The Tim9p/10p and Tim8p/13p Complexes Bind to Specific Sites on Tim23p during Mitochondrial Protein Import

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The import of polytopic membrane proteins into the mitochondrial inner membrane (IM) is facilitated by Tim9p/Tim10p and Tim8p/Tim13p protein complexes in the intermembrane space (IMS). These complexes are proposed to act as chaperones by transporting the hydrophobic IM proteins through the aqueous IMS and preventing their aggregation. To examine the nature of this interaction, Tim23p molecules containing a single photoreactive cross-linking probe were imported into mitochondria in the absence of an IM potential where they associated with small Tim complexes in the IMS. On photolysis and immunoprecipitation, a probe located at a particular Tim23p site (27 different locations were examined) was found to react covalently with, in most cases, only one of the small Tim proteins. Tim8p, Tim9p, Tim10p, and Tim13p were therefore positioned adjacent to specific sites in the Tim23p substrate before its integration into the IM. This specificity of binding to Tim23p strongly suggests that small Tim proteins do not function solely as general chaperones by minimizing the exposure of nonpolar Tim23p surfaces to the aqueous medium, but may also align a folded Tim23p substrate in the proper orientation for delivery and integration into the IM at the TIM22 translocon.

INTRODUCTION

Mitochondria have two membranes that separate the organelle into four distinct compartments: the outer membrane (OM), the inner membrane (IM), the intermembrane space (IMS), and the matrix inside the IM. Because nearly all mitochondrial proteins are synthesized in the cytosol (~99%; Attardi and Schatz, 1988), a complex molecular machinery is required to sort and deliver these proteins to their proper location in the organelle. For example, many IM proteins are recognized by the TOM complex (translocon of the OM), translocated through the OM to the IMS, and then transported through the aqueous IMS in association with complexes of Tim8p/Tim13p (hereafter designated 8-13) and Tim9p/Tim10p (hereafter termed 9-10) to the TIM22 complex (translocon of the inner membrane containing Tim22p) where polytopic membrane proteins are integrated into the IM (reviewed in Jensen and Dunn, 2002; Endo *et al.*, 2003; Koehler, 2004; Wiedemann *et al.*, 2004a, 2006; Mokranjac and Neupert, 2005). The targeting of proteins to the TIM22 complex requires multiple discrete signals in both the hydrophobic transmembrane sequences (TMSs) and the charged hydrophilic loops of the substrate polypeptide (Pfanner and Neupert, 1987; Smagula and Douglas, 1988; Davis *et al.*, 1998; Kaldi *et al.*, 1998; Koehler *et al.*, 1998b;

Luciano *et al.*, 2001; Curran *et al.*, 2002a; Vasiljev *et al.*, 2004). These different signals appear to work cooperatively for maximal import (Davis *et al.*, 1998; Wiedemann *et al.*, 2001), raising the possibility that polytopic IM proteins are partially folded during signal recognition and transport. In fact, the ATP/ADP carrier protein (Aac2p) and Tim23p, a subunit of the TIM23 translocon, appear to fold into hairpin loops and insert “loop first” into the TOM (and TIM) machinery during import (Endres *et al.*, 1999; Wiedemann *et al.*, 2001; Curran *et al.*, 2002b). After integration into the IM, the hydrophilic N-terminal domain of Tim23p faces the IMS (Bauer *et al.*, 1996; Davis *et al.*, 1998; Ryan *et al.*, 1998) where it may interact with Tim50p (Geissler *et al.*, 2002; Yamamoto *et al.*, 2002) and/or interact with matrix-directed proteins (Bauer *et al.*, 1996), whereas the four hydrophobic TMSs in the C-terminal half of the protein are in the lipid core of the IM (Bauer *et al.*, 1996; Davis *et al.*, 1998; Ryan *et al.*, 1998).

The import of polytopic IM proteins requires the essential 9-10 complex in the IMS (Koehler *et al.*, 1998a, 1998b; Sirrenberg *et al.*, 1998; Adam *et al.*, 1999). Tim9p and Tim10p form a soluble 70-kDa complex containing three copies of each protein (Luciano *et al.*, 2001; Curran *et al.*, 2002a; Vial *et al.*, 2002; Webb *et al.*, 2006) and are proposed to shuttle cargo from the TOM complex to the TIM22 complex. Fractionation and cross-linking studies have identified associations of 9-10 with both the TOM complex (Wiedemann *et al.*, 2001) and the TIM22 complex (Sirrenberg *et al.*, 1998; Adam *et al.*, 1999), and mutations in Tim9p or Tim10p block the import of IM substrates at the OM (Adam *et al.*, 1999; Truscott *et al.*, 2002; Leuenberger *et al.*, 2003). In chemical cross-linking studies, both Tim9p and Tim10p are in close proximity to Aac2p (Koehler *et al.*, 1998a, 1998b; Sirrenberg *et al.*, 1998; Adam *et al.*, 1999; Endres *et al.*, 1999) and Tim23p (Davis *et al.*, 2000; Vasiljev *et al.*, 2004) when integration at TIM22 is

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blocked by ionophore addition that dissipates the electrical potential across the IM.

Tim9p and Tim10p are thought to act as general chaperones, binding nonspecifically to hydrophobic regions in the various polytopic IM protein substrates to increase their aqueous solubility and prevent their aggregation in the aqueous IMS en route to the TIM22 complex (Koehler *et al.*, 1998b; Vial *et al.*, 2002). Given the high hydrophobicity of every TIM22 substrate, a nonspecific chaperone role for 9-10 is very attractive, although a specific binding role for 9-10 during import has been suggested (Sirrenberg *et al.*, 1998; Endres *et al.*, 1999). The 9-10 complex has also been reported to facilitate polypeptide folding (Vial *et al.*, 2002). The recent determination of the crystal structure of a 9-10 complex did not reveal how 9-10 recognizes and interacts with its substrates (Webb *et al.*, 2006). No exposed hydrophobic patches were observed in the crystallized 9-10 complex, and the small cavity formed by the subunits will not accommodate a typical full-length substrate.

Tim8p and Tim13p are also involved in the TIM22 import pathway, but their function is less clear. Tim23p was initially the only 8-13 substrate identified (Leuenberger *et al.*, 1999; Davis *et al.*, 2000), but recent reports showing that 8-13 is involved in the import of the mammalian TIM22 substrate aralar1 (Roesch *et al.*, 2004) and the insertion of OM β -barrel proteins (Hoppins and Nargang, 2004; Wiedemann *et al.*, 2004b) suggest that 8-13 may participate more extensively in trafficking than previously thought. Unlike Tim9p and Tim10p, Tim8p and Tim13p are not essential for import (Koehler *et al.*, 1999). Deletion of *TIM8* or *TIM13* reduces Tim23p import only slightly (Leuenberger *et al.*, 1999; Davis *et al.*, 2000; Paschen *et al.*, 2000) and has no effect on other TIM22 substrates (Leuenberger *et al.*, 1999; Davis *et al.*, 2000).

