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NMR analyses on the interactions of the yeast Tim50 C-terminal region with the presequence and Tim50 core domain

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ABSTRACT

The mitochondrial targeting signal in the presequence of mitochondrial precursor proteins is recognized by Tom20 and subsequently by Tim50 in mitochondria. Yeast Tim50 contains two presequence binding sites in the conserved core domain and in the fungi-specific C-terminal presequence binding domain (PBD). We report the NMR analyses on interactions of a shorter variant of PBD (sPBD), a shorter variant of PBD, with presequences. The presequence is recognized by sPBD in a similar manner to Tom20. sPBD can also bind to the core domain of Tim50 through the presequence binding region, which could promote transfer of the presequence from sPBD to the core domain in Tim50.

Structured summary of protein interactions:
Tim50 sPBD and **Tim50core** bind by nuclear magnetic resonance (View interaction)
pSu9N and **Tim50 sPBD** bind by nuclear magnetic resonance (1, 2, 3, 4, 5)
pSu9N and **dTim20** bind by nuclear magnetic resonance (View interaction)

1. Introduction

Most mitochondrial proteins are encoded by nuclear genes, synthesized in the cytosol as a precursor protein and imported into mitochondria. Protein import and subsequent sorting to the outer membrane, intermembrane space (IMS), inner membrane, and matrix are mediated by the translocator complexes in the outer and inner membranes [1–3]. Precursors to most matrix proteins and some inner membrane proteins contain an N-terminal cleavable presequence, which contains information of mitochondrial targeting. The targeting signal in a presequence is generally characterized by its ability to form a positively charged amphipathic α-helical structure, and is recognized by Tom20 and Tom22, receptor subunits of the outer membrane translocator TOM40 complex [4]. The presequence then pass through the import channel of Tom40 to reach the presequence binding site on the IMS side of the TOM40 complex. After crossing the outer membrane, the presequence is passed onto the inner membrane translocator for presequence-containing proteins, the TIM23 complex. Accumulated evidence suggests that the presequence is received by Tim50 of the TIM23 complex and transferred to the import channel of Tim23 in a manner dependent on the membrane potential ($\Delta\Psi$) across the inner membrane [1–3].

Tim50 plays a key role in the link of the translocation across the outer and inner membranes. Yeast Tim50 contains a N-terminal cleavable presequence (residues 1–43), a small matrix domain (residues 44–112), a transmembrane segment (residues 113–132) for anchoring to the inner membrane, and a large C-terminal domain exposed to the IMS (Tim50IMS: residues 133–476) [5–7]. Tim50IMS contains a well-conserved domain (residues 159–362) followed by the C-terminal tail that is conserved only among fungal species (residues 363–476). A trypsin resistant core domain (IMS-core: residues 164–361) in the conserved IMS domain was subjected to crystallization, and its X-ray structure was reported [8]. The determined X-ray structure of residues 176–361 consists of five α-helices and nine β-strands, and a negatively charged groove near the protruding β-hairpin was proposed to bind to a positively charged presequence [8]. Interestingly, the presence of a presequence binding site was reported for not only the conserved core domain (residues 164–361) but also the C-terminal "presequence binding" domain (PBD: residues 395–476) in yeast Tim50 [9,10].

Abbreviations: IMS, intermembrane space; PBD, presequence binding domain; sPBD, a shorter variant of PBD; Tim50core, Tim50 core domain; pSu9N, the N-terminal half of the presequence of subunit 9 of F_o-ATPase; pHsp60, the presequence of mitochondrial Hsp60

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In the present study, we analyzed the NMR spectra of the minimal variant of PBD of yeast Tim50 (sPBD; residues 400–450) to characterize its binding to presequence peptides. We demonstrate that the N-terminal part of sPBD is important for presequence binding. Furthermore, we found that sPBD can bind to the minimal core domain of Tim50 (Tim50core: residues 171–362) and this binding could promote transfer of the presequence from sPBD to the core domain of Tim50.

2. Materials and methods

2.1. Plasmids, yeast strains, and yeast growth condition

Construction of plasmids and yeast strains, and growth conditions are described in Supplementary material. Point mutations were introduced into the gene for sPBD by the QuikChange site-directed mutagenesis.

2.2. Preparation of yeast Tim50 sPBD variants, Tim50core, and presequence peptides

The *Escherichia coli* strain Rosetta (DE3) was used for expression of sPBD from the pET15b plasmids by 0.5 mM isopropyl-β-D-thiogalactopyranoside for overnight at 16 °C. Cells were harvested and disrupted by sonication in 20 mM Tris–HCl (pH 7.4), 300 mM NaCl. sPBD was purified from cell lysates by nickel-nitrilotriacetic acid-agarose (Qiagen) affinity chromatography followed by gel filtration chromatography with a Superdex-75 10/300 GL column (GE Healthcare). The hexahistidine-tag of purified sPBD was removed by digestion with thrombin at 23 °C for 16 h. For preparation of uniformly ^{15}N-labeled or [^{15}N, ^{13}C]-labeled sPBD, cells were grown in M9 minimal medium containing $^{15}NH_4Cl$ (1.0 g/L) and/or [U-^{13}C]-glucose (2.0 g/L). Purification of the non-labeled or uniformly labeled mutant sPBD proteins was performed by essentially the same procedure for wild-type sPBD except for omitting the thrombin digestion of [^{15}N, ^{13}C]-labeled sPBD. Preparation of Tim50core is described in Supplementary material. The non-labeled or uniformly labeled pSu9N peptide was prepared as described previously [11]. pHsp60 and pSu9NQ were purchased from CS Bio (Shanghai) Ltd.

