

Control of mitochondrial protein import by pH

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Abstract Protein import into mitochondria is inhibited by protons (IC₅₀ pH 6.5). The channels of the import machinery were examined to further investigate this pH dependence. TOM and TIM23 are the protein translocation channels of the mitochondrial outer and inner membranes, respectively, and their single channel behaviors at various pHs were determined using patch-clamp techniques. While not identical, increasing H⁺ concentration decreases the open probability of both TIM23 and TOM channels. The pattern of the pH dependences of protein import and channel properties suggests TIM23 open probability can limit import of nuclear-encoded proteins into the matrix of yeast mitochondria.

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Key words: Mitochondrion; Protein import; Translocase of inner membrane; Translocase of outer membrane; Patch clamp; pH

1. Introduction

The vast majority of mitochondrial proteins are encoded in the nucleus and synthesized in the cytoplasm. Proteins destined for the mitochondria must be targeted to, and imported across one or both of the mitochondrial membranes. These preproteins are typically synthesized as precursors carrying amino-terminal extensions, which are cleaved off after import. Since mitochondria are organelles with two membranes, the import of preproteins into the mitochondrial matrix and inner membrane depends on the transport systems of both membranes [1–6]. The translocase of the outer membrane, or Tom complex, is a multi-subunit translocation machine [7–9] and the TOM channel is integral to that complex. After translocation into the intermembrane space, preproteins interact with either the Tim23 or Tim22 complexes. Preproteins are translocated into the matrix by the Tim23 complex [10–13] through the TIM23 channel. The Tim22 complex specializes in the membrane insertion of subclasses of mitochondrial inner membrane proteins [14,15]. Recombinant Tim22 protein has channel activity [16] and a double barrel pore [17], but we have not yet detected channel activity corresponding to the Tim22 complex in native membranes.

We recently showed that TOM and TIM23 channels have

similar channel properties. The pH dependence of the open probability for the two protein import channels, as well as that of protein import, is defined in this report. Furthermore, increasing concentrations of protons inhibit protein import in a manner suggesting TIM23 open probability can limit protein import into mitochondria.

2. Materials and methods

Mitochondria were isolated from yeast grown in lactate medium and harvested in log phase as previously described [18]. Homogenization buffer was 0.6 M sorbitol, 10 mM Tris, 1 mM EDTA, 0.2% bovine serum albumin (BSA), 1 mM phenylmethylsulfonyl fluoride (pH 7.4) containing protease inhibitor cocktail (Sigma P 8215). Mitoplasts were prepared from isolated mitochondria by French pressing [19] and the outer membranes were harvested. The inner membranes were further purified according to Mannella [20]. The purity of the membrane fractions was routinely assayed as shown in Fig. 2, and cross-contamination was typically less than 5% for each membrane. Inner and outer membranes were separately reconstituted into giant proteoliposomes by dehydration–rehydration as previously described [18,21,22] using soybean L- α -phosphatidylcholine (Sigma type IV-S).

Immunoblotting was performed as previously described [23]. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electro-transferred onto polyvinylidene difluoride membranes. Indirect immunodetection employed chemiluminescence (ECL by Amersham) using horseradish peroxidase-coupled secondary antibodies. Membrane proteins (0.5–2 μ g per lane) were probed with primary antibodies against voltage-dependent anion-selective channel (VDAC) (gift of C. Mannella) and subunit IV of the ATP synthase (gift of J. Velours), and a secondary anti-rabbit antibody (Jackson Immunoresearch, 1:5000).