Although it is widely accepted that 9-10 and 8-13 act as chaperones in transporting nonpolar cargo through the aqueous IMS, the nature of 8-13 and 9-10 interactions with cargo molecules is ill defined. To minimize aggregation, the small Tim proteins could alter TMS exposure either by binding directly to exposed nonpolar surfaces and/or by forming a chaperonin-like cage that encapsulates the substrate (cf. homo-oligomeric GroEL or hetero-oligomeric TRiC, the TCP-1 ring complex). The small extent of exposed nonpolar surface area in the crystallized 9-10 complex (Webb *et al.*, 2006) may favor a variation of the latter possibility. Another unknown is whether 8-13 and 9-10 prevent aggregation by covering or binding to exposed nonpolar surfaces nonspecifically or specifically. A recent study revealed that TRiC functions nonspecifically because two different ribosome-bound nascent chains of varying lengths showed no specificity in photocross-linking to different TRiC subunits from different locations in each nascent chain (Etchells *et al.*, 2005). Thus, each nascent chain segment was proximal to and promiscuously sampled the binding sites of six different TRiC subunits instead of binding solely or preferentially to a specific subset of TRiC subunits as had been suggested earlier (Llorca *et al.*, 1999). The other alternative is that 9-10 and/or 8-13 reduce TMS exposure by binding to specific sites on each cargo molecule.

Previous studies designed to identify where specific small Tim proteins bind to Tim23p have yielded conflicting results. Both Tim8p and Tim13p were found adjacent to Tim23p during import (Leuenberger *et al.*, 1999; Davis *et al.*, 2000), and chemical cross-linking studies localized the interaction of 8-13 with Tim23p to its hydrophilic domain (Davis *et al.*, 2000; Paschen *et al.*, 2000). Thus, 8-13 was proposed to associate with the N-terminal half of Tim23p, whereas 9-10 shielded the TMSs during import (Davis *et al.*, 2000; Paschen

et al., 2000). Yet purified complexes of 8-13, but not 9-10, were found to bind to peptides within both the N-terminal and membrane-spanning domains of Tim23p (Curran *et al.*, 2002b). It was therefore proposed that 8-13 functions as the sole chaperone for Tim23p (Curran *et al.*, 2002b).

To resolve these issues, we have here positioned a single photoreactive probe at each of 27 different sites in Tim23p to identify which regions of Tim23p, if any, are adjacent to 9-10 and/or 8-13. This unusually comprehensive and high-resolution approach has identified where 9-10 and 8-13 are in proximity and presumably bind to Tim23p. In addition, these experiments have revealed that the 8-13 and 9-10 complexes bind specifically, not randomly, to Tim23p and suggest a functional role for this arrangement.

MATERIALS AND METHODS

Plasmids and tRNA

SP6-TIM23 plasmid pJE29 and SP6-TIM23C plasmid pKR35 have been described elsewhere (Ryan *et al.*, 1998), and pGEM4Z-AAC2, a plasmid that expresses the *Saccharomyces cerevisiae* Aac2 protein from the SP6 promoter, was a generous gift of N. Pfanner (University of Freiburg). Amber codons were introduced into plasmids using the Quickchange protocol (Stratagene, La Jolla, CA), and the primary sequence of each construct was confirmed by DNA sequencing. In some cases, silent mutations were also introduced near the amber codon to create or disrupt a native restriction enzyme site to facilitate screening for positive clones. The plasmids created include: SP6-TIM23 TAG-5, -14, -25, -32, -44, -55, -66, -80, -90, -100, -111, -120, -131, -143, -153, -154, -155, -156, -157, -158, -169, -181, -195, -205, -206, -207 and -208. By subcloning fragments of these SP6-TIM23 TAG plasmids containing the suppressor mutation into SP6-TIM23C, the corresponding SP6-TIM23C TAG-120, -131, -143, -153, -154, -155, -156, -157, -158, -169, -181, -195, -205, -206, -207 and -208 plasmids were constructed. In both series of mutants, the number refers to the residue in full length Tim23p that was replaced with the amber codon.

Yeast N^ε-(5-azido-2-nitrobenzoyl)-Lys-tRNA^{Lys} (ϵ ANB-Lys-tRNA^{Lys}) was prepared as before (Krieg *et al.*, 1986; McCormick *et al.*, 2003), as was the amber suppressor tRNA, ϵ ANB-Lys-tRNA^{amb}, which translates an amber stop codon (Flanagan *et al.*, 2003; McCormick *et al.*, 2003; Saksena *et al.*, 2004; Woolhead *et al.*, 2004).

Translations, Import, and Photocross-linking

Plasmid DNA containing the gene of interest was incubated (26°C, 60 min, 50 μ l) in a rabbit reticulocyte lysate-based transcription/translation system (TNT Quick; Promega, Madison, WI) in the presence of [³⁵S]Met (1.5 μ Ci/ μ l) and, as indicated, 15 pmol of either ϵ ANB-Lys-tRNA^{amb}, Lys-tRNA^{amb}, ϵ ANB-Lys-tRNA^{Lys}, or Lys-tRNA^{Lys}. After translation, 10 μ l of the sample was incubated (26°C, 20 min) with 25 μ g of mitochondria, isolated from wild-type strain D273-10B (Sherman, 1964) as described (Daum *et al.*, 1982), in 100 μ l of import buffer (0.6 M sorbitol, 50 mM HEPES-KOH, pH 7.4, 25 mM KCl, 10 mM MgCl₂, 2 mM potassium phosphate, pH 7.4, 0.5 mM EDTA, 2 mM ATP, 2 mM NADH, 1 mg/ml bovine serum albumin). Intact mitochondria or mitoplasts (mitochondria whose outer membrane was disrupted by osmotic shock; Davis *et al.*, 1998) were then treated on ice for 20 min with 50 μ g/ml proteinase K before the addition of 1 mM phenylmethylsulfonyl fluoride. To generate integration intermediates, the IM potential was dissipated by incubating mitochondria (0°C, 5 min) in import buffer lacking NADH and containing 40 μ M carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, Sigma, St. Louis, MO) before the translation was added.

Samples were photolyzed on ice for 15 min using a 500 W mercury arc lamp (McCormick *et al.*, 2003) and sedimented. Mitochondrial pellets of nonimmunoprecipitated samples were resuspended in 40 μ l of SDS-PAGE sample buffer, and 20 μ l was analyzed directly by SDS-PAGE and phosphorimaging.

Immunoprecipitations

Immunoprecipitations were generally performed on samples after import in the absence of membrane potential. Briefly, 30 μ l of translation incubation was added to 50 μ g of mitochondria in 300 μ l of import buffer lacking NADH and containing CCCP, incubated (26°C, 20 min), and photolyzed. In some experiments (Figure 1C), the IM potential was restored after import by sedimenting mitochondria, resuspending pellets in buffer containing DTT (0.1 or 0.5 mM) and respiratory substrate (2 mM malate, 2 mM pyruvate), and incubating at 26°C for 20 min before UV exposure. Samples were not treated with protease. A 30- μ l aliquot was removed from the sample, from which mitochondria were pelleted before being resuspended in 40 μ l of sample buffer and analyzed by SDS-PAGE. The remaining 300 μ l of sample was pelleted and resuspended in 50 μ l of SDS-containing buffer and immunoprecipitated as before (Davis *et al.*, 2000) with the following modifications.

