The Tim8–Tim13 Complex Has Multiple Substrate Binding Sites and Binds Cooperatively to Tim23

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The Tim8–Tim13 complex, located in the mitochondrial intermembrane space, functions in the TIM22 import pathway that mediates the import of the mitochondrial carriers Tim23, Tim22, and Tim17 into the mitochondrial inner membrane. The Tim8–Tim13 complex assembles as a hexamer and binds to the substrate Tim23 to chaperone the hydrophobic Tim23 across the aqueous intermembrane space. However, both structural features of the Tim8–Tim13 complex and the binding interaction to Tim23 remain poorly defined. The crystal structure of the yeast Tim8–Tim13 complex, reported here at 2.6 Å resolution, reveals that the architecture of the Tim8–Tim13 complex is similar to those of other chaperones such as Tim9–Tim10, prefoldin, and Skp, in which long helices extend from a central body like tentacles from a jellyfish. Surface plasmon resonance was applied to investigate interactions between the Tim8–Tim13 complex and Tim23. The Tim8–Tim13 complex contained approximately six binding sites and showed a complex binding interaction indicative of positive cooperativity rather than a simple bimolecular interaction. By combining results from the structural and binding studies, we provide a molecular model of the Tim8–Tim13 complex binding to Tim23. The regions where the tentacle helices attach to the body of the Tim8–Tim13 complex contain six hydrophobic pockets that likely interact with specific sequences of Tim23 and possibly other substrates. Smaller hydrophobic patches on the tentacles themselves likely interact nonspecifically with the substrate’s transmembrane helices, shielding it from the aqueous intermembrane space. The central region of Tim23, which enters the intermembrane space first, may serve to nucleate the binding of the Tim8–Tim13 complex, thereby initiating the chaperoned translocation of Tim23 to the mitochondrial inner membrane.

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Keywords: mitochondria; protein translocation; surface plasmon resonance; cooperativity; chaperone

Introduction

The mitochondrion has developed an elaborate translocation system with translocons on both mitochondrial outer membrane and mitochondrial inner membrane,1–3 proteins destined for the mitochondrion, termed precursors until they reach their correct location, utilize Translocase of the Outer Membrane (TOM) and Translocase of the Inner Membrane (TIM) complexes TIM23 and TIM22 to cross the outer and inner membranes, respectively. Proteins with a typical N-terminal targeting sequence use the TIM23 translocation system, whereas proteins destined for the inner membrane use the TIM22 translocation system. Components in the TIM22 translocation system include the small Tim proteins (Tim8, Tim9, Tim10, Tim12, and Tim13) and the membrane components Tim18, Tim22, and Tim54. The small Tim proteins assemble in hexameric complexes (referred to as small Tim complexes) in the intermembrane space in which three Tim9 polypeptides partner with three Tim10 polypeptides, and three Tim8 polypeptides partner with three Tim13 polypeptides. The insertion complex in

Abbreviations used: TOM, Translocase of the Outer Membrane; TIM, Translocase of the Inner Membrane; AAC, ADP/ATP carrier; SPR, surface plasmon resonance; RU, resonance units; PDB, Protein Data Bank.

the inner membrane consists of a fraction of the Tim9 and Tim10 with Tim12 and membrane proteins Tim18, Tim22, and Tim54.

The TIM22 translocation system functions through the coordinated action of the Tim8–Tim13 and Tim9–Tim10 complexes and the insertion complex in the inner membrane. The substrates of the TIM22 translocation system include the mitochondrial carrier proteins and import components Tim17, Tim22, and Tim23. These substrates cross the TOM complex as a loop in an unfolded state. The Tim17, Tim22, and Tim23. These substrates cross the TIM22 translocation system include the mitochondrial carrier proteins and import components Tim17, Tim22, and Tim23. These substrates cross the TOM complex as a loop in an unfolded state.

The Tim9 and Tim10 complex binds to the substrates to facilitate transport across the intermembrane space. Because the intermembrane space is an aqueous compartment and the substrate is unfolded, the small Tim proteins act as chaperones to maintain the hydrophobic substrates in an import-competent state, akin to cytosolic chaperones. At the inner membrane, the small Tim complexes hand the substrate over to the insertion complex. Tim22 of the insertion complex can form a channel, and insertion of the inner membrane proteins requires a membrane potential.

The Tim8–Tim13 and Tim9–Tim10 complexes display different substrate binding preferences. The Tim9–Tim10 complex can be efficiently cross-linked to carrier proteins [such as the ADP/ATP carrier (AAC) and the phosphate carrier] and the import components Tim17, Tim23, and Tim22. The Tim8–Tim13 complex can be crosslinked to Tim23 and aspartate–glutamate carriers. Peptide scans have also been utilized to determine the binding specificity of the small Tim complexes for different substrates. As reported, peptide scans for the carriers and for Tim23 were developed, in which 13-mer peptides (overlapping by 10 amino acids) for the entire sequence of the query protein were sequentially spotted on membranes; the membranes were used for far-Western analysis with the small Tim complexes. These assays showed that the Tim9–Tim10 and Tim8–Tim13 complexes bound specifically to several regions in representative substrates AAC and Tim23, respectively. Tim23 contains an N-terminal hydrophilic domain (residues 1–102), followed by four predicted membrane-spanning segments (residues 103–120, 153–160, 171–191, and 200–210). The Tim8–Tim13 complex showed the most robust binding to amino acids 80–102 of the N-terminal hydrophilic domain, as well as binding to the N-terminal side of the transmembrane domains. In addition, the results from the peptide scans were supported by a recent crosslinking study in which cysteine residues were engineered into Tim23; Tim8 preferentially bound to amino acids 80–90 in the N-terminal hydrophilic domain, and Tim13 preferentially bound to transmembrane domain 2 (residues 155–160). This study also showed that the Tim8–Tim13 complex bound to several sites throughout Tim23 as was reported in the peptide scan experiments.

