Self-association and precursor protein binding of *Saccharomyces cerevisiae* Tom40p, the core component of the protein translocation channel of the mitochondrial outer membrane

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The precursor protein translocase of the mitochondrial outer membrane (Tom) is a multi-subunit complex containing receptors and a general import channel, of which the core component is Tom40p. Nuclear-encoded mitochondrial precursor proteins are first recognized by surface receptors and then pass through the import channel. The Tom complex has been purified; however, the protein–protein interactions that drive its assembly and maintain its stability have been difficult to study. Here we show that *Saccharomyces cerevisiae* Tom40p expressed in bacteria and purified to homogeneity associates efficiently with itself. The self-association is very strong and can withstand up to 4 M urea or 1 M salt. The tight self-association does not require the N-terminal segment of Tom40p. Furthermore, purified Tom40p preferentially recognizes the targeting sequence of mitochondrial precursor proteins. Although the binding of the targeting sequence to Tom40p is inhibited by urea concentrations in excess of 1 M, it is moderately resistant to 1 M salt. Simultaneous self-assembly and precursor protein binding suggest that Tom40p contains at least two different domains mediating these processes. The experimental approach described here should be useful for analysing protein–protein interactions involving individual or groups of components of the mitochondrial import machinery.

Key words: protein translocase, self-assembly, signal sequence binding, stability of protein–protein interactions.

INTRODUCTION

Most mitochondrial proteins are nuclear-encoded as precursor proteins containing an N-terminal or internal signal sequence. Precursor proteins are translated on cytoplasmic ribosomes and imported into mitochondria. Translocation of these precursor proteins into mitochondria requires the signal sequence, and is initiated by a general protein translocase of the mitochondrial outer membrane (Tom). The yeast Tom machinery contains at least eight integral membrane proteins, which can be subdivided into two groups: (a) four receptor proteins (Tom20p, Tom22p, Tom37p and Tom70p) that serve as initial recognition sites for precursor proteins, and (b) four other proteins (Tom5p, Tom6p, Tom7p and Tom40p) that participate in subsequent translocation of precursor proteins through the outer membrane. The receptors and the small Tom proteins are postulated to be anchored to the mitochondrial outer membrane by helical transmembrane segments (for recent reviews, see [1–3]). In contrast, Tom40p is predicted to traverse the outer membrane as a series of several anti-parallel β strands that form a β barrel [4].

Tom20p–Tom22p and Tom37p–Tom70p heterodimeric complexes appear to serve as import receptors for different classes of precursor proteins [5–8]. The former preferentially binds precursor proteins with a typical positively charged N-terminal matrix targeting sequence. The latter, on the other hand, recognizes precursor proteins with internal targeting information. After associating with the import receptors, precursor proteins are somehow aided by Tom5p to move into a general insertion/import pore [9]. Tom40p is the major component of the general insertion/import pore, and is essential for cell viability and protein import [10,11]. The two other small Tom proteins, Tom6p and Tom7p, have been suggested to modulate the assembly and dissociation respectively of the Tom complex [12,13].

The Tom complex ($\sim$ 400 kDa) has been purified from *Saccharomyces cerevisiae* as well as from *Neurospora crassa* mitochondria [14–16]. In both cases, Tom40p is the key structural element and the most abundant component, with an estimated four to eight Tom40p molecules per complex [14,16,17]. In elegant experiments, Neupert and co-workers have functionally reconstituted the purified Tom complex into liposomes [15,16]. Electron microscopy and image analysis of negatively stained Tom complexes demonstrate two to three stain-filled openings, presumably representing the protein-conducting channels, with an apparent diameter of 20 Å [15,16].

Most knowledge about the Tom components, particularly how they interact with each other or with precursor proteins, has come from studies involving chemical cross-linking and co-immunoprecipitation experiments (for reviews, see [1–3]). For example, when intact mitochondria or outer-membrane vesicles were treated with cross-linking reagents, a small portion of total Tom40p was found to be cross-linked to itself, generating Tom40p dimers of different mobilities on SDS/polyacrylamide gels [18]. Likewise, Tom40p could be cross-linked to the presequence as well as to the mature portion of a precursor protein during its import into mitochondria [10,18]. In contrast with extensive studies using intact mitochondria or purified outer-membrane vesicles, only a few studies with individual purified

Abbreviations used: Tom, translocase of outer membrane; ΔNTom40p, Tom40p in which the first 93 amino acids have been deleted; Yfh1p, yeast frataxin homologue; pYfh1p, precursor of Yfh1p; Δ_{20}Yfh1p, Yfh1p lacking the first 20 amino acids; mYfh1p, mature Yfh1p, in which the entire 51-amino-acid signal sequence of Yfh1p has been deleted; Yfh1p-(1–22)–Protein A, protein in which the first 22 amino acids of Yfh1p are linked to Protein A; Ni-NTA, Ni^{2+}-nitrilotriacetate.

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Tom components have been reported. This might be due to the problems often encountered in recombinantly expressing full-length authentic integral membrane proteins and their subsequent purification in functionally active forms. The truncated versions of Tom20p, Tom22p and Tom70p, representing their corresponding cytosolic domains, have been successfully expressed in bacteria, purified and biochemically analysed for precursor protein binding [19–23]. Tom40p, to the best of our knowledge, is so far the only Tom component that has been expressed in bacteria as the full-length protein and reconstituted in a functional form, as shown by electrophysiological techniques. Remarkably, recombinant Tom40p forms a hydrophilic cation-selective channel with a diameter of ~ 22 Å through which precursor proteins are transported [24]. The channel formed by Tom40p alone therefore mimics the protein-conducting channel formed by the entire Tom complex [15,16,25]. However, it remains unclear whether single Tom40p molecules form import pores, or whether Tom40p self-associates to create these pores [16,26]. Tom40p, expressed in bacteria and purified, migrates as a monomer on Blue Native electrophoresis [24], a technique used to resolve protein complexes. In contrast, when Tom40p with a C-terminal His_6 tag was expressed in yeast mitochondria, the tagged protein, in agreement with cross-linking data (see above), was found to be associated with wild-type Tom40p [27]. Together, these results raise the possibility that Tom40p can self-associate only in the mitochondrial membrane [26]. Here we provide evidence that bacterially expressed yeast Tom40p can stably self-associate even in the absence of other Tom components or chaperones. Using purified components, we have also investigated the interaction of a mitochondrial precursor protein with Tom40p and its effect on Tom40p self-assembly.

