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Interaction of divalent metal ions with human translocase of inner membrane of mitochondria Tim50

Yongqiang Zhang [a,b], Honghua Deng [a], Xinzheng Zhang [a], Shu Jie Li [a,*]

[a] School of Physics Science, Department of Biophysics, Nankai University, Tianjin 300071, PR China
[b] Basic Department, Bengbu Automobile Non-Commissioned Officer Academy of the People's Liberation Army, Bengbu 233011, PR China

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ABSTRACT

The preprotein translocase of the inner membrane of mitochondria (TIM23 complex) is the main entry gate for proteins of the matrix and the inner membrane. Tim50 is a major receptor for transporting the precursor protein across the mitochondrial inner membrane in TIM23 complex. However, the interaction of divalent metal ions with Tim50 and the contribution in the interaction of presequence peptide with Tim50 are still unknown. Herein, we investigated the interaction of divalent metal ions with the intermembrane space domain of Tim50 ($Tim50_{IMS}$) and the interaction of presequence peptides with $Tim50_{IMS}$ in presence of Ca^{2+} ion by fluorescence spectroscopy *in vitro*. The static fluorescence quenching indicates the existence of strong binding between divalent metal ions and $Tim50_{IMS}$. The order of the binding strength is Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, and Co^{2+} (from strong to weak). Moreover, the interaction of presequence peptides with $Tim50_{IMS}$ is weakened in presence of Ca^{2+} ion, which implicates that Ca^{2+} ion may play an important role in the protein import by TIM23 complex.

1. Introduction

The vast majority of mitochondrial proteins are synthesized as precursor proteins on cytosolic ribosomes, subsequently imported into mitochondria [1–3]. The importing process is completed through the membrane-protein complexes called translocators in the outer and inner membranes and soluble factors in the cytosol, intermembrane space (IMS), and matrix [4]. TIM23 complex which is in the inner membrane mediates the translocation of mitochondrial precursor proteins with an N-terminal cleavable presequence across and insertion into the inner membrane. It contains ten subunits, Tim50, Tim23, Tim17, Tim44, Tim14, Tim16, Tim21, Pam17, mtHsp70 and Mge1, and the core complex comprises the three subunits Tim23, Tim17, and Tim50 [5].

Tim50, which is the first component to receive the translocating precursor protein from the TOM40 complex at the inner membrane [6], recognizes the presequence part of the precursors [7]. And the intermembrane space domain of Tim50 ($Tim50_{IMS}$) induces the Tim23 channel to close, while presequences overcome this effect and activate the channel for translocation [8]. The presequence is contained within precursor proteins, often in the form of an N-terminal presequence, which typically consists of 15–40 amino-acid residues, with an abundance of positively charged residues, and tends to form an amphiphilic helical conformation [9]. In our previous work, the structural properties of the intermembrane space domain of human Tim23 ($Tim23_{IMS}$) were characterized and the results showed that the protein has a limited secondary structure and a not-well defined tertiary packing [10]. Qian et al. [11] recently reported the crystal structure of the intermembrane space domain of yeast Tim50 that forms a monomer and consists of five α-helices and nine β-strands. A β-hairpin protruding out of the Tim50 molecular surface represents the largest conserved area on the Tim50. And a large groove, which contains several exposed negatively charged residues, may be ideally suited as a binding site for positively charged presequences/preproteins. In previous work, by using fluorescence and micro-Raman spectroscopy, we found that the interaction between human $Tim50_{IMS}$ and presequence peptides is mainly the electrostatic interaction under physiologically relevant conditions [12]. Though the interaction of Tim50 with presequence has been intensively studied, the details of the interaction are still unclear.