All patch-clamp experiments were carried out on reconstituted TIM23 and TOM channels of proteoliposomes containing mitochondrial inner and outer membranes, respectively. Procedures and analysis used were as previously described [18,23]. Briefly, membrane patches were excised from giant proteoliposomes after formation of a giga-seal using microelectrodes with ~ 0.4 μ m diameter tips and resistances of 10–30 M Ω (program courtesy of A.K. Dean, Sutter Instruments). Unless otherwise stated, the solution in the microelectrodes and bath was 150 mM KCl, 5 mM HEPES, pH 7.4. The buffer was 5 mM HEPES for pH 7–8.5, 5 mM CHES for pH 9–9.5, 5 mM MES for pH 5.5–6.0 and 5 mM citric acid for pH 5.0. Experiments were carried out at room temperature ($\sim 23^\circ$ C).

Voltage clamp was established with the inside-out excised configuration of the patch-clamp technique [24] using a Dagan 3900 patch-clamp amplifier in the inside-out mode. Voltages across patches excised from proteoliposomes were reported as bath potentials. The open probability, P_o , of the TIM23 and TOM channels was calculated as the fraction of the total time the channel spent in the fully open state from total amplitude histograms generated with the PAT program (Strathclyde Electrophysiological Software, courtesy of J. Dempster, University of Strathclyde, UK). Closed probability (P_c) and probability of occupying the substrate (P_s) were similarly calculated from amplitude histograms. Recordings were typically made for 15–30 min. Unless otherwise noted, curve fitting and IC₅₀ values were calculated through GrafPad Prism, V 2.01, using non-linear fit with a sigmoidal dose response.

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Abbreviations: TOM, translocase of outer membrane; TIM, translocase of inner membrane

Protein import was assessed as previously described [25] in isolated mitochondria at various pHs. Typically, import is measured by the appearance of relative amounts of mature protein and no effort is made to distinguish between translocation and maturation of the preprotein. Mitochondria were suspended to a final concentration of 1 mg/ml protein in import buffer (0.6 M sorbitol, 25 mM KCl, 20 mM MgCl₂, 4 mM KPO₄, 1 mM EDTA, 2 mg/ml BSA with 50 mM of the buffer indicated in patch-clamp experiments above). Radiolabeled dihydrofolate reductase (DHFR) with a mitochondrial targeting sequence was produced from the pGEM4 SU9(1–69)-DHFR (SP6-containing) plasmids with 1.5 mCi of [³⁵S]methionine per ml in a coupled transcription–translation system (SP6 TNT system, Promega Biotech) [25]. Each reaction contained 50–100 µg mitochondria. An aliquot of 5 µl of the lysate containing the radiolabeled protein was added to each assay mixture, and the samples were incubated at 30°C for 10 min. Import was stopped by transferring tubes to ice and adding carbonyl cyanide *m*-chlorophenyl hydrazone to a final concentration of 20 µM. Mitochondria were pelleted by centrifugation at 14000×*g* for 10 min, resuspended in sample buffer, and analyzed by SDS-PAGE. Radiolabeled proteins were detected by X-ray film. Autoradiographs were analyzed with UN-CSAN-IT gel (V 5.1, Silk Scientific, Orem, UT, USA).

3. Result and discussion

Mitochondria maintain a protonmotive force, which includes a pH gradient, across their inner membranes as a result of respiratory chain activity. While alkalization of the medium should affect the protonmotive force, protein import into mitochondria is not affected by increasing the external pH as high as pH 9.5. In contrast, accumulation of mature proteins is inhibited by increasing the proton concentration (Fig. 1). In these experiments, protein import at various pHs is normalized to the import at pH 7.4. Under these conditions, the IC₅₀ for proton inhibition of protein import into mitochondria is pH 6.5. The channels associated with the