Recently, the crystal structure of the Saccharomyces cerevisiae Tim9–Tim10 complex showed that it was similar to that of the Methanobacterium thermoautotrophicum prefoldin and Escherichia coli Skp chaperone. Although the complexes were not similar at the sequence level. The Tim9–Tim10 complex assembles as a hexamer consisting of a trimer of Tim9–Tim10 dimers. The bacterial Skp chaperone assembles as a trimeric periplasmic chaperone that assists outer membrane proteins in their folding and insertion into membranes, whereas prefoldin is a hexameric chaperone built from two related classes of subunits that functions in the cytosol of all eukaryotes and archaea to stabilize nonnative proteins. In each of these chaperones, long helices extend from a central body like tentacles from a jellyfish. In this study, we have determined the structure of the Tim8–Tim13 complex at 2.6 Å and show that it is similar to that of Tim9–Tim10. The structural analysis has offered some clues about potential substrate binding sites for Tim23. Presumably, the helices assist in protein folding by providing a cavity in which nonnative polypeptides can be enclosed and protected against intermolecular aggregation. In addition, there are six hydrophobic pockets where the tentacle helices attach to the body of the Tim8–Tim13 hexamer, providing specificity for substrate binding.

Crosslinking and peptide scan analysis showed that binding interactions between the small Tim proteins and substrates were specific. We therefore have also used surface plasmon resonance (SPR) to investigate the molecular interactions of both the Tim13 monomer and the Tim8–Tim13 complex with peptides derived from Tim23. Based on the crystal structure and these binding studies, we present a molecular model of the Tim8–Tim13 complex’s binding to Tim23 and suggest that the Tim8–Tim13 complex undergoes conformational changes to induce high-affinity multivalent substrate binding.

**Results**

**The Tim8–Tim13 structure is similar to the Tim9–Tim10 structure**

The Tim8–Tim13 complex was produced for structure determination by coexpressing the Tim8–Tim13 complex from a single transcript in E. coli and by purifying the recombinant complex to greater than 95% purity using chromatography, as described previously. Initial crystals were obtained from sparse matrix crystal screening kits, followed by customized fine-grid optimization. Diffraction from the best of these crystals was anisotropic, ranging between 3.1 and 2.5 Å resolution. The phase problem was solved using molecular replacement with a Tim8–Tim13 homology model as search model. The homology model was based on the published Tim9–Tim10 complex. The asymmetric unit of the Tim8–Tim13 crystal contained two hexamers. The structure was refined to Rwork = 24.2% and Rfree = 29.1% (Table 1). Several N-terminal residues and a few C-terminal residues were invisible on the electron density map.
due to disorder. These include Tim8 residues 1–27 and 87 and Tim13 residues 1–45 and 98–104. A similar extent of disorder was observed in the crystal structure of Tim9–Tim10, where residues 1–12 and 86–89 were disordered in Tim9, and where residues 1–12 and 78–90 were disordered in Tim10. The disorder could be a natural consequence of the lack of substrate in the crystal structure (see below).

Overall, Tim8–Tim13 and Tim9–Tim10 complexes are structurally similar. The Tim fold consists of a pair of antiparallel helices joined by two disulfide bonds and a connecting loop. The four types of Tim molecules (Tim8, Tim9, Tim10, and Tim13) are structurally superimposable over 42–58 Cα pairs, with no pairwise RMSD greater than 1.5 Å (Table 2), despite sharing less than 25% sequence identity in any pairwise comparison (Fig. 1). Three Tim8 molecules and three Tim13 molecules nestle alternately around a heterohexameric barrel, giving the barrel 3-fold rotational symmetry (pseudo-6-fold rotational symmetry) (Fig. 1). The N-terminal helices line the inside of the barrel, and the C-terminal helices line the outside of the barrel. The geometry is analogous to the Tim9–Tim10 complex. In fact, the two heterohexameric complexes can be superimposed with an RMSD of only 1.9 Å over 314 Cα pairs. Distinctively, the N- and C-terminal helices project from one end of this barrel core like tentacles from a jellyfish (Fig. 1b,14,15). This architecture grossly resembles those of prefoldin and Skp chaperones, in which amphiphilic helical tentacles emanate from an umbrella-like β-sheet domain.22,23 These tentacle regions are implicated in sequestering substrate from aggregation. The disorder observed at the tips of these tentacles (extreme N- and C-termini) in the Tim8–Tim13 and Tim9–Tim10 complexes could be the result of a lack of substrate molecule to lend an interaction surface.

The greatest structural difference between Tim8–Tim13 and Tim9–Tim10 complexes lies in the positions and lengths of their terminal helices (i.e., tentacles). Tim8 and Tim13 sequences have 12 and 30 more residues preceding the barrel compared to Tim9 and Tim10 (Fig. 1c). Conversely, Tim9 and Tim10 sequences have 9 and 17 more residues following the barrel compared to Tim8 and Tim13 (Fig. 1c). Although the extra N-terminal residues are largely disordered in the Tim8–Tim13 crystal structure, they presumably extend farther away from the barrel—partially helical, partially random coil. Notably, the N-terminal helices sit on the inner perimeter of the barrel, 12 Å closer to the center of the barrel than the C-terminal helices sitting on the outer perimeter of the barrel. It follows that these N-terminal tentacles of Tim8–Tim13 would be in closer proximity to each other compared to the C-terminal tentacles of Tim9–Tim10. If these tentacles function to encompass the substrate molecule (as has been suggested for the similarly shaped prefoldin molecule22,23), the relative differences in the positions of these tentacles suggest that Tim8–Tim13 would prefer smaller substrates to the Tim9–Tim10 tentacles. Sequence alignments of Tim13 and Tim8 proteins show variability in tentacle lengths, suggesting that such size preferences might vary among species.