Human Tim50, which spans the inner membrane with a single transmembrane segment (66–88 residues) and exposes a large hydrophilic domain in the intermembrane space (89–353 residues), is composed of 353 amino acids [13]. To fully understand the biological functions of TIM23 complex at molecular basis, detailed information about the interaction between Tim50 and presequence is necessary. In present study, the interaction of divalent metal ions with $Tim50_{IMS}$ was first found and the contribution of Ca^{2+} ion in the interaction of presequence peptides with $Tim50_{IMS}$ was further investigated by fluorescence spectroscopy. Our results showed that, the binding of divalent metal ions with $Tim50_{IMS}$ is strong, and Ca^{2+} ion exhibits the strongest binding ability among

* Corresponding author. Fax: +86 22 2350 6973.
E-mail address: shujieli@nankai.edu.cn (S.J. Li).

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all the divalent metal ions, which tends to weaken the interaction of presequence peptides with $Tim50_{IMS}$.

2. Materials and methods

2.1. Protein expression and purification

The expression and purification of the protein were carried out as described in our previous work [14]. Briefly, *Escherichia coli* strain BL21 (DE3) transformed with pIMS-$Tim50_{IMS}$ was grown to an absorbance of 1.0 at 600 nm (A_{600}) in LB medium in the presence of 100 μg ml^{-1} ampicillin at 21 °C, and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 14 °C for 24 h. The protein was purified by a Glutathione-Sepharose 4B (GE Healthcare) column, an ion exchange HiTrap Q FF column (GE Healthcare), and a Superdex 75 16/60 gel filtration column (GE Healthcare). The concentration of the protein was determined by extinction coefficient 38,515 M^{-1} cm^{-1} [15].

2.2. Presequence peptide

pCoxIV (MLATRVFSLVGKRAISTSVCVR) represents the presequence of human cytochrome c oxidase subunit IV [16]. pHsp60 (MLRLPTVFRQMRPVSRVLAPHLTRA) represents the presequence of human heat shock protein 60 [17]. pALDH (MLRAAARFGPRLGRRLL) represents the presequence of human aldehyde dehydrogenase [18]. pCoxIV, pHsp60, and pALDH were synthesized by Ketai Co. (Shanghai, China)

2.3. Fluorescence quenching

Fluorescent quenching experiments were carried out on an Edinburgh fluorescence spectrometer (NIR 301/2) at 20 °C. Titration was performed by adding 320 μM $CaCl_2$ to a cuvette containing 2 ml solution of 1.6 μM $Tim50_{IMS}$ and the volume of added $CaCl_2$ never exceeded 5% of the total volume. The mixture was incubated for 5 min after each adding of $CaCl_2$. The same measurements were repeated with different divalent metal ions, such as $MgCl_2$, $CoCl_2$, $MnCl_2$, and $CuSO_4$. Another titration was performed by adding 320 μM pCoxIV to a cuvette containing 2 ml solution of 1.6 μM $Tim50_{IMS}$ and 8 μM $CaCl_2$. The volume of added pCoxIV never exceeded 5% of the total volume and the mixture was incubated for 5 min after each adding of pCoxIV. The same measurements were repeated with different presequence peptides, such as pALDH, and pHsp60. All the titrations were performed in buffer containing 10 mM phosphate buffer, pH 7.4, and 0.15 M NaCl. The excitation wavelength was set at 295 nm with a slit width of 3 nm, and the fluorescence emission spectra were scanned from 300 to 500 nm with a slit width of 1 nm. The spectra of samples were corrected by subtracting the corresponding spectra of buffers in the absence of protein.