MATERIALS AND METHODS

Expression of precursor proteins in bacteria and their purification

The genes encoding open reading frames for Tom40p, ΔNTom40p (Tom40p in which the first 93 amino acids have been deleted), pYfh1p [precursor of Yfh1p (yeast frataxin homologue)], Δ_{20}Yfh1p (which lacks the first 20 amino acids of Yfh1p) and mYfh1p (mature Yfh1p, in which the entire 51-amino-acid signal sequence of Yfh1p has been deleted) were amplified by PCR from a yeast genomic library using appropriate primers, such that the resulting products were *Nde*I–(open reading frame)–*Xho*I–*Bam*HI. The PCR products were digested with *Nde*I and *Xho*I for subcloning into pET21b (Novagen), or with *Nde*I and *Bam*HI for subcloning into pET3a (Novagen). The former introduces a His_6 tag at the C-terminus of the protein, whereas the latter does not. The plasmid pET21b/Yfh1p-(1–22)–Protein A [where Yfh1p-(1–22)–Protein A represents the first 22 amino acids of Yfh1p linked to Protein A] has been described previously [28].

BL21 (DE3) Codon Plus cells (Stratagene) carrying different plasmids were cultured in M9 medium supplemented with 0.1 mg/ml ampicillin and 34 μg/ml chloramphenicol. Protein induction, ^{35}S radiolabelling and Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose (Qiagen) chromatography were performed as described [28,29]. Proteins were stored in 50 mM Tris/HCl, pH 8.0, containing 8 M urea at −80 °C. All proteins were centrifuged at 250000 ***g*** (Beckman TLA100 rotor) for 10 min to remove aggregates, if any, prior to use in binding assays. A bacterial extract containing the *Streptomyces lividans* potassium channel with a C-terminal His_6 tag (KscA–His_6) was a gift from Dr Zhe Lu (University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A.).

Binding assay

Tom40p–His_6 or ΔNTom40p–His_6 bound quantitatively to Ni-NTA agarose in the presence of 8 M urea. When these urea-denatured proteins were diluted with a buffer containing 1% Triton X-100 (final urea concentration 4–40 mM), the majority of the proteins remained in the supernatant upon centrifugation. Under these conditions, the efficiency of binding of Tom40p–His_6 or ΔNTom40p–His_6 to Ni-NTA agarose was $\sim$ 50–60%; this could be improved to $>$ 90% by re-addition of high concentrations of urea (4–6 M). When other urea-denatured His_6-tagged proteins were diluted with buffer containing 1% Triton X-100, their binding to Ni-NTA agarose was quantitative even at low urea concentrations. Because many protein–protein interactions may not withstand high urea concentrations, the usual binding assays were performed in the presence of low urea concentrations as follows. Urea-denatured proteins (0.1–10 μg) were added to 1 ml of binding buffer (50 mM Tris/HCl, pH 8.0, 1% Triton X-100, 10% glycerol and 40 mM potassium acetate) and incubated at room temperature for 10 min. Unless indicated otherwise, the final urea and imidazole concentrations in the usual binding assay were kept below 40 mM and 2 mM respectively. In cases where sensitivity to salt was tested, potassium acetate was replaced with NaCl, as indicated in Figure legends. Samples were centrifuged at 15000 ***g*** for 10 min to remove aggregates, if any. The supernatant was transferred to an Eppendorf tube containing 30 μl of a 50% (w/v) Ni-NTA agarose suspension and incubated at room temperature for 90 min with end-over-end mixing. The beads were washed three times with 1 ml of binding buffer, and bound protein complexes were eluted with SDS loading buffer containing 10 mM EDTA. Samples were analysed by SDS/PAGE, followed by Coomassie Blue staining and autoradiography.

Sucrose gradient analysis

Sucrose gradient ultracentrifugation was performed essentially as described previously [30]. Briefly, 3–5 μg of recombinant Tom40p–His_6 was diluted into 200 μl of binding buffer and insoluble material, if any, was removed by centrifugation. The supernatant was loaded on to a 12 ml 5–20% (w/v) linear sucrose gradient and centrifuged at 4 °C for 20 h at 285000 ***g*** (Beckman SW40.Ti rotor). Fractions (0.7 ml) were collected, and the protein was precipitated by the addition of trichloroacetic acid to 10% and analysed by SDS/PAGE followed by Coomassie Blue staining. Protein standards were centrifuged in parallel; these included carbonic anhydrase (3.1 S), BSA (4.4 S), fumarase (8.5 S) and catalase (11.3 S).

RESULTS

Principle of binding assay

Tom40p is the core component of the protein-conducting channel of the mitochondrial outer membrane. We have studied the interactions of *S. cerevisiae* Tom40p with itself and also with mitochondrial precursor proteins. The principle of our assay investigating protein–protein interactions is as follows. One protein contains a His_6 tag at its C-terminus and is unlabelled (e.g. Protein X–His_6), whereas the other component, which may or may not be same as Protein X, does not contain a His_6 tag, but is radiolabelled (e.g. [^{35}S]Protein Y). Protein X–His_6, by virtue of its His_6 tag, binds to Ni-NTA agarose. On the other hand, [^{35}S]Protein Y, lacking a His_6 tag, does not bind to Ni-NTA