Hydrophobic patches on Tim8–Tim13 suggest a mechanism for achieving substrate specificity

Over the surface of the hexameric barrel, Tim8–Tim13 and Tim9–Tim10 share similar patches of hydrophobicity. Most of the barrel’s surface is hydrophilic, including the pore in the center of the barrel, making these areas unlikely candidates for substrate interactions. But a relatively large hydrophobic region can be found where the tentacles join the barrel. This side of the barrel contains six hydrophobic cavities composed of Tim8 residues Val33, Ile37, Leu73, and Leu83, and Tim13 residues Leu49, Ile53, Leu78, Tyr81, Met82, Trp85, and Tyr92, and

Table 1. Statistics of X-ray data collection and atomic refinement

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<td>PDB deposition ID code</td>
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Numbers in parentheses refer to the outer shell of the data.

Table 2. Cα superposition of the four TIM molecules (chain A) onto one another

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<th>Tim8</th>
<th>Tim9</th>
<th>Tim10</th>
<th>Tim13</th>
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<tr>
<td>Tim8</td>
<td>0.93 (49)</td>
<td>1.17 (50)</td>
<td>1.46 (42)</td>
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<tr>
<td>Tim9</td>
<td>0.71 (58)</td>
<td>0.92 (44)</td>
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<td>Tim10</td>
<td>0.91 (43)</td>
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<tr>
<td>Tim13</td>
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</table>

1146 Tim8–Tim13 Complex Binds Cooperatively to Tim23
Ile96 (Fig. 7a). Similar hydrophobic pockets are found in the Tim9–Tim10 complex in the same locations, although they are composed of different hydrophobic residues. The sequence differences could encode substrate specificity. Due to the curvature of these pockets, any potential interaction is probably with the substrate’s interhelical loops rather than with the transmembrane helices themselves. Additional hydrophobic patches with increased accessibility exist on the tentacles, but they are smaller. These patches are located about midway down the length of the tentacles, making them the most likely contact points with the transmembrane segments of substrate molecules. They consist of Tim8 residues Ile20, Phe23, and Leu24, and Tim13 residues Ile29, Leu33, and Ile37. These patches are interspersed with positive and negative charges similar to the tentacles of prefoldin and Skp chaperones. Since the tentacle sequences are also poorly conserved among species, the amino acids forming the tentacles might also encode substrate specificity.

Fig. 1. Comparison of Tim8–Tim13 and Tim9–Tim10 structures. (a) Ribbon diagrams of the Tim8–Tim13 (TIM8–13) complex and the Tim9–Tim10 (TIM9–10) complex (PDB ID 2BSK) illustrate similarities in oligomeric assembly. In this view, the “tentacle” helices point toward the viewer. The largest differences appear in the N-terminal helices of Tim8 and Tim10 (blue and cyan helices lining the central pore). Red sticks indicate the location of conserved intrachain disulfide bonds. (b) Superposition of the Tim8–Tim13 and Tim9–Tim10 complexes. (c) Structure-based sequence alignment of Tim8, Tim9, Tim10, and Tim13. The secondary structure elements (top) are mapped to the four sequences. This figure was prepared using the programs Jalview and PyMOL.

Molecular interactions between the Tim8–Tim13 complex and peptides derived from Tim23

To determine how the Tim8–Tim13 complex binds to a substrate, SPR analysis with the Tim8–Tim13 complex, with Tim13 monomer, and with peptides derived from Tim23 was used. Tim23 contains an N-terminal hydrophilic domain (residues 1–102), followed by four predicted membrane-spanning segments (residues 103–120, 153–160, 171–191, and 200–210). Previous biochemical studies using peptide scans have shown that the Tim8–Tim13 complex bound to distinct peptides in Tim23.
by Alder et al. indicate that the Tim8–Tim13 complex can be readily crosslinked to residues in the C-terminal half of the hydrophilic domain of Tim23 (amino acids 80–90), among other regions. Because residues 75–110 of Tim23 (the C-terminal half of the hydrophilic domain and transmembrane domain 1) showed the strongest binding in peptide scan experiments, we predicted that this region may be translocated first into the intermembrane space and likely interact with the Tim8–Tim13 complex. We have employed SPR experiments with a BIACORE T100 instrument (BIACORE AB, Uppsala, Sweden) to investigate the molecular interactions in more detail, focusing on the aforementioned region. As a strategy, Tim13 or the Tim8–Tim13 complex was tethered to a Ni²⁺-coated sensor chip. The Tim8–Tim13 complex was coupled by a C-terminal 10× His tag on Tim8, and the Tim13 monomer was coupled by a C-terminal 10× His tag (Fig. 2a). The recombinant proteins were purified using Ni²⁺-agarose (Supplementary Fig. S1). This tethering approach allowed the assembled complexes to be coupled to the chip by a single defined linkage (i.e., via the C-terminus) with free rotation, rather than randomly coupled from covalent attachment via primary amines in the protein, which is typically used in SPR studies. In addition, the complex could be removed and the sensor surface could be regenerated with a fresh aliquot of complex when sensor performance deteriorated. Thus, a similar sensor chip with a new active complex was assembled for individual experiments, in contrast to generating one chip with the complex covalently coupled via random lysine residues in the protein for repeated use.