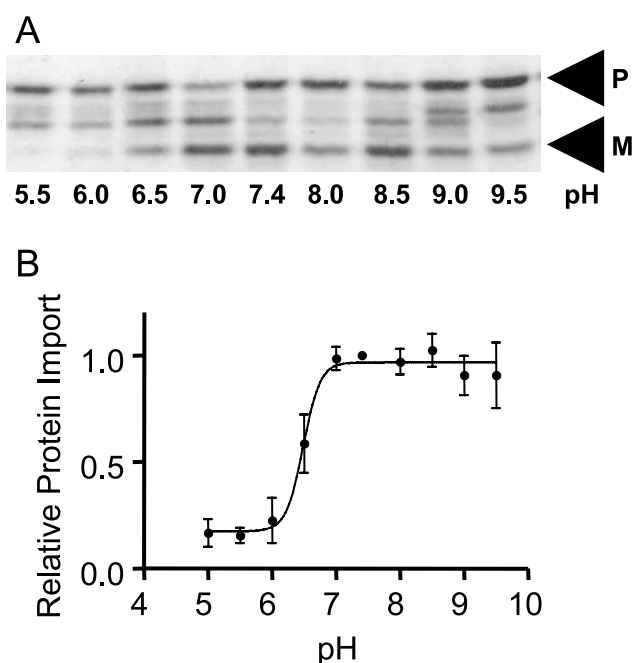


Fig. 1. The pH dependence of protein import into mitochondria. A: Autoradiographs show inhibition of protein import into mitochondria at low pH. P and M indicate preprotein and mature protein. Mature protein is processed after import and hence has a lower molecular weight. B: Protein import, as indicated by mature protein band density, was normalized to that at pH 7.4. See Section 2 for details. The IC₅₀ was pH 6.5.

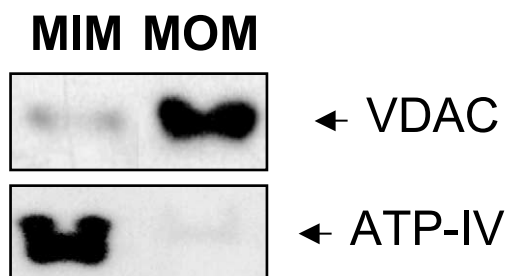


Fig. 2. Analysis of mitochondrial inner and outer membrane preparations. Immunoblots indicate the presence of ATP synthase subunit IV (ATP-IV) in the inner membrane (MIM) and VDAC in the outer membrane (MOM) preparations purified from mitochondria of wild-type yeast. Aliquots of membrane preparations were subjected to SDS-PAGE and immunoblots were decorated with antibodies to VDAC and ATP-IV proteins. Typical immunoblots showed ≤5% cross-contamination.

import machinery were examined in order to further explore this pH dependence.

TIM23 and TOM channel activities are reconstituted into proteoliposomes prepared with inner and outer membranes purified from mitochondria, respectively [18]. As shown in the Western blots of Fig. 2, the cross-contamination of the membranes is typically less than 5% as little of the outer membrane protein VDAC is detected in the inner membrane preparation and little of the inner membrane protein ATP synthase subunit IV is detected in the outer membrane preparations. TIM23 and TOM channels are normally reconstituted with their native orientation by these methods [18,22].

Recently, we reported that TIM23 and TOM channels have very similar single channel properties [26], in keeping with their function in transporting preproteins across mitochondrial membranes. The open probabilities for TIM23 and TOM channels also have similar pH dependences. Increasing proton concentration on the intermembrane or matrix faces decreases the open probability for both TOM and TIM23 channels, respectively (Fig. 3). In most experiments, the bath solution was exchanged after patching the appropriate proteoliposomes with pH 7.4 medium in the micropipette. Similar results were obtained when the proton gradient was reversed by varying the pH of the micropipette while the bath was at pH 7.4 (Fig. 3).

At neutral pH (7.4), the TIM23 and TOM channels are typically open after reconstitution. The open probability (P_o) approaches 1 and there are few transitions between the open and sub-states at +20 mV as shown in the current traces of Fig. 3. TIM23 and TOM channels are double barrel pores [26,27] and display three main conductance states. The open state corresponds to both pores open, the substate corresponds to one pore open and one closed, while neither pore is open in the closed state. While the open probabilities for TIM23 and TOM are high at alkaline pH (9.5), the noise levels of the open states are higher and there are small increases in the flickering rates. Acidification of the bath inhibits TIM23 and TOM channel activities in a proton concentration-dependent fashion (Fig. 3C). The IC₅₀ of proton-induced blockade of TIM23 is pH 6.4, while the IC₅₀ for TOM is pH 5.7.