2.3. NMR and CD measurements

NMR spectra of 0.1 mM uniformly [^{15}N, ^{13}C]-labeled sPBD in 20 mM KPi, pH 6.7, 50 mM KCl, D_2O/H_2O (7/93) were recorded on a Bruker AVANCE 600 MHz spectrometer equipped with a TCI cryogenic probe at a sample temperature of 25 °C by triple resonance experiments including HNCANNH measurements for sequential backbone resonance assignments. All spectra were processed with TopSpin 1.3 (Bruker BioSpin) and analyzed with Sparky 3.114 (http://www.cgl.ucsf.edu/home/sparky/). NMR titrations of uniformly ^{15}N-labeled sPBD with presequence peptides,

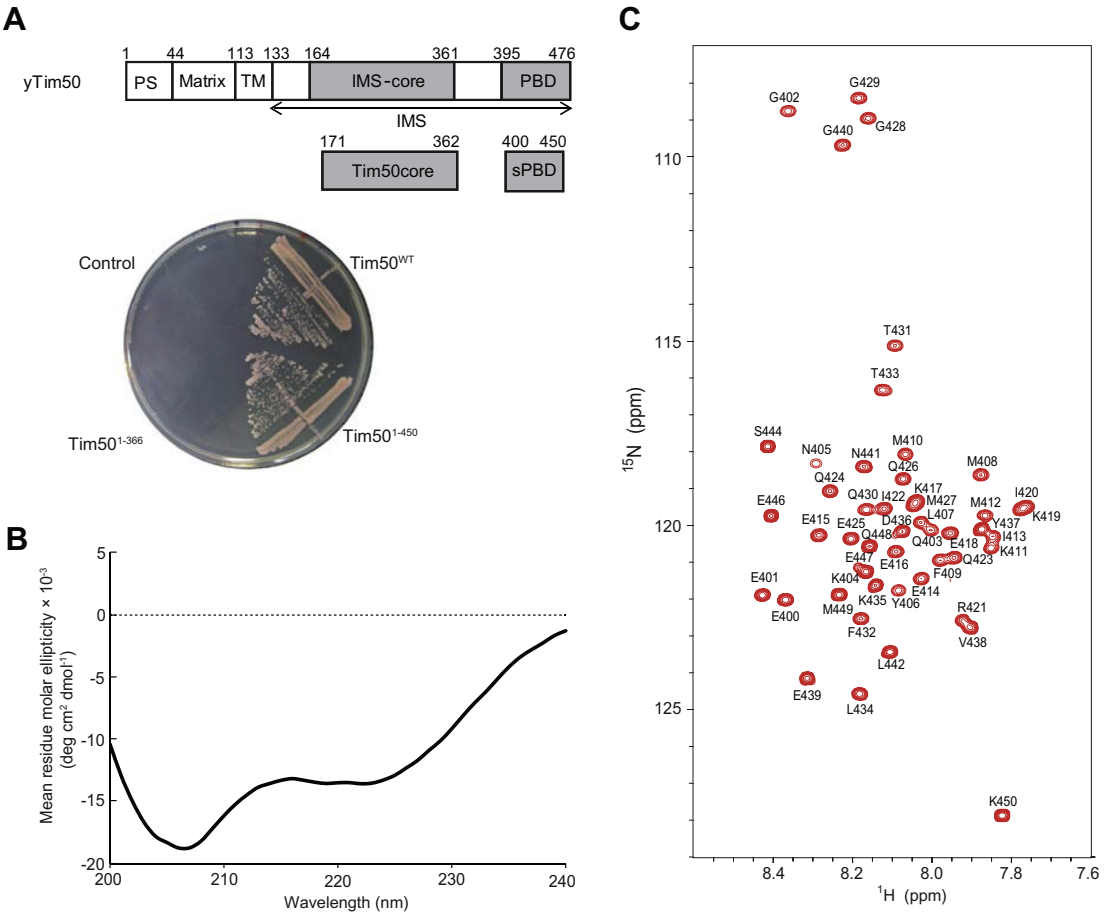

Fig. 1. NMR and CD spectra of sPBD. (A) Upper and central panels: schematic representation of yeast Tim50 (yTim50) and its segments. PS, presequence; TM, transmembrane segment; IMS, the IMS domain. Lower panel: A *TRP1* single-copy (pRS314) plasmid harboring the gene for a Tim50 truncation mutant (Tim50$^{1–366}$ or Tim50$^{1–450}$) or wild-type Tim50 (Tim50^{WT}), or empty vector (Control) was introduced into the same haploid strain (Δtim50/pRS316-Tim50), whose chromosomal disruption was complemented with a *URA3* plasmid harboring the *TIM50* gene, and the resultant strains were grown on 5-fluoroorotic acid-containing plates at 30 °C. (B) A CD spectrum of 10 μM sPBD. (C) [^{1}H, ^{15}N]HSQC spectrum of 0.1 mM sPBD with assignments for backbone amide resonances at 25 °C.