On the basis of the peptide scan results, the peptides selected (Fig. 2b) were predicted to bind to the Tim8–Tim13 complex with different affinities (Table 3). Peptides pep77–88, pep91–103, and pep98–111 were derived from the C-terminal half of the hydrophilic domain (residues 1–102 in Tim23) and transmembrane domain 1; this region showed strongest binding in the peptide scan and cross-linking experiments to Tim8 and Tim13. Peptides pep136–148 and pep181–193 were derived from regions N-terminal to transmembrane domains 2 and 3, respectively, which showed decreased binding affinity in the peptide scan experiments.

The assembly states of the Tim8–Tim13 complex and of the Tim13 monomer were investigated by blue native gel analysis (Fig. 2c). Like the endogenous Tim8–Tim13 complex in mitochondria, the Tim8–Tim13 complex migrated as a 70-kDa complex, but the Tim13 monomer migrated as a...
smaller complex. Circular dichroism (CD) analysis was used to investigate the structural properties of the monomer and the complex, as has been reported previously (Fig. 2d and e). The Tim8–Tim13 complex and Tim13 displayed similar structural properties, with approximately 70% α-helical and 6% β-sheet properties (Supplementary Fig. S1). Therefore, the Tim8–Tim13 complex was assembled, whereas the Tim13 monomer folded into a stable structure.

We first tested the Tim13 monomer in SPR studies because it is simpler than the Tim8–Tim13 complex in that it contains only one subunit of the complex. The Tim13 monomer was selected because the Tim8 monomer was problematic to purify. The Tim13 monomer was coupled to the sensor surface, and pep\(^{91-103}\) (0–500 μM) was assayed for binding. The data for the interaction were analyzed at equilibrium using different models provided with the Scrubber-2 software; a first-order binding interaction was identified, and the saturation curve was plotted (Fig. 3). Using Eq. (1),\(^{25}\) a binding stoichiometry of approximately 1 Tim13 polypeptide:1.5 peptides was calculated, suggesting that Tim13 may have approximately one peptide binding site. A dissociation constant \(K_d\) of 613 μM for the Tim13h and pep\(^{91-103}\) was calculated from a Scatchard plot of the data. This analysis suggests that the Tim13 monomer indeed binds to pep\(^{91-103}\) and most likely forms a 1:1 complex.

Because we expected the complex to behave differently from the individual Tim13 monomer when binding to substrate, interactions with the Tim8–Tim13 complex were also tested to determine (a) the number of binding sites and (b) the nature of the binding interaction (i.e., bimolecular or cooperative). In the first series of SPR studies, a concentration range (0–630 μM) of pep\(^{91-103}\) was assayed for binding to the Tim8–Tim13 complex, and a typical sensorgram is shown in Fig. 4a. Visual examination of the sensorgrams immediately showed that pep\(^{91-103}\) associated with a high on-rate and that equilibrium was reached in less than 2 s. The trace of the sensorgrams was enlarged in the range of 54–60 s to illustrate the extent of fast binding (Supplementary Fig. S2). Because the SPR instrument measures binding every 0.5 s, the on-rates for the binding of pep\(^{91-103}\) were too fast for accurate kinetic analysis; this has been documented previously.\(^{25}\) At higher concentrations of pep\(^{91-103}\), binding seemed to be saturated because the resonance units (RU) started to decline quickly upon binding. As a result, equilibrium analysis was performed. The peptide was injected over 25 s, and the binding was monitored for 4.5 min. The formed complexes also quickly dissociated from the surface, and the binding curves returned to baseline in less than 1 min.

Because the binding kinetics were too fast to be measured for curve fitting, the equilibrium sensorgram values were used to plot a saturation curve (Fig. 4b). The data for the interaction were analyzed using different models provided with the Biaevaluation 3.0 and Scrubber-2 software; however, the binding curves could not be fitted in a satisfying manner to any of these models, suggesting a complex binding mode rather than the bimolecular interaction model typically observed using SPR. Indeed, the saturation curve suggested positive cooperativity. Accordingly, we used the Hill equation to model the interaction (Fig. 4c). From the Hill plot, a straight line was obtained (\(r^2=0.87\)). The intercept at the \(x\)-axis indicated that half saturation occurred at 393 μM pep\(^{91-103}\). The slope gave an estimate of the Hill coefficient (\(n\)); the coefficient was approximately 7, confirming positive cooperativity and suggesting that the Tim8–Tim13 complex contains several binding sites. As corroboration, the binding stoichiometry (Eq. (1)) ranged from 1 Tim8–Tim13 complex:5 pep\(^{91-103}\) to 1 Tim8–Tim13 complex:5.7 pep\(^{91-103}\), also indicating that the peptide can bind to multiple sites on the Tim8–Tim13 complex.