Fluorescence quenching data were fitted to Stern–Volmer equation and the static quenching equation. Stern–Volmer equation [19] is

$$F_0/F = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] \quad (1)$$

and the static quenching equation is:

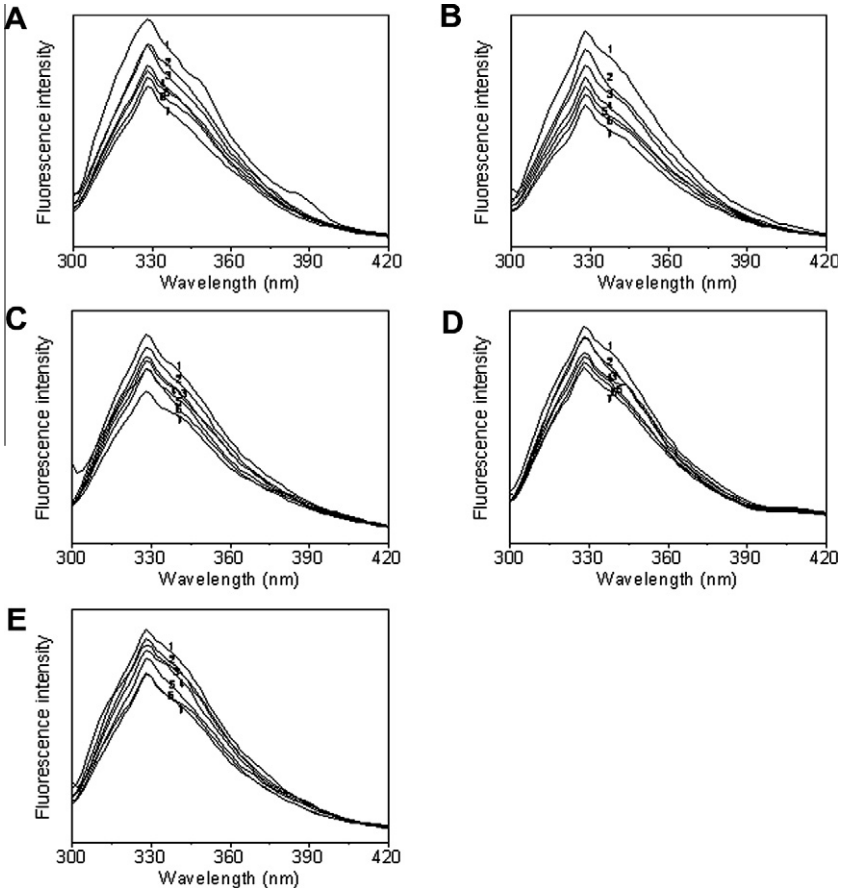

Fig. 1. The fluorescence emission spectra of $Tim50_{IMS}$ quenched by divalent metal ions. (A) Ca^{2+}; (B) Mg^{2+}; (C) Cu^{2+}; (D) Mn^{2+}; (E) Co^{2+}. 1, 0 M divalent metal ions; 2, 0.8 μM divalent metal ions; 3, 1.6 μM divalent metal ions; 4, 2.4 μM divalent metal ions; 5, 3.2 μM divalent metal ions; 6, 4.0 μM divalent metal ions; 7, 4.8 μM divalent metal ions. The concentration of $Tim50_{IMS}$ was 0.05 mg ml^{-1} (1.6 μM).

$$1/(F_0 - F) = 1/F_0 + K_D/(F_0[Q]) \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively. k_q is the quenching rate constant of the biomolecule; τ_0 is the average lifetime of molecule without quencher; $[Q]$ is the free concentration of the quencher; K_{SV} is the dynamic quenching constant; and K_D is the dissociation constant.