Figure 1. Photocross-linking of substrates of the TIM22 pathway to mitochondrial import components. (A) Photoadducts vary with stage of import. Tim23p and Aac2p were translated separately in the presence of [³⁵S]Met and either Lys-tRNA^{Lys} (−ANB) or εANB-Lys-tRNA^{Lys} (+ANB). Translation products were added to mitochondria containing (+ΔΨ) or lacking (−ΔΨ) an inner membrane potential as described in *Materials and Methods*, and samples were then photolyzed and sedimented to isolate the mitochondria. Mitochondrial proteins were separated by SDS-PAGE, and radiolabeled proteins were visualized by phosphorimaging. Lanes 1, 2, 7, and 8 contain 1/10 of the volume of the translation mix that was added to each import reaction. Arrowheads identify photoadduct bands. p, Tim23p or Aac2p. (B) Tiny Tim photoadducts. Import intermediates containing Tim23p or Aac2p were prepared as in A with εANB-Lys-tRNA^{Lys} and −ΔΨ mitochondria, photolyzed, and split into five portions before mitochondria were pelleted. One aliquot (5 μg) was resuspended directly in sample buffer (XL tot), whereas the other aliquots (50 μg) were immunoprecipitated with antibodies specific for Tim8p, Tim9p, Tim10p, or Tim13p as indicated. Other labels are the same as in A. (C) IMS complexes of Tim23p with small Tim proteins are productive intermediates. Tim23p import intermediates were prepared as in A with εANB-Lys-tRNA^{Lys} and −ΔΨ mitochondria, sedimented, and resuspended either in import buffer containing CCCP (−ΔΨ) or in import buffer containing 2 mM malate, 2 mM pyruvate, and DTT as indicated (+ΔΨ) to restore the IM potential. After photolysis, samples were immunoprecipitated using antibodies specific for Tim13p (lanes 1, 4, 7, and 10), Tim50p (lanes 2, 5, 8, and 11), and the unrelated HA epitope (lanes 3, 6, 9, and 12).

Solubilized mitochondria (50 μg) were diluted 10-fold with 0.5 ml of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% (vol/vol) Triton X-100 and centrifuged at 14,000 × *g* for 10 min. Protein A-Sepharose beads (Sigma; 15 μl of a 1:1 slurry in the same buffer) were added to the supernatants and rocked (30 min, 4°C) to adsorb material that binds nonspecifically. After bead removal by sedimentation (2 min, 14,000 × *g*), the supernatants were transferred to new tubes containing either 10 μl of antiserum to Tim10p or 20 μl of antiserum to Tim8p, Tim9p, Tim13p, and Tim50p (a generous gift of T. Endo) or anti-HA (Sigma) and then were immunoprecipitated and analyzed by SDS-PAGE as before (Davis *et al.*, 2000). In the experiments of Figures 3, 4, and 7, and their replicates, the sample aliquot was first immunoprecipitated with either Tim8p or Tim10p antiserum. After the resulting protein-antibody complexes were sedimented with protein A-Sepharose beads, the supernatants were then added to a new tube containing, respectively, either Tim9p or Tim13p antiserum and immunoprecipitated as above. Sample aliquots were therefore immunoprecipitated with antisera either to Tim8p and then Tim9p, or to Tim10p and then Tim13p. Immunoprecipitated proteins were released from the Protein A beads with 40 μl of sample buffer, and half this volume was analyzed by SDS-PAGE and phosphorimaging.

Quantification

After SDS-PAGE, ³⁵S-labeled Tim23p proteins were detected and quantified using a Bio-Rad FX phosphorimager (Hercules, CA). To eliminate concerns about screen variation and directly compare the extent of covalent complex (photoadduct) formation in parallel samples, an SDS-PAGE gel containing the directly analyzed aliquots of the cross-linked samples, and the gels containing the immunoprecipitated aliquots of those same samples were exposed to the same phosphorimaging screen for the same length of time, typically ~10 d. Although this approach means that the visual intensities of the photoadduct bands in the gel are low (e.g., Figures 3 and 4), it allows us to quantify accurately (without overexposing the unreacted bands) the relative amounts of photoadducts formed in parallel samples. The radioactivity in each unreacted Tim23p band ("p" in Figures 3 and 4) and each photoadduct band (lower panels in Figures 3 and 4) was then determined using the Bio-Rad software, and the radioactivity measured for the unreacted Tim23p band in a directly analyzed sample was multiplied by 10 because the immunoprecipitated portion of each cross-linking sample was 10 times larger than the directly analyzed aliquot. The photoadduct yield (ratio of photocross-linked to unreacted Tim23p) in each individual sample was then calculated.

RESULTS

Experimental Design

To identify which Tim23p residues are adjacent to specific IMS proteins during import, a single photoreactive probe was incorporated into a site in Tim23p by *in vitro* translation. When the resulting Tim23p molecules were imported into mitochondria that lacked an IM potential, the photoreactive substrates were trapped in the IMS in complexes with 8-13 and/or 9-10. By using a photoreactive probe, complex formation could be completed before the covalent reaction was initiated with UV light. On illumination (photolysis) of a sample, the activated probe reacted covalently with *any* nearby protein because the probe's reactivity with different amino acids in target proteins is largely indiscriminate, and the reaction was limited solely to adjacent proteins because the lifetime of the reactive state is very short (nanoseconds). By locating probes at different Tim23p locations, the close juxtaposition of Tim23p to the small Tim proteins was mapped.

To position photoreactive probes in Tim23p, we used an aminoacyl-tRNA analogue approach that we originated (Johnson *et al.*, 1976; Krieg *et al.*, 1986). Wild-type Tim23p mRNAs were translated *in vitro* in the presence of [³⁵S]Met and εANB-Lys-tRNA^{Lys} to yield radioactive Tim23p molecules in which photoreactive εANB-Lys probes replaced ~25% of the lysines incorporated (Krieg *et al.*, 1989) at each of the 14 lysine codons in Tim23p. Alternatively, to obtain site-specifically labeled Tim23p molecules, Tim23p mRNAs in which a single amber stop codon replaced a natural codon in the sequence were translated as above in the presence of εANB-Lys-tRNA^{amb}, an amber suppressor tRNA that recognizes an amber stop codon and typically suppresses with an efficiency of 30–50% (Flanagan *et al.*, 2003; McCormick *et al.*,

2003; Saksena *et al.*, 2004; Woolhead *et al.*, 2004; Davis, unpublished data). The resulting radioactive and photoreactive Tim23p molecules were then imported into mitochondria added to the sample. In most cases reported here, the IM potential was dissipated by CCCP before the mitochondria were added to the sample. After photolysis, the extent of photocross-linking to individual IMS proteins was quantified from the amount of radioactive protein immunoprecipitated by antibodies specific for Tim8p, Tim9p, Tim10p, or Tim13p (Davis *et al.*, 2000).