### Table 3. Peptides derived from Tim23 for SPR studies

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<tr>
<th>Tim23-derived peptide</th>
<th>Peptide sequence</th>
<th>Binding interaction from peptide scan*</th>
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<tr>
<td>Pep7–48</td>
<td>N-KREEQSLSCQGLC</td>
<td>++</td>
</tr>
<tr>
<td>Pep91–103</td>
<td>N-KRGWDDLCYTGCA</td>
<td>+++</td>
</tr>
<tr>
<td>Pep106–120</td>
<td>N-KREECYGTAVVLYLLG</td>
<td>+++</td>
</tr>
<tr>
<td>Pep136–148</td>
<td>N-KREEGLIGFSGMMQG</td>
<td>++</td>
</tr>
<tr>
<td>Pep181–193</td>
<td>N-KTVLHITKRGFRL</td>
<td>++</td>
</tr>
<tr>
<td>Pep103</td>
<td>N-KAGAGALTGALFK55KG</td>
<td>++</td>
</tr>
</tbody>
</table>

* Binding interaction based on peptide scan results.\(^{4}\)

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**Fig. 3.** Tim13 bound to pep\(^{91-103}\) in a bimolecular interaction. For SPR analysis, the Tim13 monomer was coupled to the Ni\(^{2+}\)-coated sensor surface at 1500 RU. Pep\(^{91-103}(0–500\,\mu M)\) was analyzed for binding to the Tim13 monomer (described in Materials and Methods), and the saturation curve is shown.
complex. Therefore, the Tim8–Tim13 complex has approximately six binding sites. The binding mechanism was not that of a simple bimolecular interaction, suggesting that the individual Tim subunits may acquire an increased affinity for binding as increasing peptide binding sites are occupied.

We investigated the binding of the Tim8–Tim13 complex with other peptides derived from Tim23 (Fig. 2b), as was performed with pep91–103. The binding data were plotted on a saturation graph (Fig. 5a), and the saturation data from pep91–103 were also included for comparison. Whereas the peptides derived from the C-terminal half of Tim23 (pep98–111, pep108–120, pep136–148, and pep181–193) did not show appreciable binding, pep77–88 seemed to display cooperative binding similar to that of pep91–103. The interaction between pep77–88 and Tim8–Tim13 was analyzed with the Hill equation (Fig. 5b), and pep77–88 also showed positive cooperativity with a correlation of $r^2 = 0.85$. The intercept with the x-axis indicated that half saturation occurred at 363 μM pep77–88 and that the Hill coefficient (n) was approximately 7, indicating again that the Tim8–Tim13 complex has multiple binding sites. In addition, this region of Tim23 seems to induce an increased binding affinity in the Tim8–Tim13 complex.

The positive cooperativity implies that binding at one site of the complex may influence binding at other sites. Further support of this concept comes from two additional experiments. In the first approach, we mixed a fixed concentration of pep91–103 with a peptide that has a low binding affinity (pep136–148) to determine whether binding...
by pep^{91–103} could influence the binding of other peptides. A constant low concentration of pep^{91–103} (63 μM) that could slightly induce binding was added with increasing amounts of pep^{136–148}, and the saturation curve was plotted (Fig. 6a). For comparison, the binding of pep^{136–148} alone was included on the plot. The addition of pep^{91–103} with pep^{136–148} clearly increased the binding of pep^{136–148} approximately three times above that of pep^{136–148} alone. After analysis of the data, the binding interaction was fitted to a first-order binding interaction. The equilibrium dissociation constant \( K_d \) was 350 μM. In the second assay, we kept the total peptide concentration constant at 500 μM and inversely varied the pep^{91–103}/pep^{136–148} ratio (Fig. 6b). The saturation curve was plotted, and the saturation curve for pep^{91–103} was included for comparison. Inverting the ratio of the peptides resulted in a sigmoidal curve that showed an affinity for binding higher than that with pep^{91–103} alone. The interaction was plotted using the Hill equation (\( r^2 = 0.97 \)) (Fig. 6c). The half saturation was 212 μM, and the Hill coefficient (n) was approximately 4. The binding stoichiometry of approximately 1 Tim8–Tim13 complex:5.9–6.8 peptides was calculated from Eq. (1). In addition to pep^{91–103} enhancing pep^{136–148} binding (Fig. 6a), it seems that binding of pep^{136–148} enhances binding of pep^{91–103} (Fig. 6b).

Thus, the Tim8–Tim13 complex displays a different binding affinity, depending on the presence of different peptides, thereby implying that the Tim8–Tim13 complex may undergo conformational changes as a substrate is being bound, allowing it to bind multiple regions of the incoming substrate.

### Discussion

Previous studies employing crosslinking and peptide scans suggest that the Tim8–Tim13 complex binds to substrates to shield the hydrophobic regions in the aqueous intermembrane space and that the complex may contact several sites in the substrate.\(^4\,^9\,^{13}\) Our study builds on these previous reports by determining the structure of the Tim8–Tim13 complex and by addressing the mechanism by which the Tim8–Tim13 complex may bind to substrates. As expected, the Tim8–Tim13 structure is similar to that of Tim9–Tim10; however, the organization of the tentacles with respect to the positions and lengths of their terminal helices differed between the two complexes. This difference may be important for determining substrate specificity.

We have used SPR technology to investigate the interactions with the Tim8–Tim13 complex. We predicted that the central region (amino acids 80–110) of Tim23 most likely entered the intermembrane space first because constructs with dihydrofolate reductase molecules appended to the N- and C-termini of Tim23, which block translocation across the TOM complex, were crosslinked to Tim8 and Tim13, demonstrating that this region has entered the intermembrane space. Studies by Alder et al. also showed prevalent crosslinking of the Tim8–Tim13 complex to Tim23 in the same region.\(^13\) As expected when the complex is composed of six subunits (each with the ability to bind the substrate), the Tim13 monomer showed a typical bimolecular interaction, with the peptide derived from the aforementioned region of Tim23, pep^{91–103}. However, the dissociation constant (\( K_d = 613 \) μM) indicated that the interaction was of low affinity compared to that of the Tim8–Tim13 complex (\( K_d = 393 \) μM).