3. Results

3.1. Divalent metal ions interact with $Tim50_{IMS}$

To estimate the interaction between divalent metal ions and $Tim50_{IMS}$, we performed the fluorescent quenching experiment. The fluorescence emission spectra of $Tim50_{IMS}$ upon addition of divalent metal ions were shown in Fig. 1A–E. K_{SV} were obtained from the Stern–Volmer plots in Fig. 2A–E by using the Eq. (1). K_{SV} is equal to $k_q\tau_0$ and the fluorescence lifetime of the biopolymer τ_0 is 10^{-8} s [20], so the quenching constant k_q can be calculated (Table 1). However, the maximum scatter collision quenching constant of various quenchers with the biopolymer is 2.0×10^{10} l mol^{-1} s^{-1} [21], and the rate constants of protein quenching procedure initiated by divalent metal ions were much greater than the k_q of the scatter procedure, so the quenching should be initiated by the formation of complex rather than the dynamic collision, and the Eq. (2) should be used. As shown in Fig. 3A–E, the dissociation constants (K_D) estimated from the slopes (Table 1) suggested that the binding of divalent metal ions with $Tim50_{IMS}$ is strong and Ca^{2+} ion exhibits the strongest binding ability among all the divalent metal ions. In summary, the results in Table 1 showed that the fluorescent quenching should result from the strong binding of divalent metal ions with $Tim50_{IMS}$, rather than the collisional encounters between divalent metal ions and $Tim50_{IMS}$. What is more, the order of the binding strength between divalent metal ions and $Tim50_{IMS}$ from strong to weak is Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, and Co^{2+}.

Table 1
Dissociation constants of divalent metal ions with $Tim50_{IMS}$ measured by fluorescence quenching.

	K_{SV} (l mol^{-1})	k_q (l mol^{-1} s^{-1})	K_D (μM)
$Tim50_{IMS}$-Ca	7.13×10^4	7.13×10^{12}	2.09 ± 0.01
$Tim50_{IMS}$-Mg	1.03×10^5	1.03×10^{13}	5.16 ± 0.03
$Tim50_{IMS}$-Cu	5.36×10^4	5.36×10^{12}	5.64 ± 0.17
$Tim50_{IMS}$-Mn	4.45×10^4	4.45×10^{12}	6.46 ± 0.02
$Tim50_{IMS}$-Co	5.52×10^4	5.52×10^{12}	15.26 ± 0.23

3.2. Ca^{2+} tends to weaken the interaction of $Tim50_{IMS}$ with presequence peptide

To further determine the contribution of Ca^{2+} ion in the interaction of presequence peptides with $Tim50_{IMS}$, we used the static quenching equation. Dissociation constants of presequence peptides with $Tim50_{IMS}$ obtained from Fig. 4A–C were showed in Table 2. The results showed that $Tim50_{IMS}$ still interacts with