We have previously used this approach to examine nascent chain environment during translation inside the ribosome (Woolhead *et al.*, 2004), chaperonin-mediated folding (McCallum *et al.*, 2000; Etchells *et al.*, 2005), glycosylation (Nilsson *et al.*, 2003; Karamyshev *et al.*, 2005), targeting to the membrane (Krieg *et al.*, 1986; Karamyshev and Johnson, 2005), and translocation across (Krieg *et al.*, 1989) and integration into (Thrift *et al.*, 1991; Do *et al.*, 1996; McCormick *et al.*, 2003; Saksena *et al.*, 2004) the membrane of the endoplasmic reticulum (ER). This approach has also been used successfully in a comprehensive study that identified the TOM and TIM23 complex proteins adjacent to multiple sites in a matrix-directed substrate during import (Kanamori *et al.*, 1997, 1999).

Photocross-linking of Tim23p and Aac2p to IMS Proteins

To determine whether the photocross-linking approach would work with substrates of the TIM22 pathway, full-length Tim23p and Aac2p were translated *in vitro* in the presence of [³⁵S]Met and either Lys-tRNA^{Lys} or ϵ ANB-Lys-tRNA^{Lys}. On incubation with mitochondria, each of these substrates was imported, as evidenced by their presence in the mitochondrial pellet in each sample (p in lanes 3–6 and 9–12 in Figure 1A). Based on the approximately equal amounts of imported substrate observed in these lanes, the presence of the probe does not inhibit the import of Tim23p or Aac2p significantly, if at all.

The photoreactive probes also survive the import conditions, because photolysis yields radioactive species with higher apparent molecular masses than the precursor protein in the samples containing the ANB probe. When imported into mitochondria lacking an IM potential, both photoreactive Tim23p and Aac2p reacted covalently with proteins with an apparent molecular mass of ~10 kDa (Figure 1A, lanes 6 and 12). When fully imported into the IM in functional mitochondria, Tim23p reacted covalently with a ~50-kDa protein (Figure 1A, lane 4), whereas Aac2p did not detectably react with any protein (Figure 1A, lane 10). Thus, the proteinaceous environments appeared similar for the two TIM22 substrates when their import was arrested in the IMS due to the absence of a $\Delta\Psi$, but were different after integration into the IM. Based on previous studies, the likely photocross-linking targets are the small Tim proteins in $-\Delta\Psi$ mitochondria (Davis *et al.*, 2000) and Tim50p in the $+\Delta\Psi$ mitochondria (Geissler *et al.*, 2002; Yamamoto *et al.*, 2002); these assignments are confirmed below.

In principle, the absence of a $\Delta\Psi$ could trap an 8-13 Δ Tim23p Δ 9-10 complex either in the IMS and/or at the TIM22 translocon. Because eliminating the $\Delta\Psi$ did not cause a detectable formation of a ~45-kDa radioactive species (the expected apparent molecular mass of a Tim23p-Tim22p photoadduct; Figure 1A), it seems likely that most of the Tim23p molecules are solubilized in the IMS, but we cannot rule out the possibility that the photocross-linked Tim23p in our samples are membrane-bound and arrested at TIM22 (see Discussion).

To identify the ~10-kDa proteins that photocross-link to Tim23p and Aac2p, aliquots of the photolyzed samples containing $-\Delta\Psi$ mitochondria were immunoprecipitated with antibodies specific for Tim8p, Tim9p, Tim10p, and Tim13p (α Tim8p, α Tim9p, α Tim10p, or α Tim13p, respectively). As shown in Figure 1B, Tim23p reacts covalently with each of these proteins (lanes 3–6; the doublets in the Tim8p and Tim13p lanes are explained below). In contrast, Aac2p photocross-linked only to Tim9p and Tim10p (lanes 9–12). These results are consistent with earlier data (Davis *et al.*, 2000; Paschen *et al.*, 2000) showing that only the N-terminal half of a full-length Tim23p chemically cross-links to Tim8p and Tim13p.

To determine whether Tim23p complexes in the IMS are on-pathway or dead-end intermediates, Tim23p was imported into $-\Delta\Psi$ mitochondria as above. After sedimentation, mitochondria were resuspended either in buffer containing CCCP to maintain the $-\Delta\Psi$ state or in a buffer containing respiratory substrate and DTT to restore the IM electric potential and Tim23p integration. After photolysis, analysis of the parallel samples revealed that Tim23p photocross-linking to Tim13p was much less in the $+\Delta\Psi$ than in the $-\Delta\Psi$ sample (Figure 1C, compare lanes 1 and 7 with 4 and 10), whereas Tim23p photocross-linking to Tim50p was observed only in $+\Delta\Psi$ mitochondria (Figure 1C, compare lanes 5 and 11 with 2 and 8). This marked transition in photocross-linking targets indicates that Tim23p is associated with 8-13 and 9-10 in the IMS in the absence of the $\Delta\Psi$, but that restoration of the $\Delta\Psi$ allows Tim23p to integrate into the IM and associate with Tim50p. The observed changes varied with DTT concentration because DTT facilitates inactivation of CCCP (Yaffe, 1991) and hence restoration of the $\Delta\Psi$, but it also chemically inactivates the photoreactive probe and thereby reduces photoadduct formation. Yet each set of samples showed that IMS-trapped Tim23p was chased into the IM upon restoring its potential. The Tim23p complexes with 8-13 and 9-10 in the absence of $\Delta\Psi$ are therefore productive intermediates in the TIM22 pathway.

Singly labeled Tim23 Proteins Exhibit Normal Mitochondrial Biogenesis

The above results were obtained with the photoreactive probes distributed randomly in place of Lys residues throughout the primary sequences of Tim23p and Aac2p. To obtain higher resolution information about which residues of Tim23p are adjacent to or contact specific small Tim proteins in the absence of a $\Delta\Psi$, photoreactive derivatives of Tim23p were prepared by introducing a single amber stop codon into the Tim23p mRNA coding sequence, initially about every 10 codons in the 222-residue protein (Figure 2A). In addition, a set of constructs was prepared that had a single amber stop codon at each of 4–6 adjacent codons in TMS 2 and 4. Each modified Tim23p construct was designated TAG-#, where # identifies the wild-type residue substituted with the amber codon and hence the site at which the ϵ ANB-Lys probe was incorporated. Translation of these coding sequences in the presence of ϵ ANB-Lys-tRNA^{amb} (Flanagan *et al.*, 2003; McCormick *et al.*, 2003; Saksena *et al.*, 2004) yielded Tim23p proteins with only a single probe located at a defined site.

