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Assembly of the Mitochondrial Tim9–Tim10 Complex: A Multi-step Reaction with Novel Intermediates

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Protein assembly is a crucial process in biology, because most proteins must assemble into complexes to perform their function in the cell. The mitochondrial Tim9–Tim10 translocase complex, located in the mitochondrial intermembrane space, plays an essential chaperone-like role during the import of mitochondrial membrane proteins. The complex consists of three molecules of each subunit arranged alternately in a ring-shaped structure. While structural and functional studies have indicated a dynamic nature of the complex, little is known about the assembly process and the mechanism of its function. Here we investigated the assembly process of yeast Tim9–Tim10 complex in real time, using stopped-flow fluorescence coupled with Trp mutagenesis, and stopped-flow light scattering techniques. We show that different parts of the proteins are assembled at different rates; also assembly intermediates consisting four subunits arise transiently before formation of the final hexameric Tim9–Tim10 complex. Interestingly, the assembly intermediate has more organised N-terminal helices that form an inner layer of the complex, but not the C-terminal helices, which form the outer layer of the complex. In addition, using analytical ultracentrifugation techniques, we show that Tim9 forms a homo-dimer while Tim10 is a monomer. A four-step assembly pathway of Tim9–Tim10 complex, involving formation of hetero-dimer and tetramer assembly intermediates, is proposed. This study provides the first description of the assembly pathway of this translocase complex, and insight into the mechanism of its function.

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Introduction

Understanding complex assembly of proteins is an important research focus in biochemistry and cell biology, since the biological functions of most proteins are carried out by protein complexes, and complex formation or disassociation often causes activation or inhibition of the proteins.[1–3] The mitochondrial Tim9–Tim10 complex, located in the mitochondrial intermembrane space (IMS), plays an essential chaperone-like role during the import of mitochondrial membrane proteins, presumably to prevent the aggregation of the hydrophobic membrane proteins in the aqueous environment of the IMS.[4–7] Both Tim9 and Tim10 belong to the evolutionarily conserved small Tim family (in yeast that also includes Tim8, Tim12 and Tim13), which are homologous with about 25% sequence identity, 50% similarity, and have a molecular mass of approximately 10 kDa each.[8,9] All the small Tim proteins contain a strictly conserved twin CX_3C zinc-finger motif, which can coordinate a zinc ion in the Cys-reduced state.[7,10,11]

We have previously shown that both Tim9 and Tim10 are imported individually from the cytosol in a reduced unfolded form,[12] and zinc-binding can protect the protein from oxidative folding.[13] Recent studies show that this import process is mediated by the newly identified MIA40 import machinery.[14–17] While keeping the protein in a reduced form is essential for its mitochondrial import, only the oxi-

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Abbreviations used: IMS, intermembrane space; Tim, translocase of inner membrane; AAC, ADP/ATP carrier; RCF, relative centrifugal field.

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dised (disulphide bonded) proteins can form the functional Tim9–Tim10 complex in the mitochondrial IMS. The complex consists of three molecules of oxidised Tim9 and three oxidised Tim10.[12,18] Both *in vivo* and *in vitro* studies show that oxidation of the conserved CX_3C-X_n-CX_3C ($n=15$ for Tim9, $n=16$ for Tim10) motif forming two pairs of intra-molecular disulphide bonds in juxtaposition are necessary for formation of the Tim9–Tim10 complex.[12,19,20] The importance of such a structure is illustrated by the observation that a single point mutation of a Cys in the motif of human deafness-dystonia peptide 1 (DDP1, homologue of yeast Tim8) causes Mohr-Tranebjaerg syndrome in humans.[21,22] This congenital neurodegenerative condition has been shown to result from impaired assembly of the Tim8–Tim13 complex. Hot13, another protein of the mitochondrial IMS, seems to play a mediating role in the assembly or recycle and function of the small TIM complex in the IMS.[23] Mitochondria without Hot13 (Δ*hot13)* are competent in import of the small Tim proteins, but formation of the Tim9–Tim10 complex is impaired presumably due to the effect on the redox state of Tim9 and Tim10.