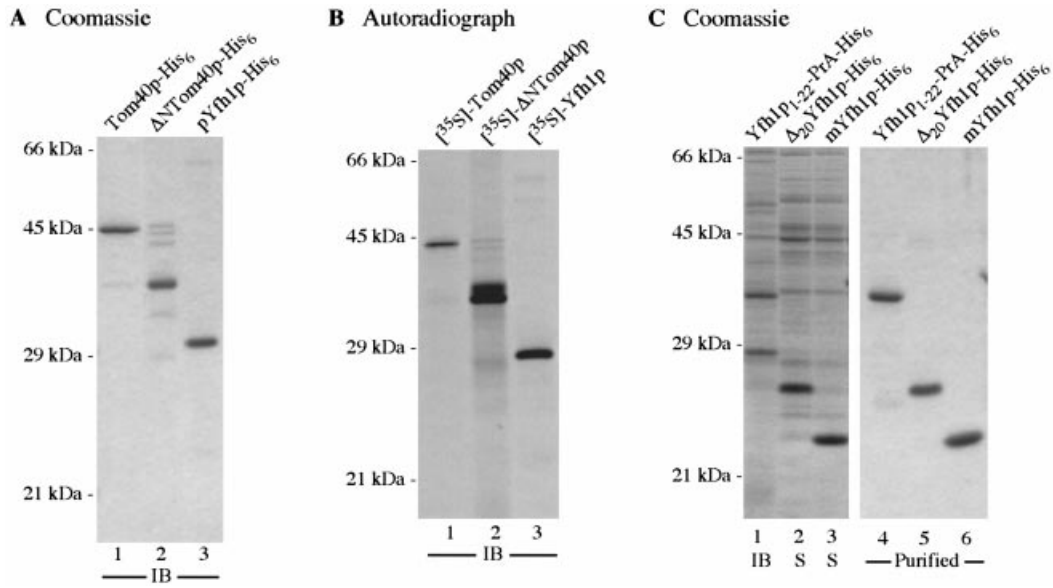

Figure 1 Analysis of recombinant proteins

Proteins with or without a C-terminal His_6 tag were expressed in bacteria in the absence or presence of a mixture of [^{35}S]Met and [^{35}S]Cys. Most of these overexpressed proteins were found to be sequestered in inclusion bodies (IB); only two, Δ_{20}Yfh1p–His_6 and mYfh1p–His_6, were found in soluble fractions (S). Proteins were analysed by SDS/PAGE followed by Coomassie Blue staining for unlabelled proteins (**A** and **C**) or by autoradiography for ^{35}S-labelled proteins (**B**). Chimaeric or N-terminally truncated Yfh1p proteins with His_6 tags were analysed before and after purification ('Purified') on Ni-NTA agarose (**C**). PrA, Protein A. The positions of molecular mass markers are shown on the left of each panel.

agarose. When the two proteins are mixed together, [^{35}S]Protein Y becomes associated with Ni-NTA agarose only through its interaction with Protein X–His_6. The two main advantages of this system are as follows. (i) Tom protein with a C-terminal His_6 tag is functionally active [16]. Likewise, mitochondrial precursor proteins with C-terminal His_6 tags are imported efficiently into isolated mitochondria [28,29,31]. Thus the C-terminal His_6 tags do not interfere with the interaction of the precursor protein's N-terminal signal sequence with components of the mitochondrial import machinery. (ii) The binding of His_6-tagged proteins to Ni-NTA agarose is unaffected by the presence of most non-ionic detergents, high salt or strong denaturants such as 8 M urea [29,31]. Therefore the stability of protein–protein interactions can be examined under stringent conditions.

Expression of proteins in bacteria

Proteins used in the present study were expressed in bacteria with or without a C-terminal His_6 tag. They included: (i) wild-type Tom40p and ΔNTom40p (first 93 amino acids of Tom40p deleted); (ii) wild-type Yfh1p and N-terminally truncated forms of Yfh1p: Δ_{20}Yfh1p (lacking the first 20 amino acids of Yfh1p) and mYfh1p (entire 51-amino-acid signal sequence of Yfh1p deleted); and (iii) a fusion protein, Yfh1p-(1–22)–Protein A (first 22 amino acids of Yfh1p linked to Protein A). Unlabelled or ^{35}S-labelled proteins that were used for binding assays were at least 90% homogeneous, as determined by Coomassie Blue staining (Figures 1A and 1C) or autoradiography (Figure 1B) of SDS/polyacrylamide gels. Tom40p, ΔNTom40p and Yfh1p were found to be sequestered in inclusion bodies which were solubilized in 8 M urea and used directly in the binding assays without prior purification on Ni-NTA agarose (Figures 1A and 1B). However, the inclusion bodies containing Yfh1p-(1–22)–Protein A–His_6 or the soluble fractions containing Δ_{20}Yfh1p–His_6 and mYfh1p–His_6 had multiple contaminating bacterial proteins, and were therefore purified to homogeneity on Ni-NTA agarose prior to their use in binding assays (Figure 1C).

Stable self-association of recombinant Tom40p

Protein folding is often a prerequisite for protein–protein interactions. To investigate Tom40p self-association using urea-denatured components, we took advantage of an established property of the protein. An earlier study demonstrated that, when urea-denatured recombinant Tom40p was diluted in buffer containing a non-ionic detergent, the protein was able to refold efficiently, as determined by CD analysis. Furthermore, when Tom40p was reconstituted into liposomes by removing the detergent, the CD spectrum of liposome-inserted Tom40p was almost identical with that of Tom40p in the detergent [24].

In our binding assay containing a non-ionic detergent (Triton X-100), increasing amounts of [^{35}S]Tom40p (no His_6) were incubated in the absence or presence of a constant amount of Tom40p–His_6. Samples were then subjected to Ni-NTA agarose chromatography, and proteins bound to the resin were analysed by SDS/PAGE followed by Coomassie Blue staining (Figure 2A, upper panel) and autoradiography (Figure 2A, lower panel). In the absence of added Tom40p–His_6, practically no non-specific association of [^{35}S]Tom40p with Ni-NTA agarose was detected. When Tom40p–His_6 was included, the affinity eluate contained not only unlabelled Tom40p–His_6, but also [^{35}S]-Tom40p. The Coomassie Blue staining of bound unlabelled Tom40p–His_6 served as an internal control. While the amount of unlabelled Tom40p–His_6 remained fairly constant, increasing