When the percentage of amber codon suppression (full-length Tim23p/[full-length Tim23p + Tim23p terminated at the amber codon]) was examined for each of the constructs, we found that the amount of suppression ranged between 20 and 50% for the individual mutants. This suppression efficiency did not correlate with the distance of the amber

Figure 2. Tim23p derivatives containing a single ϵ ANB-Lys are import competent and form stage-specific photoadducts. (A) Schematic representation of the integrated Tim23p protein and the locations of photoreactive probes. The numbered rectangles indicate the four hydrophobic regions proposed to be membrane-spanning segments of Tim23p. Small numbers along the length of the Tim23p sequence denote the amino acid positions at which an amber codon was substituted for the natural codon. (B) Suppressor tRNAs translate amber codons located far into the coding sequence. Tim23p containing amber codons at amino acid positions 111, 156, 169, 181, 195, or 206 were translated in vitro with [35 S]Met and in the presence or absence of Lys-tRNA^{amb}. The full-length (suppressed) nascent chains are ~27 kDa and are marked by an arrowhead; the nonsuppressed nascent chains terminated at the amber codon are indicated by the bracket. (C) Tim23p containing an ϵ ANB-Lys probe is imported into mitochondria and integrated into the IM. Wild-type Tim23p, TAG-131, and TAG-156 were translated in the presence of ϵ ANB-Lys-tRNA^{amb} and then exposed to 10 mM DTT to chemically inactivate the azido group. Each precursor was imported into isolated mitochondria, and the samples were split into three 25- μ g aliquots. One aliquot was not treated further (lanes 2, 7, and 12), one aliquot was treated with 50 μ g/ml proteinase K on ice for 20 min (lanes 3, 8, and 13), and the mitochondria in the third aliquot were converted to mitoplasts by osmotic shock and then treated with proteinase K (lanes 4, 9, and 14). Each precursor was also imported into mitochondria (25 μ g) lacking a membrane potential ($-\Delta\Psi$), converted to mitoplasts, and treated with proteinase K (lanes 5, 10, and 15). In all samples, mitochondria or mitoplasts were pelleted before analysis by SDS-PAGE and phosphorimaging. Arrowheads indicate the protease-protected fragment of Tim23p in mitoplasts, indicative of successful integration of Tim23p into the IM. (D) Photocross-linking to Tim23p varies with probe location and the stage of integration. Tim23p TAG-25, TAG-32, and TAG-66 were translated with either ϵ ANB-Lys-tRNA^{amb} or Lys-tRNA^{amb} (+ or -ANB), imported into mitochondria in the presence or absence of a membrane potential (+/- $\Delta\Psi$), and then photocross-linked and analyzed as above. Prominent photoadducts are indicated by arrowheads (lanes 4 and 8) and an asterisk (lane 10).

codon from the initiator codon and instead appeared to vary according to the codon context flanking the amber codon. Evidence for this conclusion is provided by comparing the intensities of the full-length (suppressed) protein bands in Figure 2B.

It is conceivable that the incorporation of ϵ ANB-Lys into Tim23p at a particular position may interfere with its targeting to or import into mitochondria. For example, TAG-131 is located in one of the positively charged loops required for the integration of Tim23p into the IM (Davis *et al.*, 1998), whereas TAG-156 is located in the middle of the second transmembrane segment where it may affect the ability of Tim23p to fold or insert into the IM. Yet when 35 S-labeled Tim23p, TAG-131, or TAG-156 polypeptides containing ϵ ANB-Lys were chemically inactivated by a 10-min, 26°C incubation in 10 mM DTT and then incubated with isolated mitochondria, each was imported across the OM and protected from externally added protease (Figure 2C, arrowheads, lanes 3, 8, and 13), thereby demonstrating that import across the OM was not compromised by the probe. Furthermore, after disruption of the OM by osmotic shock, a ~12-kDa fragment of Tim23p, diagnostic for its insertion in the IM (Davis *et al.*, 1998; Ryan *et al.*, 1998), was generated by protease treatment for each protein (Figure 2C, lanes 4, 9, and 14). Although these fragments contain fewer [35 S]Met and hence gave less intense phosphorimager bands, the important point is that the extent of import was approximately the same for these three proteins. In contrast, 35 S-labeled Tim23p, TAG-131, or TAG-156 precursors incubated with mitochondria lacking a membrane potential ($-\Delta\Psi$) were not inserted into the IM because no ~12-kDa fragment

of Tim23p was generated after protease treatment of mitoplasts (Figure 2C, lanes 5, 10, and 15). Each of the other ϵ ANB-Lys-containing Tim23p derivatives was also properly targeted to mitochondria and inserted into the IM (data not shown). Thus, the single ϵ ANB-Lys probes incorporated into Tim23p did not interfere with its biogenesis.

Import Stage- and Site-specific Photoadduct Formation

The photocross-linking of singly labeled Tim23p derivatives was examined at two different stages of import: before insertion into the IM ($-\Delta\Psi$) and after insertion ($+\Delta\Psi$). To distinguish between photoadduct and nonphotoadduct radioactive bands, two samples of each derivative that contained either ϵ ANB-Lys-tRNA^{amb} or Lys-tRNA^{amb} were run in parallel. The results of three representative single-probe photocross-linking experiments are shown in Figure 2D. The non-probe-dependent bands of unknown origin were ignored, and we focused on the probe-dependent high-molecular-mass bands in the gel. For some probe locations (e.g., TAG-25 and TAG-32), photoadducts were observed only when IM insertion was blocked by the absence of a $\Delta\Psi$ (Figure 2D, arrowheads, lanes 4 and 8). In other cases (e.g., TAG-66), photoadducts were observed only after insertion into the IM ($+\Delta\Psi$; Figure 2D, asterisk, lane 10), whereas no significant photoadduct formation was observed at other probe locations after IM integration (data not shown). Furthermore, the target proteins differ because the size of the photoadducts are substantially different when probes are located at residue 66 instead of 25 or 32 (compare lanes 4, 8, and 10 in Figure 2D). These marked differences serve as an important internal control for this approach, because it is

Figure 3. Site-specific photocross-linking of Tim23p to Tim8p and Tim9p in the IMS. *TIM23* constructs with single amber codons located at 27 different positions (aa#) were transcribed and translated in the presence of [³⁵S]Met and ϵ ANB-Lys-tRNA^{amb}, imported into de-energized mitochondria, and photolyzed. Samples were split into two aliquots and pelleted: one aliquot (5 μ g) was resuspended in sample buffer and the other (50 μ g) was immunoprecipitated as described in *Materials and Methods*, first with antisera to Tim8p (α Tim8p) and subsequently with antisera to Tim9p (α Tim9p) before analysis by SDS-PAGE and phosphorimaging. (A) Total radioactive species; (B) photoadducts immunoprecipitated with α Tim8p or α Tim9p. 23p, Tim23p; nonsup., nonsuppressed Tim23p (these proteins are not visible in most lanes because the earlier location of the amber stop codons created shorter terminated polypeptides that ran off the gel).

clear that the differing photoadduct yields and identities that are both probe- and import stage-specific do not result from some random or systematic photocross-linking, but instead must reflect the local environments of the probes at the time of illumination.