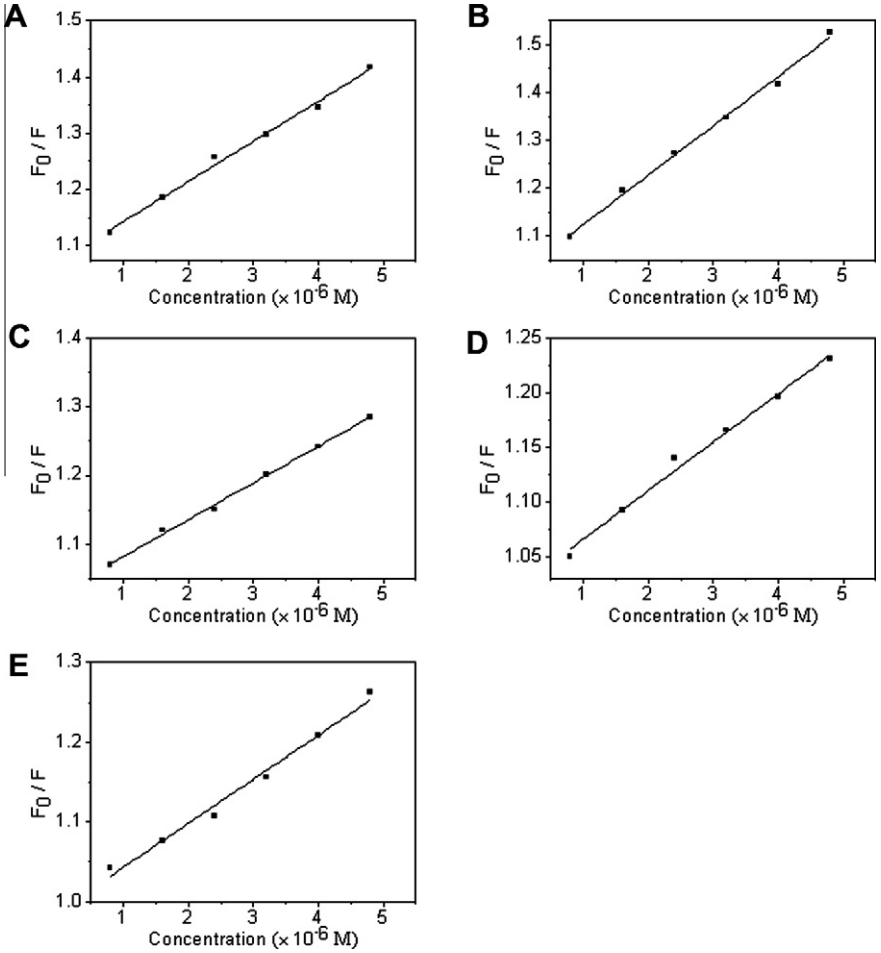

Fig. 2. The Stern–Volmer plots for the fluorescence quenching of $Tim50_{IMS}$ by divalent metal ions at 20 °C. The K_{SV} values were obtained from the slope of a linear dependence of F_0/F versus the free concentration of divalent metal ions. (A) Ca^{2+}; (B) Mg^{2+}; (C) Cu^{2+}; (D) Mn^{2+}; (E) Co^{2+}. The concentration of $Tim50_{IMS}$ was 0.05 mg ml^{-1} (1.6 μM).

presequence peptides in the presence of Ca^{2+} ion but the interaction is weaker than that in the absence of Ca^{2+} ion. Therefore, it indicated that the interaction of presequence peptides with $Tim50_{IMS}$ is weakened in presence of Ca^{2+} ion.

4. Discussion

As a receptor, Tim50 is crucial for transporting the precursor protein across the mitochondrial inner membrane in TIM23 complex, and the dysfunction of Tim50 induces cell apoptosis and several diseases. Though the interaction of Tim50 with presequence has been intensively studied, the interaction details between Tim50 and presequence peptide are still unknown. The fluorescence spectroscopy has been widely used to study conformational changes of proteins. A valuable feature of intrinsic protein fluorescence is the high sensitivity of tryptophan in response to the change of its local environment, which can be used to frequently observe the changes in the emission spectra of tryptophan in response to protein conformational transitions, subunit association, substrate binding, or denaturation [22–23]. But the reports about using this method to investigate the interaction of proteins with peptides are rare. In our manuscript, we have first found the strong binding of divalent metal ions with $Tim50_{IMS}$ by static fluorescence quenching and further investigated the contribution of Ca^{2+} ion in the interaction of presequence peptides with $Tim50_{IMS}$. The results revealed that the binding of divalent metal ions with $Tim50_{IMS}$ is strong and Ca^{2+} ion exhibits the strongest binding ability among all the divalent metal ions, which tends to weaken the interaction of presequence peptides with $Tim50_{IMS}$. The results implicated that Ca^{2+} ion may play an important role in the protein import by TIM23 complex.

TIM23 complex is the main entry gate for proteins of the matrix and the inner membrane. It has been intensively studied on the functions of yeast TIM23 complex [24–25]. Tim50, as a receptor, is essential for precursor proteins to be imported into the mitochondrial matrix [26]. It is known that precursors which carry an N-terminal, positively charged matrix-targeting signal (a presequence) are directed to the TIM23 complex [7], and presequences selectively override the Tim50-induced closure and activate the TIM23 channel [8], in which Tim50 promotes oligomerization and voltage-dependent closure of the channel. Many studies showed that the interaction between presequence peptide and Tim50 is hydrophobic interaction [17,18,27]. In our previous work [12], we found that there are both electrostatic interaction and hydrophobic interaction between human $Tim50_{IMS}$ and presequence peptide, and the interaction is mainly electrostatic interaction under physiologically relevant conditions. Qian et al. [11] reported that Tim50 contains a large groove as putative binding site for presequences and the groove contains several exposed negatively charged residues, which may be ideally suited as a binding site for positively charged presequences/preproteins. Many previous studies showed that the electrostatic interaction may be important for the import of the precursor proteins.

