys mutants with wt Tim9 in terms of their in vitro chaperone activity (28). Fig. 5B shows a histogram of the mutant complexes chaperone activity with all values normalized against the wt. When a complex between wt Tim9 and the C40S mutant was used, chaperone activity was reduced by 25%. However, this chaperone activity could be restored to wild type levels by doubling the amount of complex. Similar results were obtained with the C65S mutant protein; when used at wild type concentrations, the chaperone activity of this mutant was reduced by 21%, but doubling the concentration restored the chaperone activity to wt levels. In sharp contrast, this pattern was not observed when the two inner cysteine Tim10 mutants, C44S and C61S, were tested in the assay. In this case, chaperone activity was reduced, and impor-
tantly, it was not concentration-dependent, suggesting that mutation of the two inner cysteine residues affects the structure to a clearly greater extent than mutations of the two outer cysteines.

Defects of the Tim10 Cys Mutations in Organello—Isolated mitochondria provide a very useful system for testing the defects of the Cys mutants under physiological conditions. However, for testing for any such defects, wild type mitochondria present the difficulty that they have a functional TIM10 complex, and any analysis might be influenced by the presence of the wt complex. In contrast, mitochondria completely devoid of any endogenous Tim9 and Tim10 proteins from a tim9ts strain (16) grown at the nonpermissive temperature are a more appropriate system. Import into these mitochondria could thus be followed by using chemical amounts of pure Tim10 (or the Cys mutants) and immunoblotting (shown in Fig. 6). The wt protein was imported efficiently without any measurable dependence on preimporting pure Tim9. In sharp contrast, the import of all Cys mutants was clearly impaired. Interestingly, the import levels of the mutants were significantly increased when pure wt Tim9 was imported into these mitochondria first (Fig. 6A, compare lanes 2 and 3). This indicates that preimporting Tim9 in the intermembrane space allows the mutant proteins to be more efficiently retained because of their interaction, albeit weak, with their partner Tim9 in the trans-side of the outer membrane. In contrast, the wt Tim10 can be retained efficiently in the intermembrane space even without preimported Tim9 because it can fold properly on its own. Similar experiments using 35S-labeled precursors gave similar results (data not shown).

DISCUSSION

The presence of the strictly conserved twin CX3C motif in the small Tim9s suggested that it is important for their assembly and/or function. However, detailed characterization of specific Cys mutants of this motif, an understanding of their molecular defects, the relative importance of the different Cys, and elucidation of their intrachain disulfide connectivities are still limited. Here we addressed these issues by undertaking a systematic Cys mutagenesis for Tim10.

We have mutated all Cys to Ser one by one and compared their properties to those of the wt protein. The molecular defect of these mutations is impaired folding, as supported by several data. First, although the surface charge of the mutants is the same as the wt (Fig. 1A), they are less soluble. Second, their secondary structure is clearly affected as evidenced by far-UV CD measurements (Fig. 1B). Because the molar ellipticity is lower, but the two characteristic minima for α-helix are still present, we conclude that the mutants are less folded than the wt, but they still maintain elements of α-helical structure, as opposed to the fully unfolded wt protein by guanidine. Third,
limited trypsinolysis indicates that the Cys mutants are less stable than the wt protein (data not shown). Fourth, gel filtration analysis shows that the Cys mutants have an increased tendency to form aberrant homo-oligomers. Nonreducing SDS-PAGE and AMS thiol trapping assays show that these arise mainly from intermolecular, disulfide-bonded covalent species generated because a Cys to Ser exchange left its “partner” Cys-free and hence rendered the polypeptide more prone to form non-native intermolecular disulfides (Fig. 3).

Having established that the underlying molecular defect of Cys mutations in Tim10 is defective folding, we investigated its functional consequences in terms of (i) complex assembly with Tim9 in vitro, (ii) chaperone activity, and (iii) import into mitochondria and complex formation in organello. As expected, assembly with Tim9 was defective for all mutants. Interestingly though, it was not completely abolished. This would indicate that there are regions other than the CX3C motifs that are involved in contact sites in the complex assembly. These might stay unaffected in the Cys mutants but would not be at an ideal topology to interact with each other.