Identification of Site-specific Cross-links to 8-13 and 9-10

To identify residues of Tim23p that are in close proximity to the small Tim proteins, 27 different singly labeled derivatives of Tim23p were individually imported into mitochondria lacking a $\Delta\Psi$. After photolysis, a sample was immunoprecipitated either with α Tim8p and then α Tim9p (Figure 3) or with α Tim10p and then α Tim13p (Figure 4). The total sample content of radioactive polypeptide species for each of the entire set of Tim23 single-probe substrates is shown in

panel A of both Figures 3 and 4. These gels reveal a range of high-apparent-molecular-mass photoadducts that contain the photoreactive Tim23p species and target proteins of ~ 10 kDa (e.g., TAG-25, 32, 80, 90, and 156), ~ 50 kDa (e.g., TAG-66, 120, and 131), ~ 70 kDa (e.g., TAG-120, 131, and 181), and ~ 80 kDa (TAG-80). As is evident by comparing the photoadduct patterns from the two independent sets of samples shown in Figures 3A and 4A, the photocross-linking experiments were quite reproducible.

Immunoprecipitations of the samples with small Tim-specific antisera identified which probe sites were able to react covalently with small Tim proteins in the IMS (Figures 3B and 4B). An abrupt increase in the apparent molecular mass of Tim23p photoadducts to Tim8p and Tim13p was observed when the probe location moved from residue

Figure 4. Site-specific photocross-linking of Tim23p to Tim10p and Tim13p in the IMS. Experiments were performed exactly as in Figure 3, except that samples were first immunoprecipitated with α Tim10p and then with α Tim13p. (A) Total radioactive species; (B) photoadducts immunoprecipitated with α Tim10p or α Tim13p.

80–90 in Tim23p (Figures 3B and 4B). This marked change in electrophoretic mobility most likely resulted from a change in the site of covalent linkage between Tim23p and the smaller Tim protein that yielded a different mobility for the photoadduct, as has been observed previously by us (McCormick *et al.*, 2003) and others (Plath *et al.*, 1998). A similar effect, photocross-linking of Tim50p from different sites on Tim23p, most likely explains the multiple photoadduct bands in Figure 1C (lanes 5 and 11). The mobilities of the Tim9p and Tim10p photoadducts did not change.

Quantification of Photocross-linking Efficiencies from Different Probe Locations

The efficiency of amber codon suppression differed for the various Tim23p TAG constructs, and hence the amounts of full-length Tim23p produced, imported, and available for photocross-linking varied for the different Tim23p derivatives (compare intensities of “23p” in different lanes of Figures 3A or 4A). Thus, to directly compare the efficiencies of covalent reaction from different probe locations to the different small Tim proteins, the gels showing the total imported radioactive protein content in each sample were exposed for the same length of time and to the same phosphorimager plate as the gels that showed the amount of material immunoprecipitated from the same sample. The ratio of photoadduct to unreacted Tim23p was determined from these gels for each Tim23p derivative and small Tim protein. Although this approach (not exposing the gels of the immunoprecipitated material longer) meant that some of the weaker photoadduct bands would not be detected, it provided a reliable and reproducible mechanism for comparing the proximity of individual Tim23p sites to the different small Tim proteins in the IMS.

The results of three independent experiments are shown in Figure 5. The observed low yields of Tim23p photocross-linking to individual IMS proteins are explained by a combination of effects that includes probe reaction with water or reducing agents, the proximity of probe and target, and inefficient immunoprecipitation. Because of their nucleophilic properties, reducing agents and water react efficiently with electrophilic aryl azides. Hence, photocross-linking efficiencies to nearby proteins are significantly reduced when the probes are in a largely aqueous environment, especially one that contains reducing agents, and are higher when the probes are located in a hydrophobic environment (McCormick *et al.*, 2003; Saksena *et al.*, 2004). Hence, when the binding of a probe-carrying molecule to a target protein reduces its exposure to the local aqueous space and/or localizes the probe in close proximity to the target, the chances of the probe reacting covalently with the target before reacting with water increase significantly (e.g., Krieg *et al.*, 1986, 1989). Although the reason(s) for a low photocross-linking efficiency from a particular Tim23p site cannot be conclusively determined from the available data, it would appear that most of the Tim23p probes are in a largely aqueous environment and are not trapped at a protein interface in a Tim23p complex with small Tim proteins, presumably because the probes at the end of the long flexible lysine side chains can be squeezed out of and bend away from such an interface.

Despite the low efficiency of detected covalent reaction, the photocross-linking results reveal a number of important structural features of Tim23p complexes with 8-13 and/or 9-10. Most strikingly, the efficiencies of covalent reaction with a small Tim protein varied tremendously for the 27 different probe locations in Tim23p. If the nonpolar surfaces of the small Tim proteins associated more-or-less randomly

Figure 5. Quantification of photoadduct formation. The photoadduct yield (the ratio of Tim23p photoadduct to unreacted Tim23p imported into mitochondria) was determined after immunoprecipitation with α Tim8p-, α Tim9p-, α Tim10p-, or α Tim13p-specific antiserum as described in *Materials and Methods*. Bars show the averaged data and SDs from three independent experiments. Probe locations in the Tim23p sequence are indicated on the x-axis, and the putative locations of the TMSs in Tim23p are indicated in the linear representation of the Tim23p sequence at the top of the figure.

with Tim23p only to cover up its hydrophobic surfaces, one would expect to see each probe site in Tim23p photocross-linking to each of the small Tim proteins. Yet we found that each Tim23p residue was reproducibly adjacent only to one and sometimes two small Tim proteins (Figure 5), suggesting that the interactions of Tim9p, Tim10p, Tim8p, and Tim13p with Tim23p are not random events, and that the various nonpolar regions of Tim23p are not simply being protected from aqueous exposure by relatively nonspecific hydrophobic interactions with nonpolar surfaces on the small Tim proteins acting as nonspecific chaperones.

In general, residues in the N-terminal half of Tim23p photocross-link primarily to Tim8p and Tim13p (Figures 3B, 4B, and 5), whereas residues in the C-terminal half of Tim23p react covalently primarily with Tim9p and Tim10p (Figures 3B, 4B, and 5), results consistent with earlier chemical cross-linking data (Davis *et al.*, 2000; Paschen *et al.*, 2000). Furthermore, because the photoreactive probe is located at the end of the long, flexible lysine side chain, it is not surprising that a few Tim23p sites are situated where they photocross-linked to more than one small Tim protein. Yet, because some Tim23p sites photocross-linked to only a sin-

Figure 6. Photocross-linking of sites in Tim23p TMS2 to the IMS proteins. The data shown in Figure 5 have been normalized as described in the text to depict the relative photocross-linking efficiencies of probes positioned at residues 153–158 in Tim23p to each of the small Tim proteins.

gle small Tim protein, both 8-13 and 9-10 associate with Tim23p in a specific arrangement. For example, Tim13p appears to be in contact primarily with residues 90, 80, and 32, whereas Tim8p is photocross-linked mostly to residue 156 and secondarily to residues 32, 100, 55, and 80 (Figure 5). Also, although several sites photocross-link to both Tim9p and Tim10p, residue 80 and the sites in TMS4 react almost solely with Tim10p and very little with Tim9p (Figure 5). We therefore conclude that the small Tim proteins associate with specific sites on the Tim23p substrate to form structurally well-defined complexes.