The ADP/ATP carrier (AAC) has been used extensively in studies of import of the mitochondrial inner membrane proteins.[24,25] AAC is a model substrate of the Tim9–Tim10 complex with six transmembrane helices forming three internal repeats.[26] Peptide spot binding array studies suggest that the small TIM complex binds to the hydrophobic transmembrane regions and the flanking hydrophilic segments of the protein.[19,27,28] In addition, Tim9 and Tim10 seem to play different roles in the assembly of the Tim9–Tim10 complex, and in the binding to its substrate AAC. It was suggested that Tim9 serves mainly a structural role, and substrate recognition is achieved largely by Tim10.[28] Furthermore, protein import studies have revealed that Tim9 accumulates with Mia40 in the IMS when the assembly partner Tim10 is not available.[16] However, at present, little is known about the process of association and dissociation of the small TIM complex.

The first high resolution crystal structure of the human Tim9–Tim10 complex was reported recently by Webb *et al.*,[18] and reveals a conformation consistent with that obtained by small-angle solution X-ray scattering structure of yeast Tim9–Tim10 complex which we described previously.[10] The high resolution structure of human TIM complex shows an α-propeller topology with three Tim9 and three Tim10 subunits arranged alternately, giving a pseudo-6-fold molecular symmetry and a central ring-shaped structure as viewed orthogonally (see Figure 2(c), below).[18] Each subunit consists of a helix-loop-helix structure. The two antiparallel helices are linked by the central loop between the two CX_3C motifs on one end and radiate like tentacles at the other end. The six N-terminal helices together form the inner layer and the six C-terminal helices form the outer layer of the ring-shaped structure. As expected, two intramolecular disulphide bonds in juxtaposition are formed in each subunit, which may play a supportive role for the flattened conformation of the central loop between the two CX_3C motifs.[18] Interestingly, almost all hydrophobic surfaces of the proteins are concealed between the interfaces of the two layers of the helixes of the complex, and there is no obvious binding pocket for hydrophobic substrates. Therefore, the authors suggested that some reconfiguration is required during the Tim9–Tim10 chaperone function of binding hydrophobic substrates.[18] Clearly, understanding the molecular mechanism of the Tim9–Tim10 complex assembly is fundamentally important for understanding the mechanism of its function.

Here we investigated the assembly process of yeast Tim9–Tim10 complex in real time using stopped-flow fluorescence coupled with mutagenesis approaches introducing a single Trp probe at various sites in the proteins, and a stopped-flow light scattering method. We show that the assembly process involves four observable kinetic steps, in which different parts of Tim9 and Tim10 are assembled with different rates. The N-terminal helices and the central loop forming the inner layer of the complex, assemble more rapidly than that of the C-terminal helices that form the outer layer of the complex. Furthermore, stopped-flow light scattering studies show that an assembly intermediate consisting of four subunits exists transiently before formation of the final hexameric Tim9–Tim10 complex. In addition, we show that Tim9 is a homo-dimer while Tim10 is a monomer using analytical ultracentrifugation. A four-step model is proposed describing the Tim9–Tim10 complex assembly pathway.

Results

The assembly process of the hexameric Tim9–Tim10 complex studied using the wild-type proteins

The hexameric Tim9–Tim10 complex is formed from three molecules of each protein as shown in the reaction:

$$3\,\mathrm{Tim9} + 3\,\mathrm{Tim10} \rightarrow \mathrm{Tim9_3Tim10_3}$$

The complex $Tim9_3Tim10_3$ is normally referred as Tim9–Tim10 or TIM10 complex in the research field. There is one Tyr residue in Tim9 (Y28) and three Tyr in Tim10 (Y41, Y49, Y69), but no Trp residues in either protein. Thus, we studied the complex formation process by following Tyr fluorescence intensity change, and assembly was initiated by rapid mixing of Tim9 and Tim10 at a molar ratio of 1:1 using the stopped-flow technique. An excitation wavelength of 275 nm was used, and emission above 295 nm was recorded (Figure 1(a)). The complex formation was confirmed by gel filtration (Figure 1(b)). In all the experiments performed proteins were prepared in 150 mM NaCl, 50 mM

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