The chaperone activity data mirror those on complex formation. All four mutants have a weaker activity than the wt, but a clear difference is seen between C40S and C65S on the one hand and C44S and C61S on the other. The activity of the external C40S and C65S mutants can be increased to reach almost wt levels by doubling their concentration compared with the wt protein. In sharp contrast, activity of the internal C44S and C61S mutants is not concentration-dependent and cannot reach wt levels even by increasing their concentration significantly. This would indicate that there are drastic alterations in these mutants (see below).

Are these defects also manifested in vivo? To address this point we used import into isolated mitochondria, which is a more physiological system. Import of the mutants was defective when performed into tim9ts mitochondria that lack the endogenous wt Tim9. This could be explained by the fact that in the absence of endogenous Tim9, the misfolded Tim10 mutants can still be targeted to mitochondria but cannot be retained there efficiently because they cannot properly fold on their own, and their partner Tim9 is absent. When these mitochondria were supplemented with Tim9 first and then allowed to import the mutant Tim10, interaction with Tim9, albeit very weak, increased the amount of mutant Tim10 that was in a protease protected mitochondrial location. These results suggest that correct folding and/or assembly in the trans-side of the outer membrane could be a generic mechanism for the retention, hence targeting of intermembrane space proteins like the small Tims. Previously, it was reported that targeting of cytochrome c in the intermembrane space was dependent on association with the cytochrome c heme lyases (that functions as a trans-side “receptor”) and final co-factor binding in the intermembrane space (37). Taken together these data would thus support a common underlying mechanism for protein targeting to the mitochondrial intermembrane space whereby folding and stabilization (by assembling with another protein or by co-factor binding) would be an essential requirement that renders this process unidirectional. Results similar to our data for Tim10 were published for its homologue Tim8 that assembles to a different 70-kDa complex together with Tim13. The Tim8-Tim13 complex is dispensable in yeast cells, in contrast to the essential TIM10 complex. Mutation of the fourth Cys residue of DDP1 (the human homologue of the yeast Tim8 protein) led to significantly reduced TIMM8/TIMM13 complex formation in human cells (33, 34). Although these studies were limited to only one cysteine, they are in agreement with our data on Tim10. We suggest that maintaining the folding of the individual subunits via the conserved Cys residues is an important and generally applicable principle for the organization of all small Tims in specific assemblies in mitochondria.

We determined the precise organization of the four Cys residues in Tim10 by TOF-MS. Generation of distinct sets of tryptic fragments for the fully oxidized (intramolecularly disulfide-bonded) compared with the fully reduced and alkylated species followed by a precise mass determination with MALDI-TOF-MS allowed us to map the disulfide pairs. In the wt protein, there is one disulfide pair connecting the inner Cys44 and Cys61 (confirmed by sequencing of tryptic peptides). The remaining Cys can be accounted for as a Cys44-Cys61 heterodisulfide. The cysteine connectivities and mutational Tim10 folding defects are shown in Fig. 4. The Cys pairs in the wt complex are indicated in red. The connectivities were determined by TOF-MS in the oxidized form and MALDI-TOF-MS on reduced TIM10. The missing disulfide pairs were limited to only one cysteine, they are in agreement with our data on Tim10. The cysteine connectivities and mutational Tim10 folding defects are shown in Fig. 4.

Fig. 5. Functional defects of the Tim10 Cys mutants in complex formation and chaperone activity. A, gel filtration analysis of complex formation using wt Tim10 or the Tim10 cysteine mutants with wt Tim9. Quantification of the immunoblotting showed that 55–60% of the total protein shifted to fraction 10 for the wild-type complex compared with 20–30% for C40S, C61S, and C65S and 7% for C44S (data not shown). B, histogram showing the in vitro chaperone activity of the TIM10 complex. The mutant complexes were assayed at either the same concentration or double that of the wt TIM10 complex (2X). All of the values are normalized against wild type TIM10 complex.
with Cys$^61$ and another one connecting the outer Cys$^{40}$ with Cys$^{65}$. This is an interesting arrangement that requires the two distal CX3C motifs that are 15 residues apart in the linear sequence to be juxtaposed in the Tim10 native structure and lock onto each other like a “press stud” via the two disulfide pairs. This folding mechanism results in a pouch-like structure (Fig. 4) where the N-terminal, C-terminal, and loop region (the 15-aminocacid region between the two CX3C motifs) could be structurally separate. In agreement with this, our previous limited proteolysis results suggested that the N and C termini are rather flexible and protease-sensitive (28). Also, this arrangement would explain the fact that DTT strongly unfolds the protein (data not shown) as the disulfide pairs are crucial for the stability of the folded conformation of the protein. Intramolecular disulfides between long distance Cys stabilize the structure of naturally occurring, small Cys-rich proteins like the knotin family (or cysteine knots), whereas disulfides between vicinal Cys usually misfold these proteins and very rarely occur naturally (38–40).