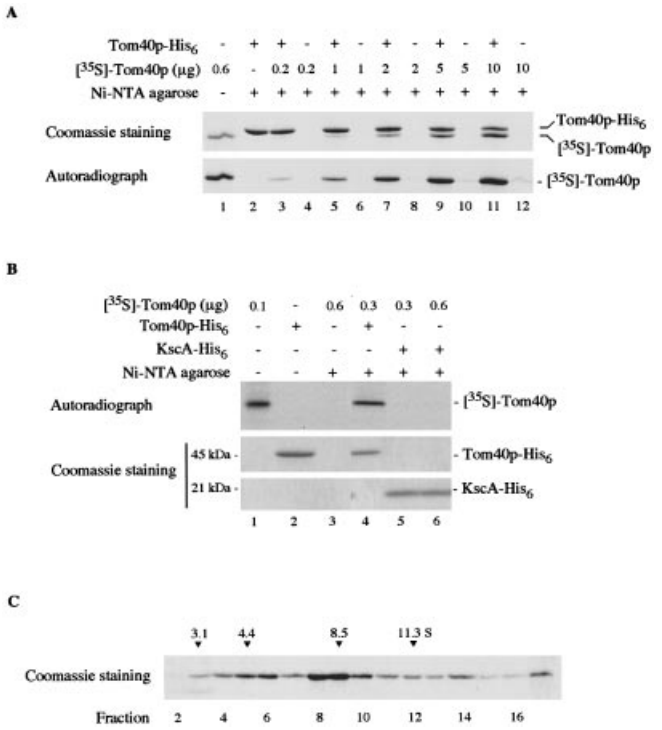

Figure 2 Recombinant Tom40p can stably associate with itself

(**A**) Increasing amounts of [^{35}S]Tom40p were incubated with or without a fixed amount of unlabelled Tom40p–His$_6$ (1 μg). Reaction mixtures were then added to Ni-NTA agarose; proteins bound to the resin were analysed by SDS/PAGE followed by Coomassie Blue staining (upper panel) and autoradiography (lower panel) of the same gel. (**B**) The specificity of [^{35}S]Tom40p binding was tested as described above, except that, where indicated, Tom40p–His$_6$ was replaced with KscA–His$_6$. Note that the Coomassie Blue-stained gel is not continuous; only the regions of interest are shown. (**C**) Purified Tom40p–His$_6$ was centrifuged through a linear 5–20% (w/v) sucrose gradient and fractions were analysed by SDS/PAGE and Coomassie Blue staining. The migration positions of protein standards and their corresponding sedimentation coefficients are indicated.

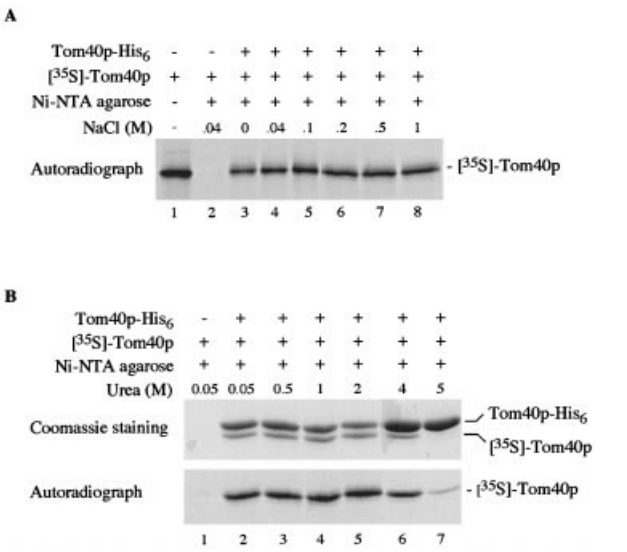

Figure 3 Stability of self-associated Tom40p

(**A**) Binding of [^{35}S]Tom40p to Tom40p–His$_6$ was carried out as in Figure 2(A), except that the assay buffer contained increasing concentrations of NaCl. Samples were analysed by SDS/PAGE followed by autoradiography. (**B**) Binding of [^{35}S]Tom40p to Tom40p–His$_6$ was carried out as in Figure 2(A), but in the presence of increasing concentrations of urea. Samples were analysed by SDS/PAGE followed by Coomassie Blue staining (upper panel) and autoradiography (lower panel) of the same gel.

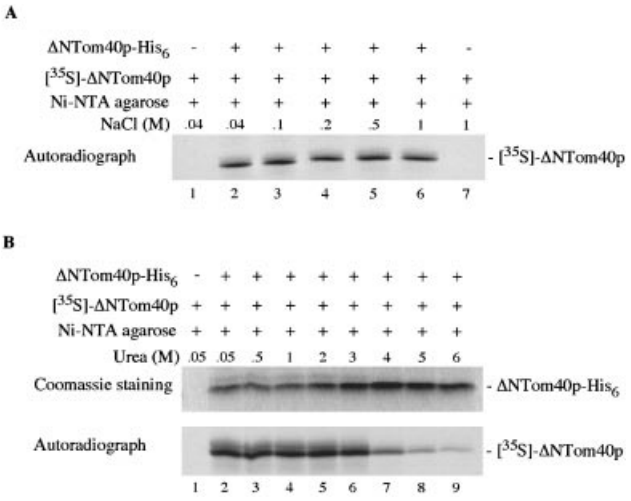

Figure 4 The N-terminal segment of Tom40p is not critical for its self-assembly

(**A**) Binding of [^{35}S]ΔNTom40p to ΔNTom40p–His$_6$ was carried out as in Figure 2(A), except that the assay buffer contained increasing concentrations of NaCl. Samples were analysed by SDS/PAGE followed by autoradiography. (**B**) Binding of [^{35}S]ΔNTom40p to ΔNTom40p–His$_6$ was carried out as in Figure 2(A), but in the presence of increasing concentrations of urea. Samples were analysed by SDS/PAGE followed by Coomassie Blue staining (upper panel) and autoradiography (lower panel) of the same gel.

amounts of [^{35}S]Tom40p were detected in a dose-dependent manner at lower concentrations. These results demonstrate that [^{35}S]Tom40p associates with unlabelled Tom40p–His$_6$, and that this association is essential for [^{35}S]Tom40p to be retained by the Ni-NTA resin. The self-association was efficient; approx. 35% of added [^{35}S]Tom40p was associated with Tom40p–His$_6$, e.g. compare bound [^{35}S]Tom40p when 2 μg of protein was used (Figure 2A, lower panel, lane 7) with a direct load of 0.6 μg (Figure 2A lower panel, lane 1). As expected, the binding of [^{35}S]Tom40p was saturable, and reached a plateau at the highest concentrations tested. An interesting conclusion from these experiments is that recombinant Tom40p can self-associate efficiently in the absence of other Tom components or mitochondrial/cytosolic chaperones.