Mitochondria depend on the Ca^{2+} signal for their own functionality, in particular for their capacity to produce ATP. Under physiological conditions, mitochondrial Ca^{2+} uptake occurs by a uniport mechanism driven electrophoretically by the mitochondrial

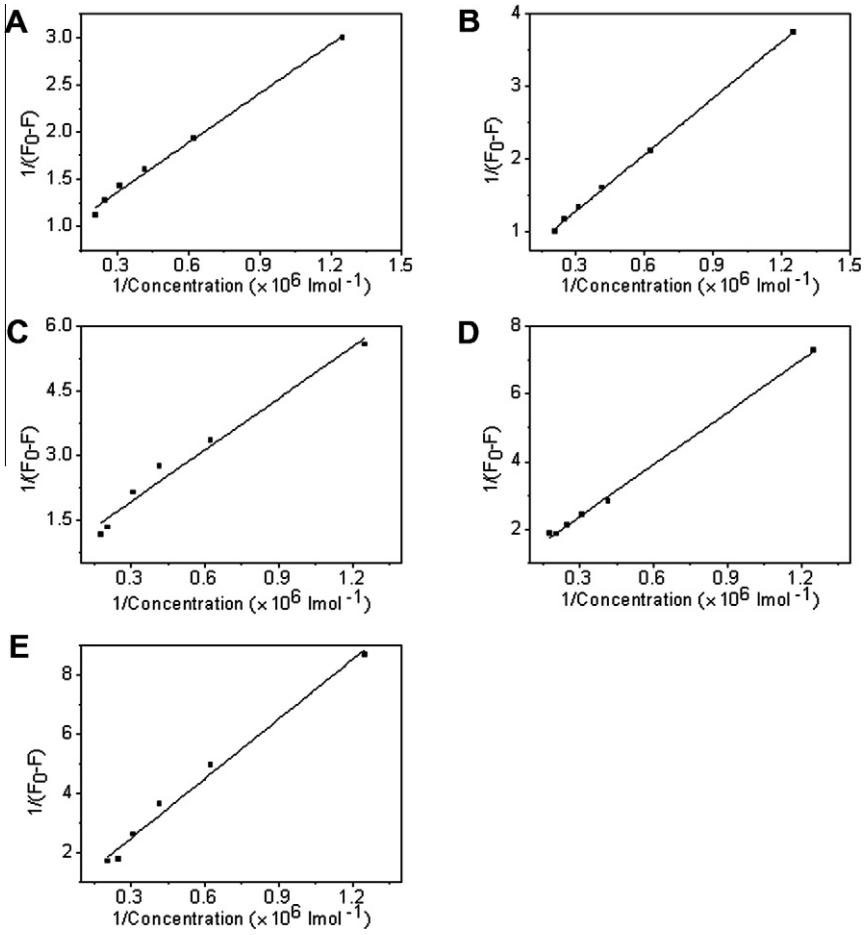

Fig. 3. The static quenching plots for the fluorescence quenching of $Tim50_{IMS}$ by divalent metal ions at 20 °C. (A) Ca^{2+}; (B) Mg^{2+}; (C) Cu^{2+}; (D) Mn^{2+}; (E) Co^{2+}. The concentration of $Tim50_{IMS}$ was 0.05 mg l^{-1} (1.6 μM).