We investigated the binding of the Tim8–Tim13 complex and predicted that the Tim8–Tim13 complex...
complex should have multiple binding sites because
the complex contains three Tim13 monomers and
three Tim8 monomers. Indeed, SPR analysis showed
that the complex contains approximately six binding
sites. The binding, however, was not a simple
bimolecular interaction. The Tim8–Tim13 complex’s
interaction with the substrate was cooperative for
peptides derived from amino acids 77–103 of Tim23,
whereas the complex did not bind appreciably to
peptides from the C-terminus of Tim23. We suggest
that the Tim8–Tim13 complex may undergo a
conformational change in which the complex has
increased affinity for the substrate. Thus, the central
region of Tim23 (amino acids 77–103) may serve as a
nucleation point to induce the complex to bind to the
substrate, preparing it for transport across the
intermembrane space.

The quick on-rates and off-rates may reflect
properties of the Tim8–Tim13 complex interacting
with the substrate because the complex binds to the
Tim23 substrate as it enters the intermembrane
space and then releases it to the insertion complex in
the inner membrane. The specific mechanism by
which the small Tim complexes release the substrate
is not understood. \(^1\) The small Tim proteins do not
have an ATP requirement like other chaperones, but
release of the substrate at the inner membrane may
be due to increased affinity for the Tim22 insertion
complex or oxidation–reduction chemistry. Alterna-
tively, there may be an internal timing mechanism

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**Fig. 7.** Model showing the Tim8–Tim13 complex binding to Tim23. (a) Surface hydrophobicity diagrams of the Tim8–Tim13 complex. The hydrophobic residues are shown in pink, and hydrophilic residues are shown in green. N- and C-
terminal residues disordered in the Tim8–Tim13 structure are modeled here as α-helices. (b) Model proposing how Tim8–Tim13 binds to the Tim23 substrate. Tim23 folding is based on a weak level of homology to AAC2. Magenta color segments correspond to peptide sequences shown to bind Tim8–Tim13, and dark magenta segments mark conserved residues that are predicted to bind to the substrate. Evolutionarily conserved residues in Tim13 Ile37, Glu40, Ala42, Ala44, Asn45, Ala46, and Leu49 are represented as sticks (see Discussion for details).
such that, as soon as the binding sites in the Tim8–Tim13 complex are loaded, the complex may quickly switch conformations to unload the substrate.

**Molecular model of Tim8–Tim13 binding to Tim23**

Our structural and binding data suggest a model for the binding of the Tim8–Tim13 complex to Tim23 that differs from that proposed for the binding of the Tim9–Tim10 complex to the AAC complex.\(^7\) In the Tim9–Tim10 model, Tim9 subunits are successively displaced from the hexameric complex as the transmembrane-spanning helices of AAC compete for interaction with the remaining Tim10 subunits. Thus, dissociation of the hexameric barrel is a central feature of the chaperone mechanism. On the contrary, evidence that Tim8–Tim13 binds cooperatively to six Tim23 peptide molecules suggests instead that the Tim8–Tim13 complex does not dissociate, but acts cooperatively, to bind multiple distinct sites on a single Tim23 molecule. To test whether such a mechanism was feasible in terms of accommodating a molecule of 23 kDa within the circumference of the Tim8–Tim13 tentacles, we constructed a model of Tim23 and investigated how it might fit with the Tim8–Tim13 crystal structure. The model of Tim23 is based on a weak sequence similarity with AAC2 using the program Modeller (Fig. 7b),\(^2^7\) but its accuracy is important here only in so much as it reflects the true dimensions and secondary structure content. The dimensions of Tim23 (cyan) fit well, wedged within the N-terminal helical tentacles of the Tim8–Tim13 complex (gray). The Tim23 model like AAC2 has a pseudo-3-fold rotational symmetry that coincides with the 3-fold symmetry of the Tim8–Tim13 complex. Additionally, the regions that are implicated to bind to the Tim8–Tim13 complex (amino acids 79–89, 91–103, and 136–148; marked in magenta) contact the Tim8–Tim13 complex. Notably, Trp93, which derives from the strongest binding of the Tim23 pep\(^{140}\)\(^{140}\) closely nestled into a pocket formed by Tim8 (Leu73) and Tim13 (Tyr92). At this stage of analysis, nonhydrophobic interactions most likely play an important role in substrate binding. Gentle et al. have used bioinformatics to identify residues Ile37, Glu40, Ala42, Ala44, Asn45, Ala46, and Leu49 as conserved among different Tim13 species.\(^2^8\) These residues have been highlighted by sticks in our model in Fig 7b, and these conserved residues are largely in contact with the modeled Tim23 substrate. By combining the structural analysis with the binding studies, the Tim8–Tim13 complex most likely encompasses one Tim23 monomer (making contact at several sites) to create a system in which the hydrophobic substrate can be escorted to the inner membrane. This model begins to define how the Tim8–Tim13 complex interacts with its substrates.

It is also possible that the Tim8–Tim13 binds to other substrates such as AAC and porin. However, our modeling attempts were not successful (data not shown) because of incompatibilities in the sizes and charges of amino acid side chains at the proposed region of contact at the helical ring (Tim13 residue W85; Fig. 7a). From crosslinking studies with radiolabeled AAC precursor, a direct interaction between Tim8 and Tim13 has also not been detected,\(^7\) so the biochemical evidence does point towards specificity in interactions between the Tim8–Tim13 complex and substrates. Porin is a β-barrel protein and, therefore, has a shape different from those of Tim23 and AAC. Experiments pointing to freezing substrate–complex interactions for structural studies are ultimately required to determine how the Tim8–Tim13 complex binds to its substrates.