To rule out the possibility that the self-association of Tom40p was due to non-specific interactions, we used the potassium channel KscA as a control (Figure 2B). KscA is an integral membrane protein consisting of four identical subunits of $\sim$ 15 kDa that interact strongly with each other [32]. [^{35}S]Tom40p was incubated with unlabelled Tom40p–His$_6$ or unlabelled recombinant potassium channel with a C-terminal His$_6$ tag (KscA–His$_6$), and the assay mixtures were subjected to Ni-NTA agarose chromatography. Although both Tom40p–His$_6$ and KscA–His$_6$ were retained efficiently by the Ni-NTA resin, [^{35}S]Tom40p was found to be associated only with the former. No non-specific association of [^{35}S]Tom40p with KscA–His$_6$ was detected. These results suggest that the self-association of Tom40p is mediated by specific protein–protein interactions.

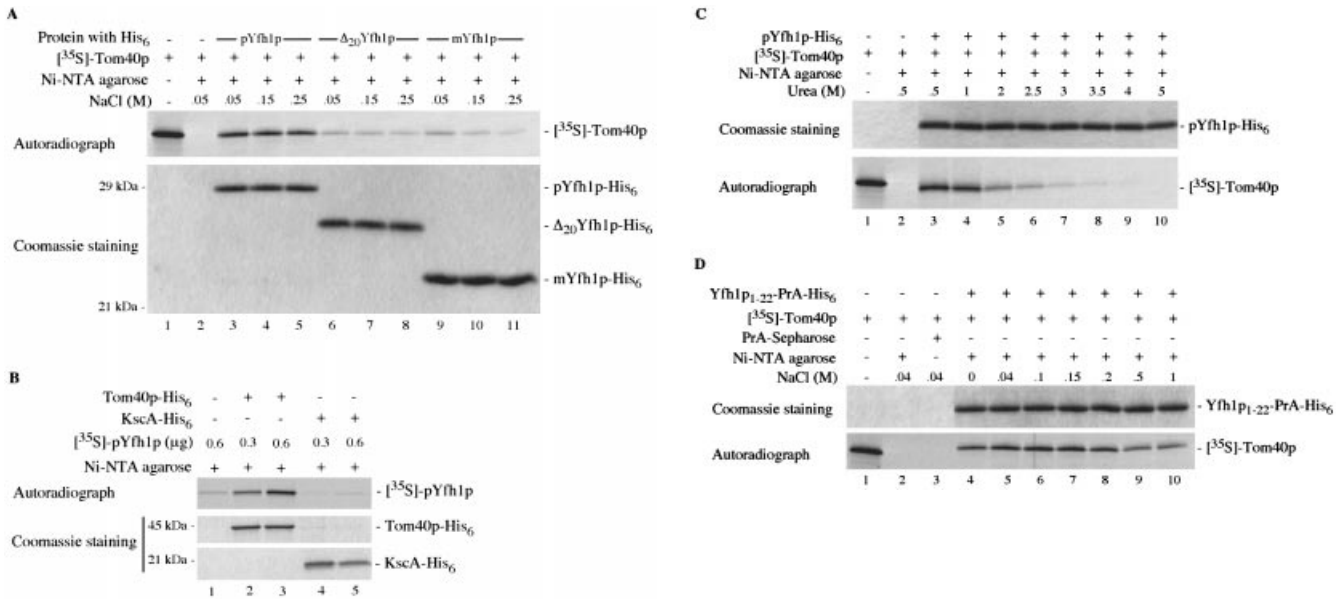

Figure 5 Interaction of a mitochondrial targeting sequence with Tom40p

(**A**) [^{35}S]Tom40p (0.3 μg) was incubated with pYfh1p–His$_6$, Δ_{20}Yfh1p–His$_6$ or mYfh1p–His$_6$ (1 μg each) in binding buffer containing 0.05–0.25 M NaCl. Samples were then added to Ni-NTA agarose, and proteins bound to the resin were analysed by SDS/PAGE followed by Coomassie Blue staining (lower panel) and autoradiography (upper panel) of the same gel. (**B**) Binding of [^{35}S]pYfh1p (0.3 and 0.6 μg) to Tom40p–His$_6$ or KscA–His$_6$ (0.5 μg each) was carried out as described in Figure 2(A). Samples were analysed by SDS/PAGE followed by Coomassie Blue staining (lower panels) and autoradiography (upper panel) of the same gel. Note that the Coomassie Blue-stained gel is not continuous; only the regions of interest are shown. (**C**) [^{35}S]Tom40p (0.3 μg) was incubated with pYfh1p–His$_6$ (1 μg) in the presence of increasing urea concentrations and subjected to Ni-NTA agarose chromatography. Resin-bound proteins were analysed by SDS/PAGE followed by Coomassie Blue staining (upper panel) and autoradiography (lower panel) of the same gel. (**D**) [^{35}S]Tom40p (0.3 μg) was incubated with purified Yfh1p-(1–22)–Protein A–His$_6$ (1 μg) in the presence of increasing salt concentrations and subjected to Ni-NTA agarose chromatography. Proteins bound to the resin were analysed by SDS/PAGE followed by Coomassie Blue staining (upper panel) and autoradiography (lower panel) of the same gel. PrA, Protein A.

To determine the oligomeric state of recombinant Tom40p, we performed sucrose gradient centrifugation experiments. A significant portion of the loaded protein was detected in fractions corresponding to $\sim$ 8.3 S, suggesting a tetrameric form (Figure 2C). Interestingly, the protein was also found in other fractions. In a way, these results are in agreement with earlier observations by others that Tom40p may exist in different oligomeric states (see the Introduction). However, our sucrose density gradient centrifugation experiments cannot rule out the possibility that heterogeneous Tom40p oligomers were generated as a result of subunit dissociation from a higher-order complex (e.g. an octamer) during centrifugation through a gradient of increasing viscosity.