This conclusion is further supported by comparing the differential photocross-linking observed from each of six consecutive probe locations in the middle of the second hydrophobic TMS (TMS2). Each of the four small Tim proteins is photocross-linked to a residue within this span, but each Tim protein has a distinct reactivity and hence proximity pattern with the six adjacent residues (Figure 5). To facilitate this comparison, the relative amounts of photocross-linking from these sites to a particular small Tim protein (Figure 5; residues 153–158) were normalized and are shown in Figure 6, where the maximum photoadduct yield from these six sites to a given IMS protein was designated as 100%. When the data are expressed in this manner, the resulting histograms clearly show the variations both in photocross-linking to a given IMS protein from the six adjacent sites and also in the proximities of the different IMS proteins to a single site on Tim23p.

For example, the photocross-linking data indicate that Tim8p is primarily adjacent to residue 156 of these six, whereas Tim10p is primarily adjacent to residue 158 and Tim9p to 154 (Figure 6). Tim13p photocross-linking to these sites is relatively weak compared with its photocross-linking at other sites (Figure 5), but a reduced covalent reaction was

observed at residues 154 and 157 (Figure 6). Comparing the data of Figure 6 vertically rather than horizontally, residue 158 is exposed primarily to Tim10p, 156 to Tim8p, 155 to Tim13p, and 153 to Tim9p. Although the photocross-linking targets from a particular Tim23p site show some overlap, perhaps because the probe is on a long tether, the distribution of photoadducts is clearly not random.

Photocross-linking Dependence on the N-terminal Half of Tim23p

Tim8p was photocross-linked more efficiently to a probe located in TMS2 than to any other probe, and both Tim8p and Tim13p were photocross-linked to other sites in the C-terminal half of Tim23p (Figures 3B and 5). Although peptide binding experiments indicated that 8-13 associates with the TMSs in Tim23p (Curran *et al.*, 2002b), previous chemical cross-linking data suggested that 8-13 was only proximal to the N-terminal half of Tim23p and that 9-10 interacted with the C-terminal half (Davis *et al.*, 2000; Paschen *et al.*, 2000; Vasiljev *et al.*, 2004). To clarify the nature of the photoadducts between 8 and 13 and the C-terminus of Tim23p, we repeated the photocross-linking experiments using Tim23Cp, a Tim23p derivative that lacked its N-terminal 94 residues (Ryan *et al.*, 1998).

Tim23Cp photocross-linking to Tim9p and Tim10p (Figure 7) was similar to that observed with full-length Tim23p (compare Figures 3B and 4B with 7B, and Figure 5 with Figure 7C). The association of 9-10 with Tim23p therefore appears to be independent of the N-terminal domain of Tim23p and is mediated almost entirely by interactions between 9 and 10 and the C-terminal half of Tim23p.

In contrast, no photoadducts to Tim8p or Tim13p were detected, even after very long exposures (>4 wk) of the gels to the phosphorimager screens (data not shown). Because no photocross-linking to 8-13 was observed from Tim23Cp residues 155 and 156, it appears that 8-13 does not have significant affinity for binding to the putative TM2 of Tim23Cp. Instead, it seems likely that the proximity of 8-13 to these sites in Tim23p (Figure 6) is dictated by something other than a direct, high-affinity interaction between 8 and 13 and Tim23Cp. We therefore conclude that any positioning of 8-13 adjacent to the C-terminal half of Tim23p is dependent on the presence of the N-terminal half of the protein. Presumably the N-terminal portion of Tim23p binds 8-13 and then folds so as to position 8-13 in close proximity to residues in the C-terminal portion of Tim23p. The three-dimensional arrangement of proteins in the complex may also explain why Tim10p photocross-links with relatively high efficiency to residue 80 and Tim9p to residue 100 in the N-terminal domain of Tim23p (Figure 5).

Variations in Photocross-linking Due to Putative Substrate Conformation

To what extent are the observed photocross-linking patterns dictated by substrate conformation? Photocross-linking to Tim8p is much higher from position 156 than from either 155 or 157 (Figure 6), which suggests that this segment of Tim23p is folded in the complex. If Tim23p were unfolded and extended more-or-less linearly at this point, then one would expect the extended, flexible εANB-Lys probe attached at either 155 or 157 to react with significant frequency to Tim8p because the lysine side-chain tether would allow the probes at 155 or 157 in a linear polymer to reach much of the space within reach of a probe at 156. The site specificity of the photocross-linking therefore suggests that TMS2 is folded, perhaps into a helical conformation. If true, probes at 155, 156, and 157 would extend from the helix surface in

Figure 7. Site-specific photocross-linking of Tim23p to Tim9p or Tim10p. Experiments were performed exactly as in Figure 3, except that samples were first immunoprecipitated with α Tim9p and then with α Tim10p. (A) Total radioactive species; (B) photoadducts immunoprecipitated with α Tim9p or α Tim10p; (C) the photoadduct yield was determined after immunoprecipitation with α Tim9p or α Tim10p as in Figure 5.

different directions, and the space accessible to adjacent probes would be reduced. Interestingly, probes at 154 and 157 would be positioned on the same face of the putative helix, and Tim13p is photocross-linked least efficiently from these two sites (Figure 6). Similarly, Tim10p reacts covalently most efficiently with probes located on the same side of the putative helix, 158 and 155.

Importantly, there are precedents for such variations in photoadduct formation. Major differences in photocross-linking yields and targets have been observed previously with probes positioned at adjacent residues in the α -helices formed by transmembrane sequences during integration into a membrane bilayer at a translocon (McCormick *et al.*, 2003; Saksena *et al.*, 2004). Thus, some of the photocross-linking data are consistent with Tim23p being folded when bound to 8-13 and 9-10. However, other experimental approaches will be required to ascertain the actual extent of Tim23p folding in its IMS complex (e.g., Woolhead *et al.*, 2004).

DISCUSSION

Shortly after their discovery, Tim9p and Tim10p were found to be required for carrier protein import into mitochondria (Koehler *et al.*, 1998a, 1998b; Sirrenberg *et al.*, 1998; Adam *et al.*, 1999), perhaps by binding to specific sequences common to all TIM22 substrates (Sirrenberg *et al.*, 1998). However, because carrier proteins consist primarily of hydrophobic amino acids that are ultimately embedded in the nonpolar IM bilayer, 9-10 complexes are generally thought to function as chaperones by keeping carrier protein TMSs soluble in the aqueous IMS and preventing their aggregation before integration into the IM at TIM22. Such a role would require 9-10 to cover the exposed hydrophobic TMS surfaces of an IM substrate and minimize their exposure to the aqueous IMS. However, the structure(s) of 9-10 (and 8-13) complexes with full-length substrates has (have) not yet been sufficiently characterized to identify the nature and role of 9-10 (8-13) in complexes.

We have here examined the proximity of 12% (27 of 222) of the residues in Tim23p to the small Tim proteins in complexes formed after import into mitochondria in the absence of a $\Delta\Psi$. The somewhat surprising result of this comprehensive study is that each of these sites was adjacent to a single or sometimes two small TIM proteins, but not to

all four. If the small Tim proteins associate nonspecifically with Tim23p to cover its exposed TMS surfaces, then a probe located in a specific TMS site would be predicted to photocross-link to each of the small Tim proteins. However, because the photocross-linking targets (Figures 4–7) differed greatly and reproducibly from one TMS (and non-TMS) residue to the next and because most sites examined were proximal to only a single small Tim protein, we conclude that Tim8p, Tim9p, Tim10p, and Tim13p each occupy specific sites in the IMS complexes with Tim23p.