Extending our Cys connectivity analysis to the mutants, we find that C40S and C65S, which cannot form the outer disulfide, still maintain the native inner disulfide Cys$^{44}$–Cys$^{61}$. In contrast, C44S, which cannot form the inner disulfide pair, has a non-native disulfide Cys$^{61}$–Cys$^{65}$. This is drastically different from the native one, because it connects the two neighboring Cys rather than juxtaposing them to their native distal partner Cys.

These results also explain the interesting observation that the different Cys are not equally important, and the corresponding mutants present distinguishable functional differences. On the one hand, the outer cysteine mutants C40S and C65S maintain one of the two native disulfide bonds (the inner Cys$^{44}$–Cys$^{61}$ pair) and can at least partially keep the protein structure together. On the other hand, the internal Cys mutants C44S (and presumably C61S) contain a wrong, non-native disulfide, thus displaying more pronounced functional defects. Indeed, C44S shows a distinctly higher tendency to form aberrant oligomers (Fig. 3B), it is mostly affected in its interaction with Tim9 (Fig. 5A), whereas both C44S and C61S display the strongest defect in chaperone activity.

Taken together these data have uncovered that the precise organization of the four conserved Cys in two pairs connecting the distal CX3C motifs is the mechanism of folding of Tim10 and suggest that the inner disulfide bond may have a predominant role in this process.

Acknowledgments—We thank members of our laboratory for discussions and comments on the manuscript and Dr. Frederick van Deursen for help with isoelectric focusing.