To test the stability of self-associated Tom40p, we performed the binding assay in the presence of increasing salt (Figure 3A) or increasing urea (Figure 3B) concentrations. [^{35}S]Tom40p was able to associate with unlabelled Tom40p–His$_6$ even in the presence of 1 M NaCl. In fact, the association was stimulated slightly with increasing salt concentrations. Urea titrations also revealed a strong Tom40p–Tom40p interaction; binding of [^{35}S]Tom40p to unlabelled Tom40p–His$_6$ was slightly affected by exposure to 4 M urea and was almost completely ($> 90\,\%$) abolished by 5 M urea. Note that the binding of Tom40p–His$_6$ to Ni-NTA agarose was significantly greater at higher urea concentrations. We have observed that the binding of many His$_6$-tagged proteins to Ni-NTA agarose is often enhanced when the proteins are completely denatured by 6–8 M urea [29,31], thereby making the tag available for efficient binding. Results from salt and urea titrations together suggest that Tom40p self-association is unlikely to be mediated by ionic interactions, but rather that it is mediated by a hydrophobic type of interaction.

The N-terminal segment of Tom40p is not essential for self-association

In vitro import studies demonstrated that, when the N-terminal segment of Tom40p was deleted, the protein failed to be assembled into the endogenous Tom complex [26]. We therefore tested the effect of N-terminal truncation of Tom40p on self-association. [^{35}S]ΔNTom40p behaved like the wild-type protein, and was found to be stably associated with unlabelled Tom40p–His$_6$ (results not shown). In fact, stable self-association was observed even when both components were N-terminally truncated (i.e. [^{35}S]ΔNTom40p and unlabelled ΔNTom40p–His$_6$), as determined by sensitivity to salt (Figure 4A) and urea (Figure 4B). Thus the N-terminal segment of Tom40p is unlikely to mediate the self-association of this protein.

Interaction of Tom40p with a mitochondrial precursor protein

Earlier studies have demonstrated that Tom40p can be cross-linked to the signal sequence as well as to the mature portion of a precursor protein during its import into mitochondria [10,18]. To investigate the specificity of the interaction of recombinant Tom40p with mitochondrial precursor proteins, we chose Yfh1p as a model substrate. Yfh1p is the yeast homologue of human frataxin, a protein that is deficient in patients with Friedreich ataxia [33]. Yfh1p is a nuclear-encoded mitochondrial matrix protein, and its import into mitochondria is well characterized. Upon import into mitochondria, the N-terminal 51-amino-acid signal sequence of the Yfh1p precursor protein (pYfh1p) is processed in two steps by matrix processing peptidase. An initial proteolytic cleavage removes the first 20 amino acids, generating

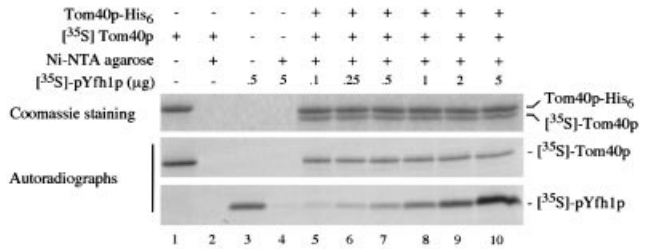

Figure 6 Tom40p self-assembly is unaffected by pYfh1p binding

A saturating amount of [^{35}S]Tom40p (6 μg) was incubated with a limiting amount of unlabelled Tom40p–His$_6$ (0.8 μg) in the presence of Ni-NTA agarose. Resin-bound complexes were then incubated with increasing amounts (0.1–5 μg) of [^{35}S]pYfh1p. Following extensive washing, the bound proteins were analysed by SDS/PAGE followed by Coomassie Blue staining (top panel) and autoradiography (middle panel, [^{35}S]Tom40p; bottom panel, [^{35}S]pYfh1p) of the same gel. At the highest amount tested, [^{35}S]pYfh1p was in $\sim$ 3–5-fold molar excess over total Tom40p present in the assay. Note that the Coomassie Blue staining of [^{35}S]pYfh1p is not shown in the top panel. Likewise, the autoradiograph is not continuous; only the regions of interest are shown, and no bands were detected in other regions of the autoradiograph.

an intermediate fragment (Δ_{20}Yfh1p), and a subsequent cleavage removes an additional 31 amino acids from the N-terminus of the intermediate, generating the final mature form of Yfh1p (mYfh1p). Residues 1–20 serve as a typical matrix targeting signal; residues 21–51 do not contain any targeting information, but ensure the efficient import of Yfh1p [28,31]. This detailed and precise information allowed us to design constructs to study their interactions with recombinant Tom40p.

pYfh1p–His$_6$, Δ_{20}Yfh1p–His$_6$ and mYfh1p–His$_6$ were expressed in bacteria and purified. These unlabelled proteins were added to the assay buffer containing [^{35}S]Tom40p (no His$_6$) and subjected to Ni-NTA agarose chromatography, and proteins bound to the resin were analysed by SDS/PAGE followed by Coomassie Blue staining (Figure 5A, lower panel) and autoradiography (Figure 5A, upper panel) of the same gel. The data show that pYfh1p was able to bind more than 25 % of [^{35}S]Tom40p used in the assay (Figure 5A, upper panel; compare lanes 3–5 with lane 1). On the other hand, Δ_{20}Yfh1p and mYfh1p were very inefficient in their binding to Tom40p. A portion of the Coomassie Blue-stained gel (21–30 kDa region; Figure 5A, lower panel) indicated that, indeed, comparable amounts of all three His$_6$-tagged unlabelled proteins were bound to Ni-NTA agarose. Note that the total amount of [^{35}S]Tom40p used in the assay was low, and was faintly detected by Coomassie Blue staining only when it was loaded directly (Figure 5A, lower panel, lane 1; 40–45 kDa region not visible); no other band was detected in the entire gel (see Figures 1A and 1B). Thus efficient binding to Tom40p requires the presence of the mitochondrial targeting sequence.

To rule out a non-specific interaction between pYfh1p and Tom40p, we performed additional control experiments. First, we tested three non-mitochondrial proteins for binding to Tom40p. No detectable binding was observed with any of these proteins; the data for one are included in Figure 5(D) (lane 3). Secondly, as demonstrated in Figure 5(B), [^{35}S]pYfh1p showed dose-dependent binding to the core component of the import channel (Tom40p–His$_6$), but no binding to the potassium channel (KscA–His$_6$). Thus binding of the signal sequence to Tom40p is quite specific.