**Materials and Methods**

**Instrumentation and reagents**

All experiments were performed using a BIACORE T100 biosensor developed by BIACORE AB. Series S NTA sensor chips and coupling reagents were also purchased from BIACORE AB.

**Peptides, plasmids, and strains**

For structural studies, recombinant Tim8–Tim13 complex from S. cerevisiae, which lacked affinity tags, was constructed and purified as described previously.\(^3\) For SPR studies, TIM13 was cloned into pET28a (Novagen) with a C-terminal 10× histidine tag to generate recombinant Tim13. For the Tim8–Tim13 complex for SPR studies, TIM8 was cloned into pET28a with a C-terminal 10× histidine tag as Ncol/Sall fragment, and TIM13 was cloned into pET28a as Ncol/Ndel fragment. Tim13 with the ribosomal binding site was then removed as XbaI/Ndel fragment and cloned into the XbaI site of the pET28a-TIM8His plasmid. As a result, a single transcript in which both Tim8 and Tim13 were translated from their own ribosomal binding site was synthesized.

His-tagged recombinant proteins were purified using Ni\(^{2+}\)-NTA agarose. The Tim8–Tim13 complex was purified under native conditions, whereas Tim13 was purified under denaturing conditions in the presence of 8.0 M urea. Assembly of the proteins was tested by blue native PAGE, as described previously.\(^4\) The purified proteins were dialyzed in TBST buffer (5 mM Tris pH 7.4, 150 mM KCl, and 0.05% Tween-20) overnight at 4 °C. Tim23 peptides for the SPR studies were selected from peptide scan results with the Tim8–Tim13 complex interactions for Tim8–Tim13 complex and synthesized under native conditions. The peptides are designated according to the amino acids numbers in the Tim23 protein from S. cerevisiae.

**Crystallization**

The purified Tim8–Tim13 complex was concentrated in a Centricon 10 device (Millipore), and the buffer was exchanged three times with 10 mM Tris (pH 8.0), 10 mM
NaCl, and 3% 2-methyl-2,4-pentanediol. The addition of 2-
methyl-2,4-pentanediol to the protein buffer was important in
preventing phase separation at high protein concentration.
The final protein concentration (30 mg/ml) was measured using absorbance reading at a wavelength of 280 nm (extinction coefficient $\varepsilon = 10,010 \text{ M}^{-1} \text{ cm}^{-1}$).

Crystals were prepared by mixing 2.0 $\mu$l of protein with 2.0 $\mu$l of reservoir solution in a sitting-drop vapor-
diffusion tray at room temperature. The reservoir solution
contained 25% polyethylene glycol 2000 MME and 0.11 M
4-morpholineethanesulfonic acid (pH 6.5). Rhomboid-
shaped crystals appeared after 2 weeks. The ability to obtain
these crystals varied with different protein pre-
parations. The largest of these crystals was only about
50 $\mu$m on edge. The crystals belong to space group P1,
with cell dimensions very close to a primitive hexagonal
(see Table 2 below). Crystals were cryoprotected by a
quick swipe through a solution containing 75% reservoir
solution and 25% glycerol.

Data collection
X-ray diffraction data to 2.3 Å were collected at
Advanced Light Source beamline 8.2.2 using an ADSC
Quantum 315 3×3 charge-coupled device array. Three
hundred sixty 1.0° oscillation frames were collected at a
wavelength anomalous dispersion. Following the pub-
derivative prevented us from obtaining phases by multi-
plex crystallographic methods. Other derivative was foreseen to be extremely challenging for
these small and poorly reproducible crystals. Difficulties
in producing a sufficient quantity of selenomethionyl
derivative prevented us from obtaining phases by multi-
wave length anomalous dispersion. Following the pub-
licaton of the Tim9–Tim10 complex,17 the structure could be solved with molecular replacement, using the program Phaser.31 A homology model of the Tim8–Tim10 hetero-
examer was created by SWISS-MODEL32 based on the
structure of the human Tim9–Tim10 heterohex-
amer [Protein Data Bank (PDB) code 2BSK].17 The
sequence alignments are shown in Fig. 1. Unexpectedly,
the homology model produced better rotation and
translation function Z-scores than did the crystallographic coordinates of the Tim9–Tim10 complex; a low success rate is typical of the use of homology models in molecular replacement when the template sequence identity is 30%
less.33,34 SWISS-MODEL was capable of producing a
sufficiently accurate alignment in this case.

First, refinement steps were performed with CNS,35
using simulated annealing and conjugate gradient algo-
rithms, and with the aid of a hydrogen bond potential function.36 Six-fold noncrystallographic symmetry
restraints were used throughout (i.e., there were two
heterohexamers in the asymmetric unit; six copies of Tim8
were restrained to be geometrically similar to each other,
and six copies of Tim13 were similarly restrained). After
each refinement step, the model was visually inspected in
Coot,37 using both 2Fo−Fc and Fo−Fc difference maps. All
hydrogen atoms connected to carbon atoms and backbone
nitrogen atoms were included at their geometrically
calculated positions and refined using a riding model.
The hydrogen atoms provide a useful constraint to
prevent violation of van der Waals contacts and do not
contribute to $F_{calc}$. Later rounds of refinement were
performed with REFMAC5 to benefit from TLS para-
meterization of domain disorder.38,39 The model was
validated with the following structure validation tools:
PROCHECK, ERRAT, and VERIFY3D.40