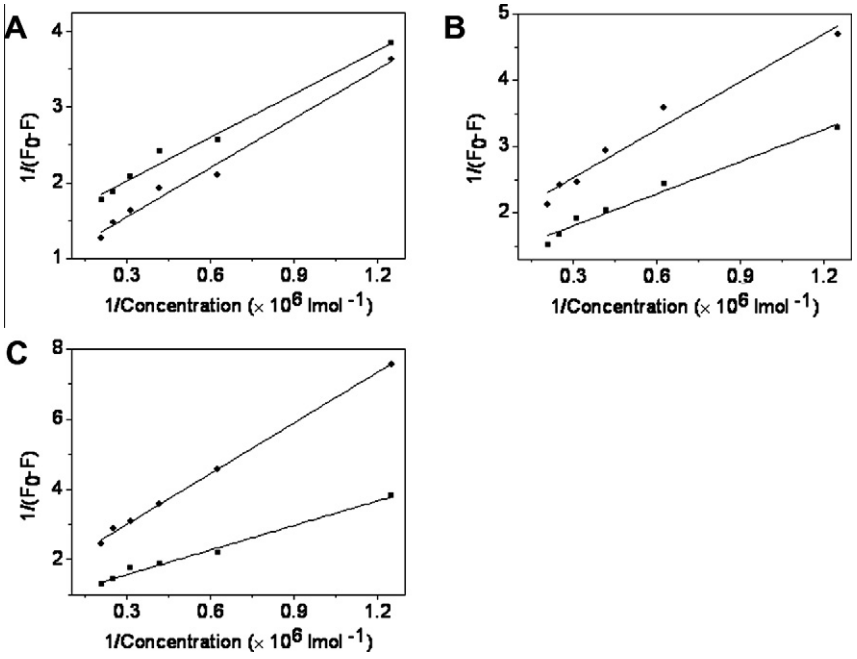

Fig. 4. (A) The static quenching plots for the fluorescence quenching of $Tim50_{IMS}$ by pCoxIV. (B) The static quenching plots for the fluorescence quenching of $Tim50_{IMS}$ by pALDH. (C) The static quenching plots for the fluorescence quenching of $Tim50_{IMS}$ by pHsp60. (■) 0 M $CaCl_2$; (●) 8 μM $CaCl_2$. The concentration of $Tim50_{IMS}$ was 0.05 mg ml^{-1} (1.6 μM).

Table 2
Dissociation constants of presequence peptides with $Tim50_{IMS}$ measured by fluorescence quenching.

	K_D (μM)	
	$CaCl_2$ (+)[a]	$CaCl_2$ (−)[b]
$Tim50_{IMS}$-pHsp60	3.09 ± 0.04	2.70 ± 0.03
$Tim50_{IMS}$-pALDH	1.33 ± 0.03	1.22 ± 0.02
$Tim50_{IMS}$-pCoxIV	2.42 ± 0.03	1.32 ± 0.02

[a] $CaCl_2$ (+): 1.6 μM $Tim50_{IMS}$ containing 8 μM $CaCl_2$ in 10 mM phosphate buffer (pH 7.4) with 0.15 M NaCl.
[b] $CaCl_2$ (−): 1.6 μM $Tim50_{IMS}$ containing 0 M $CaCl_2$ in 10 mM phosphate buffer (pH 7.4) with 0.15 M NaCl.

membrane potential (Δφ). For the translocation of the N-terminal presequence across the inner membrane, mitochondrial membrane potential is also required [28]. In our case, we found that there is strong binding of divalent metal ions with $Tim50_{IMS}$ and the binding of Ca^{2+} ion with $Tim50_{IMS}$ is the strongest one of all, which implicated that Ca^{2+} ion may play an important role in the protein import by TIM23 complex. Therefore, the contribution of Ca^{2+} ion in the interaction of presequence peptides with $Tim50_{IMS}$ should be considered. Our results further showed that the interaction of presequence peptides with $Tim50_{IMS}$ is weakened in presence of Ca^{2+} ion.

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