Bacterially expressed and urea-denatured pYfh1p [31], like many other mitochondrial precursor proteins [29], can be imported efficiently into isolated mitochondria in the presence of urea concentrations up to 0.5 M. We therefore measured the binding of [^{35}S]Tom40p to unlabelled pYfh1p–His$_6$ in the presence of increasing concentrations of urea. The binding was unaffected up to 1 M urea, and became progressively sensitive at higher concentrations of urea. For example, binding was inhibited by more than 50 % at 2 M urea, and inhibition was almost complete at 3 M urea (Figure 5C, lower panel). As a control, binding of pYfh1p–His$_6$ to Ni-NTA agarose remained unaffected, regardless of the urea concentration (Figure 5C, upper panel). These results show that the binding of the Yfh1p precursor to Tom40p can withstand reasonably high concentrations of urea. Other steps in the mitochondrial import process may be more sensitive.

We have shown previously [28] that the targeting signal of pYfh1p contains necessary and sufficient information for mediating mitochondrial import of a passenger protein (Protein A). To investigate further the interaction of Tom40p specifically with the Yfh1p targeting sequence, we took advantage of a chimaeric protein, Yfh1p-(1–22)–Protein A–His$_6$, in which residues 1–22 of the Yfh1p precursor are linked to Protein A–His$_6$. [^{35}S]Tom40p was incubated with unlabelled Yfh1p-(1–22)–Protein A–His$_6$ in the presence of increasing salt concentrations and then subjected to Ni-NTA agarose chromatography, and proteins bound to the resin were analysed by SDS/PAGE followed by Coomassie Blue staining (Figure 5D, upper panel) and autoradiography (Figure 5D, lower panel). As expected, $\sim$ 25 % of [^{35}S]Tom40p added in the binding assay was bound to the Yfh1p targeting signal (Figure 5D, lower panel; compare lanes 1 and 5). As controls, [^{35}S]Tom40p did not bind non-specifically to Ni-NTA agarose (lane 2) or to Protein A–Sepharose (lane 3). Interestingly, binding of Tom40p to the Yfh1p targeting signal was practically independent of salt concentrations up to 0.2 M, and was inhibited only by $\sim$ 40 % at the highest salt concentration (1 M) tested (Figure 5D, lower panel). Coomassie Blue staining served as an internal control, and demonstrated that the binding of Yfh1p-(1–22)–Protein A–His$_6$ to Ni-NTA agarose was almost quantitative ($\sim$ 90 %), regardless of salt concentration (Figure 5D, upper panel). These results suggest that binding of Tom40p to the mitochondrial targeting signal is not mediated primarily through ionic interactions.

Different domains of Tom40p mediate self-assembly and precursor protein binding

During import, interactions of a precursor protein with the Tom40p import pore may lead to changes in Tom40p self-association as part of the translocation process. To investigate this possibility, we tested whether precursor protein binding would dissociate preformed Tom40p complexes (Figure 6). A limiting amount of Tom40p–His$_6$ was incubated with large excess of [^{35}S]Tom40p (no His$_6$) in order to saturate the binding of the latter to the former (see Figure 2A). The self-assembled complexes were then allowed to bind to the Ni-NTA resin, and unbound Tom40p molecules were removed. The beads were then incubated with increasing concentrations of [^{35}S]pYfh1p (no His$_6$) to test whether binding of Yfh1p would release [^{35}S]Tom40p from the self-assembled complex bound to the beads. Analysis of the samples by autoradiography revealed that [^{35}S]pYfh1p was able to bind to the preformed complex in a dose-dependent manner. However, the binding of Yfh1p did not cause any significant release of [^{35}S]Tom40p from the complex; the quantity of [^{35}S]Tom40p bound remained fairly constant, even in the presence of excess unbound [^{35}S]pYfh1p. Note that, at the highest concentration tested, [^{35}S]pYfh1p was present in $\sim$ 3–5-

fold molar excess over total Tom40p used in the assay. These results suggest that Tom40p contains at least two domains; one mediates its self-assembly, and the other mediates precursor protein binding. Further experiments will be needed to ascertain whether self-assembly is a prerequisite for precursor protein binding.

DISCUSSION

Tom40p is the core component of the protein-conducting channel of the mitochondrial outer membrane. Here we have provided biochemical evidence that (i) purified recombinant Tom40p stably associates with itself; (ii) it interacts preferentially and strongly with the mitochondrial targeting signal; and (iii) its ability to self-associate and to bind precursor protein are not mutually exclusive. Neither self-association nor precursor protein binding requires the presence of other Tom components or chaperones.

Recombinant Tom40p has been analysed previously by biophysical and electrophysiological techniques [24]. It was shown that, when urea-denatured recombinant Tom40p was diluted in buffer containing a non-ionic detergent, it was able to fold properly, as judged by CD analysis indicating a predominantly β sheet structure. Furthermore, when Tom40p was reconstituted by removing the detergent, the CD spectrum of liposome-inserted Tom40p was almost identical with that of Tom40p in non-ionic detergent. More importantly, reconstituted recombinant Tom40p formed a channel [24] that resembled the protein-conducting channel formed by the purified Tom complex containing Tom40p and other Tom components [15,16,25]. Recombinant Tom40p was detected only as a monomer by Blue Native PAGE [24]. In contrast, Tom40p in intact mitochondria or outer-membrane vesicles could be cross-linked to itself [27]. These studies raised two questions: (i) whether Tom40p can oligomerize only in the mitochondrial outer membrane, and (ii) whether single Tom40p molecules or Tom40p oligomers are the functional unit of the outer membrane translocation channel. Here we show that recombinant Tom40p (in the absence of other mitochondrial proteins) was able to self-associate, forming an oligomeric unit. The self-association of recombinant Tom40p was remarkably efficient and stable; it could withstand up to 4 M urea or 1 M salt. These results suggest that self-associated Tom40p subunits, not individual molecules, are the functional unit of the import channel. Note that our binding assay conditions described here are entirely different from those employed for Blue Native PAGE [24,34], and we suspect that the conditions for Blue Native analysis interfered with the self-association of recombinant Tom40p.