Structure determination and refinement
Solving the phase problem by using heavy-atom
derivative was foreseen to be extremely challenging for
these small and poorly reproducible crystals. Difficulties
in producing a sufficient quantity of selenomethionyl
derivative prevented us from obtaining phases by multi-
wave length anomalous dispersion. Following the pub-
lication of the Tim9–Tim10 complex,17 the structure could be solved with molecular replacement, using the program Phaser.31 A homology model of the Tim8–Tim10 hetero-
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heterohexamers in the asymmetric unit; six copies of Tim8
were restrained to be geometrically similar to each other,
and six copies of Tim13 were similarly restrained). After

Model of the Tim8–Tim13 complex bound to Tim23
First, a model of the Tim23 protein was constructed with
the program Modeller,7 since there are no published structures of Tim23 to date. The sequence alignments
between Tim23 and potential structural templates were
very weak. One of the suggested templates corresponded
to bovine AAC2 (PDB code 1OKC). It was selected because AAC2 is known to be a substrate of
Tim9–Tim10 complex. Since Tim8–Tim13 is structurally similar to Tim9–Tim10, it seems plausible that their
substrates might resemble each other. Furthermore, the
AAC2 has a pseudo-3-fold rotational symmetry that
conveniently coincides with the 3-fold symmetry of the
Tim–Tim13 complex. Docking the Tim23 model to the
Tim–Tim13 crystal structure was accomplished with the
graphics program "O."41 The N- and C-terminal helices of
Tim8–Tim13 were adjusted to fit around the Tim23
molecule, interacting with the transmembrane helices.
The loops that connect Tim23 helices fit neatly into
hydrophobic pockets where the N- and C-terminal helices of
Tim8–Tim13 branch away from the barrel. The model was energy-minimized using CNS.35

CD analysis
CD analysis was performed on a JASCO J-600 spectropolarimeter. A scan speed of 10 nm/min, a time constant
of 4 s, and a bandwidth of 1.0 nm were used to acquire the
data. In addition to baseline correction, three scans were
averaged for each spectrum. The proteins were scanned
from 260 to 200 nm at 4 °C in a 1-mm pathlength cell with
protein concentrations of 0.2–0.4 mg/mL. Spectra were
analyzed for secondary structure using the convex
constraint algorithm for secondary structure prediction.42

SPR analysis
Recombinant Tim8–Tim13 complex and Tim13 mono-
er were immobilized on the SPR Ni2+ chip at 25 °C.43
The chip was activated by injecting 1 mM NiCl2 over the
chip for 2 min at 5 µl/min. Protein in TBST buffer at 6 µM
for Tim8–Tim13 complex and at 9.8 µM for Tim13
monomer was immobilized on the chip with two
sequential 30-min injections at 5 µl/min to yield 3500–
4000 RU for the Tim8–Tim13 complex and 1500 U for the
Tim13 monomer. Note that these concentrations were
selected as recommended by the BIACORE website, with
the assumption that up to six substrate binding sites
may be present on the Tim8–Tim13 complex because it consists of
three Tim8 monomers and three Tim13 monomers.
Mock-derivatized flow cells served as reference surfaces,
and nonspecific binding was subtracted.
The binding analysis was performed at 25 °C and 30 μl/min flow rate in TBST buffer. Peptides in TBST buffer at concentrations of 0–60 μM were analyzed for binding to the sensor chip coupled with Tim8–Tim13 or Tim13h. Binding ran for 25 s, followed by at least 3 min of dissociation phase to allow the baseline to return to the starting level. The chip was typically regenerated with fresh complex after one to two concentration series of a particular peptide.

SPR data for each concentration of peptide were, at minimum, duplicated. Control experiments were conducted to ensure that mass transport and bulk flow limitations were absent in the analyte regime. In addition, Eq. (1) was used to calculate binding stoichiometry between the peptide and the coupled protein (MW peptide) (MW complex). The Tim8–Tim13 complex has a molecular mass of 63.2 kDa, and the Tim13 monomer has a molecular mass of 12.4 kDa:

$$\text{Binding Stoichiometry} = \frac{\text{RU}_{\text{max}}/\text{RU} - \text{bound complex}}{\text{MW peptide/MW complex}}$$

The binding data for the Tim8–Tim13 complex were not analyzed kinetically with the Bioevaluation 3.0 software. Attempts at kinetic analysis were unsuccessful because the on-rate proceeded too quickly. Therefore, equilibrium binding analysis was employed to analyze the interaction. Furthermore, Tim8–Tim13 and pep91 were not fitted with either the Bioevaluation 3.0 or the Scrubber-2 software because simple models involving noncooperative interactions could not explain the clearly sigmoidal data. Accordingly, the data were analyzed with a Hill plot with GraphPad Prism 4 to generate a theoretical fit of the data.44

**Blue native gel electrophoresis**

Wild-type mitochondria and recombinant proteins were solubilized (20 mM HEPES, 50 mM NaCl, 10% glycerol, 2.5 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid pH 7.4, and 0.2% n-dodecylmaltoside) at 4 °C for 30 min. The lysate was centrifuged at 14,000 rpm for 30 min at 4 °C to pellet any insoluble material. The solubilized proteins were analyzed via blue native gel electrophoresis on a 6–16% linear polyacrylamide gradient.24

**Accession code**

The coordinates of the final model and the merged structure factors have been deposited in the PDB. The corresponding PDB code is 3CJH.

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**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.07.069

**References**