REFERENCES

1. Pfanner, N., and Geissler, A. (2001) Nat. Rev. Mol. Cell. Biol. 2, 339–349
2. Pfanner, N., and Wiedemann, N. (2002) Curr. Opin. Cell Biol. 14, 400–411
3. Koebsch, C. M. (2006) FEBS Lett. 576, 27–31
4. Bauer, M. P., Hofmann, S., and Neupert, W. (2002) Int. Rev. Neurobiol. 53, 57–80
5. Schatz, G. (1996) J. Biol. Chem. 271, 31763–31766
6. Ryan, K. R., and Jensen, R. E. (1995) Cell 83, 517–519
7. Wiedemann, N., Pfanner, N., and Ryan, M. T. (2001) EMBO J. 20, 951–960
8. Endres, M., Neupert, W., and Brunner, M. (1999) EMBO J. 18, 2214–2221
9. Pfanner, N., Hoeben, P., Tropfschug, M., and Neupert, W. (1987) J. Biol. Chem. 262, 14851–14854
10. Pfanner, N., Tropfschug, M., and Neupert, W. (1987) Cell 49, 815–823
11. Brix, J., Rudiger, S., Bukau, B., Schneider-Mergener, J., and Pfanner, N. (1999) J. Biol. Chem. 274, 16522–16530
12. Ryan, K. R., Leung, R. S., and Jensen, R. E. (1998) Mol. Cell. Biol. 18, 178–187
13. Sirrenberg, C., Bauer, M. F., Gruau, B., Neupert, W., and Brunner, M. (1996) Nature 384, 582–585
14. Kerscher, O., Holder, J., Srinivasan, M., Leung, R. S., and Jensen, R. E. (1997) J. Cell Biol. 139, 1663–1675
15. Kerscher, O., Sepuri, N. B., and Jensen, R. E. (2000) Mol. Biol. Cell 11, 103–116
16. Koehler, C. M., Merchant, S., Opplinger, W., Schmid, K., Jarosch, E., Dolfini, L., Junne, T., Schatz, G., and Tokatlidis, K. (1999) EMBO J. 18, 6477–6486
17. Sirrenberg, C., Endres, M., Folsch, H., Stuart, R. A., Neupert, W., and Brummer, M. (1998) Nature 391, 912–915
18. Koehler, C. M., Jarosch, E., Tokatlidis, K., Schmid, K., Schweyen, R. J., and Schatz, G. (1998) Science 279, 369–373
19. Adam, A., Endres, M., Sirrenberg, C., Lottspeich, F., Neupert, W., and Brunner, M. (1999) EMBO J. 18, 313–319
20. Luciano, V., Vial, S., Vergnolle, M. A., Dyall, S. D., Robinson, D. R., and Tokatlidis, K. (2001) EMBO J. 20, 4099–4106
21. Davis, A. J., Sepuri, N. B., Holder, J., Johnson, A. D., and Jensen, R. E. (2000) J. Cell Biol. 150, 1271–1282
22. Leuenberger, D., Bally, N. A., Schatz, G., and Koehler, C. M. (1999) EMBO J. 18, 4816–4822
23. Koehler, C. M., Leuenberger, D., Merchant, S., Renold, A., Junne, T., and Schatz, G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2141–2146
24. Tokatlidis, K., and Schatz, G. (1999) J. Biol. Chem. 274, 35285–35288
25. Curran, S. P., Leuenberger, D., Opplinger, W., and Koehler, C. M. (2002) EMBO J. 21, 942–953
26. Truscott, K. N., Wiedemann, N., Rehling, P., Muller, H., Meisinger, C., Pfanner, N., and Guiraud, B. (2002) Mol. Cell. Biol. 22, 7780–7789
27. Tokatlidis, K., Vial, S., Luciano, P., Vergnolle, M., and Clemence, S. (2000) Biochem. Soc. Trans. 28, 495–499
28. Vial, S., Lu, H., Allen, S., Savory, P., Thornton, D., Sheehan, J., and Tokatlidis, K. (2002) J. Biol. Chem. 277, 36100–36108
29. Pfanner, N., and Neupert, W. (1999) J. Biol. Chem. 274, 7528–7536
30. Wachter, C., Schatz, G., and Glick, B. S. (1992) Science 255, 478–4794
31. Rehling, P., Model, K., Brandner, K., Kevermann, P., Sickmann, A., Meyer, H. E., Kohlbrandt, W., Wagner, R., Truscott, K. N., and Pfanner, N. (2003) Science 299, 1747–1751
32. Dyall, S. D., Agius, S. C., De Marcos Lousa, C., Trezeguet, V., and Tokatlidis, K. (2003) J. Biol. Chem. 278, 35675–35674
33. Roesch, K., Curran, S. P., Tranebjærg, L., and Koehler, C. M. (2002) Hum. Mol. Genet. 11, 477–486
34. Hofmann, S., Rothbauer, U., Muhlenbein, N., Neupert, W., Gerbitz, K. D.
Cysteine Connectivities and Mutational Tim10 Folding Defects

38513

Brunner, M., and Bauer, M. F. (2002) J. Biol. Chem. 277, 23287–23293
35. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Anal. Chem. 68, 850–858
36. Joly, J. C., and Swartz, J. R. (1997) Biochemistry 36, 10067–10072
37. Mayer, A., Neupert, W., and Lill, R. (1995) J. Biol. Chem. 270, 12390–12397
38. Wang, X., Connor, M., Smith, R., Maciejewski, M. W., Howden, M. E., Nicholson, G. M., Christie, M. J., and King, G. F. (2000) Nat. Struct. Biol. 7, 505–513
39. Cemazar, M., Zahariev, S., Lopez, J. J., Carugo, O., Jones, J. A., Hore, P. J., and Pongor, S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5754–5759
40. Carugo, O., Lu, S., Luo, J., Gu, X., Liang, S., Strohl, S., and Pongor, S. (2001) Protein Eng. 14, 639–646