It was shown previously that Tom40p lacking the first 60 amino acids (ΔN_{60}Tom40p) was imported into isolated mitochondria, but failed to be assembled into the endogenous pre-existing Tom complex. This could be due to the impaired ability of ΔN_{60}Tom40p to interact stably with endogenous Tom40p and/or other Tom components, e.g. Tom22p or Tom6p [26]. We found that deletion of as many as the first 93 amino acids of Tom40p had no significant effect on its self-association. Thus the failure of ΔN_{60}Tom40p to be properly assembled into the Tom complex may be due to impaired interactions with other partner proteins, Tom22p or Tom6p. The assay described here now offers a direct way of evaluating these interactions using purified recombinant proteins.

Although Tom40p could be cross-linked to the targeting signal as well as to the mature portion of a precursor protein during its import into mitochondria [10,18], our data suggest that the targeting signal, and not the mature portion, mediates an efficient interaction with recombinant Tom40p. This conclusion is based on two observations. First, the binding of pYfh1p (containing the targeting signal) to Tom40p was at least 10 times more efficient than that of Δ_{20}Yfh1p–His_6 or mYfh1p–His_6 (both lacking the targeting signal). Secondly, the binding of a fusion protein, in which the targeting signal of pYfh1p was linked to an unrelated non-mitochondrial protein [Yfh1p-(1–22)–Protein A–His_6], to Tom40p was almost as efficient as that observed for the pYfh1p precursor.

We have demonstrated previously that urea-denatured recombinant Yfh1p, as well as other precursor proteins, are imported efficiently into isolated mitochondria in the absence of added cytosolic chaperones [28,29,31]. In agreement with our earlier reports, we have now demonstrated that urea-denatured recombinant Yfh1p can bind efficiently to purified Tom40p in the absence of cytosolic chaperones or other factor(s). A recent study by others also demonstrated that the binding of recombinant precursor proteins to an isolated Tom complex is independent of chaperones [35]. In view of these results, it would be interesting to determine if recombinant precursor proteins can bind to purified receptors in the absence of cytosolic chaperones.

Unlike the efficient precursor protein binding to recombinant Tom40p presented here, binding of precursors to recombinant import receptors (Tom20p, Tom22p and Tom70p) was found previously to be less efficient ($< 5\%$) [21]. However, different precursor proteins were used in the previous study, and a quantitative comparison can only be made using identical precuror proteins and performing binding assays under identical conditions. Furthermore, only the cytosolic domains of the import receptors were used for the earlier binding studies, and it is possible that recombinant full-length receptors would be more efficient in precursor binding than the corresponding truncated proteins. Alternatively, binding of precursors to Tom40p may indeed occur with higher affinity compared with binding to receptors, and this may be mechanistically important for the transfer of precursor proteins from the receptors to the core component of the import channel as a prerequisite for translocation to occur. The 'acid chain hypothesis' proposes a similar mechanism in which protein translocation across the outer membrane is driven by binding of the basic targeting signal to acidic receptors of increasing avidity [22,36]. The binding of the targeting signal to Tom40p, however, is unlikely to be mediated mainly by ionic interactions (see below).

The salt-sensitivity of precursor binding to various Tom components has been studied extensively. These studies have led to an alternative '*cis*-site/*trans*-site' model to explain what drives the translocation of precursor proteins across the mitochondrial outer membrane [5,8,37]. The Tom20p–Tom22p heterodimeric receptor complex probably forms a presequence recognition site, termed the *cis* site. Translocation of the precursor protein bound to receptors is initiated by its interaction with a *trans* binding site on the intermembrane space side of the outer membrane. Co-immunoprecipitation and cross-linking studies by Rapaport et al. [18] suggested that Tom40p may be involved in *trans* binding. The *cis* and *trans* bindings are distinguished by their sensitivity to salt; the former is completely abolished by 50–120 mM salt, while the latter remains unaffected [5,8,18,38,39]. Using purified components, we have demonstrated directly that the binding of a mitochondrial targeting signal to Tom40p is quite salt-resistant; it was only partially sensitive to 1 M salt. These results now make it a distinct possibility that Tom40p is a major component of the *trans* precursor binding site of the mitochondrial outer membrane. Whether or not Tom40p alone constitutes the *trans* binding site remains to be determined.

Tom40p subunits were not dissociated as a result of precursor binding, even when excess unbound precursor protein was present in our assay. Thus Tom40p contains at least two different domains: one mediating self-assembly and the other mediating precursor binding. An interesting implication of this finding is that, on the one hand, Tom40p forms a protein-conducting channel through which precursor proteins are translocated, whereas on the other hand Tom40p subunits are not dissociated as a prerequisite for translocation to occur. Tom40p subunits may somehow re-organize themselves in order to accommodate the translocating polypeptide chain, and more than one self-association domain may exist to allow such re-organization during the translocation process. This notion is in agreement with a cross-linking study using intact mitochondria. Rapaport et al. [27] tested the influence of a precursor protein bound to the *trans* side of the mitochondrial outer membrane on the cross-linking of Tom40p. The formation of Tom40p dimers was not influenced by precursor binding when a cross-linker containing a long spacer arm (20 Å) was used. However, a decreased amount of Tom40p dimer was noted in the presence of a cross-linker containing a short spacer arm (7.6 Å). The fact that the extent of Tom40p–Tom40p cross-linking was dependent on cross-linkers that differed in the length of their spacer arms also suggests structural re-organization of Tom40p as a result of precursor binding.

Using Tom40p mutants, the method presented here may allow the identification of domains that mediate self-association or precursor protein binding, and may also reveal whether self-assembly is a prerequisite for precursor binding. The method can be extended for studying other components of the mitochondrial import machinery; it provides a general applicability for protein–protein interactions: self, pairwise or groups of proteins. Interestingly, it has been estimated that as many as 1000 proteins could reside in mitochondria. So far, only about half of these proteins have been identified (MITOP database; [40]). Because translocation of most, if not all, precursor proteins into or across the mitochondrial outer membrane is initiated through interactions with the Tom complex, the Tom40p binding assay described here should prove useful for identifying new mitochondrial proteins.

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