This specificity is best evidenced by the data of Figure 6, where the photocross-linking target differed markedly for a probe at each of six successive locations in the Tim23p sequence. Given the fact that the probe is attached to the Tim23p polypeptide backbone by a ~ 13 -Å tether, the dramatic differences in photocross-linking seen from adjacent residues can only be explained if the probes and tethers that extend from adjacent residues are oriented in different directions by the folding of the Tim23p backbone. Such a variation in photocross-linking targets for adjacent probe sites has been observed previously with TMSs in the ER translocon (McCormick *et al.*, 2003; Saksena *et al.*, 2004). Thus, because residues 153–158 are predicted to be part of transmembrane segment 2 in Tim23p, it is likely that the TMSs of Tim23p are folded, perhaps into helices, in its complexes with 8-13 and 9-10.

Two very different experimental approaches, photocross-linking (this study) and peptide binding (Curran *et al.*, 2002b; Vasiljev *et al.*, 2004), have been used to determine where 8-13 and 9-10 bind to Tim23p. The formation of a covalent photoadduct shows conclusively that a Tim23p probe site is in close proximity to a small Tim protein in the IMS, but one cannot assume that the cross-linked site is directly involved in binding the small Tim protein. In contrast, the peptide binding approach does discriminate between peptides based on their binding affinity for 8-13 or 9-10, but does not distinguish between specific and nonspecific binding. These approaches also differed dramatically in the type of substrate species being examined. The photocross-linking experiments detected small Tim protein proximity to Tim23p that had been imported into the IMS of active mitochondria. The full-length substrate had therefore folded into whatever secondary and tertiary structure it adopts after traversing the TOM complex and binding to small Tim proteins. In contrast, each peptide binding exper-

iment examined 8-13 or 9-10 affinity for 13-residue segments of Tim23p. Because such short peptides will not fold into stable secondary or tertiary structures, the peptide-binding experiments detected primarily small Tim affinity for short primary sequences of Tim23p, though it is possible that binding to 8-13 or 9-10 may stabilize the partial folding of some 13-residue peptides. The peptide binding approach also cannot detect any interaction that requires the simultaneous association of 8-13 or 9-10 with noncontiguous stretches of Tim23p primary sequence. Because the 9-10 crystal structure suggests that it must undergo a conformational change to bind a substrate (Webb *et al.*, 2006), a multiplicity of substrate interactions with 9-10 may be required to initiate such a conformational change and hence binding.

Despite the above caveats, both approaches show that 8-13 associates with the N-terminal hydrophilic half of Tim23p (Figure 5; Curran *et al.*, 2002b), a result consistent with earlier studies (Davis *et al.*, 2000; Paschen *et al.*, 2000). The combined data therefore demonstrate that 8-13 binds to the N-terminal half of Tim23p. However, there is no consensus on the extent of 8-13 and 9-10 binding to the hydrophobic TMSs in the C-terminal half of Tim23p. One peptide binding study observed no binding of yeast Tim23p peptides to yeast 9-10 (Curran *et al.*, 2002a) and found instead that yeast 8-13 bound preferentially to the C-terminal peptides of yeast Tim23p (Curran *et al.*, 2002b). Another group found that *Neurospora crassa* 9-10 bound C-terminal *N. crassa* Tim23p peptides (Vasiljev *et al.*, 2004), whereas a third study reported chemical cross-links between yeast 9-10 and the C-terminal half of Tim23p (Davis *et al.*, 2000). These dramatically different conclusions about 9-10 binding to Tim23p are difficult to reconcile if they are not species-specific. The photocross-linking data support 9-10 binding to the C-terminal half of Tim23p because most C-terminal sites photocross-linked either Tim9p or Tim10p (Figures 5 and 7). A few C-terminal Tim23p sites photocross-linked specifically to Tim8p or Tim13p, but because these photoadducts disappeared when the N-terminal half of Tim23p was absent (see *Results*), it appears that 8-13 bound only weakly, if at all, to the C-terminal Tim23p. Taken together, the data indicate that 8-13 binds primarily to the N-terminal half of Tim23p and 9-10 binds primarily to the C-terminal half of Tim23p. However, these putative binding domains are not completely distinct, as evidenced by 8-13 binding of C-terminal Tim23p peptides (Curran *et al.*, 2002b) and photocross-linking to C-terminal residues (Figure 5) and also by 9-10 binding of N-terminal Tim23p peptides (Vasiljev *et al.*, 2004) and photocross-linking to N-terminal residues (Figure 5). It therefore seems likely that in assembled complexes of Tim23p and small Tim proteins, 8-13 is in close proximity to and may interact with specific C-terminal Tim23p residues, whereas 9-10 is similarly juxtaposed to N-terminal Tim23p residues.

Differences in the functional states of Tim23p complexes in the IMS may also explain, at least in part, some of the differing observations. Specifically, the Tim23p molecules in the chemical and photochemical cross-linking samples may be distributed between two or more populations in the mitochondria in the absence of a $\Delta\Psi$: those that are docked with small Tim proteins at a TIM22 complex but are unable to integrate and those that are associated with small Tim proteins in the IMS. It is currently impossible to determine experimentally what fraction of the imported full-length Tim23p proteins are in each state in the IMS and hence to determine whether the photoadducts originate from complexes that are soluble or membrane-bound. But the two

different views of 9-10 interactions with Tim23p could be accommodated if 8-13 were solely associated with Tim23p in the IMS, as proposed by Curran *et al.* (2002b), and associated with 8-13, 9-10, and Tim12p (9-10 in *N. crassa*) after docking at the TIM22 translocon.

Despite the uncertainty in whether complexes detected by photocross-linking are soluble or membrane-bound, one important fact is clear from the very specific photocross-linking data reported in this comprehensive study: the small Tim proteins associate with the Tim23p substrate to form a multicomponent complex that is very well defined in terms of protein placement within the complex. Such a specific arrangement would not be predicted for a nonspecific chaperone whose sole function was to promote substrate transport through the aqueous IMS by covering the hydrophobic surfaces of the substrate to maintain its solubility and prevent its aggregation. Our data instead strongly suggest that both 8-13 and 9-10 are positioned at precise locations around the full-length Tim23p substrate before integration. Because correctly inserting a polypeptide with multiple hydrophobic TMSs into the IM is mechanistically difficult and complex, it seems likely that the specific arrangement of the small Tim proteins around the substrate accomplishes a functional goal. Such a well-defined protein complex would presumably promote the efficient and accurate integration of a substrate at the TIM22 complex by delivering the substrate in the proper alignment and directing its entry into the translocon. The small Tim proteins would thereby ensure that the interactions of a particular substrate would occur with the desired translocon components and in the correct sequence to achieve successful integration.

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