As indicated by the differences in IC₅₀, acidic pH more dramatically influences the TIM23 channel than the TOM channel, as shown in Fig. 3A,B. At pH 5.5, the TIM23 chan-

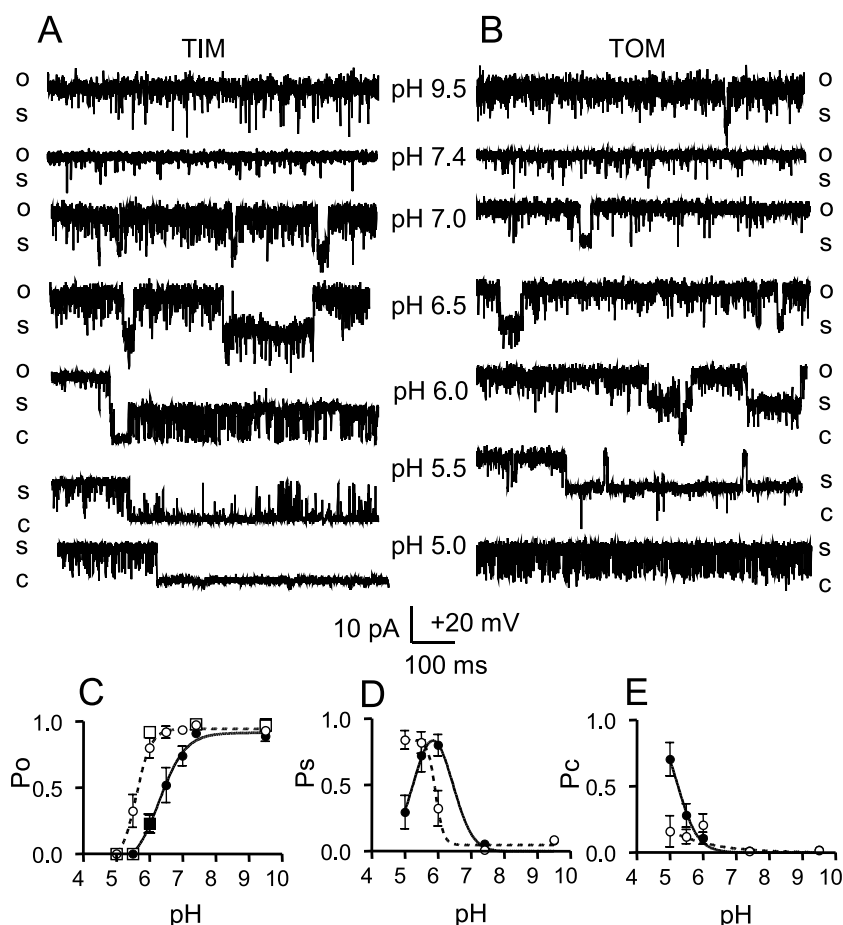


Fig. 3. The TIM23 and TOM channels are pH-dependent. Current traces of TIM23 (A) and TOM (B) channels were recorded from excised patches after perfusion of the bath with media at the indicated pH with the microelectrodes filled with 150 mM KCl, 5 mM HEPES, pH 7.4. See Section 2 for details. O, S, C corresponds to the open, sub-, and closed states, respectively. C: The open probability (P_o) of the TIM23 (●, ■) and TOM (○, □) channels was calculated from total amplitude histograms of 30 s of current traces at +20 mV after perfusion of the bath with media at the indicated pH (●, ○). Alternatively, pH indicates that inside the pipette with pH 7.4 medium in the bath (■, □). The proton IC_{50} is pH 6.5 and 5.7 for TIM23 and TOM, respectively. Means $\pm$ S.E.M. with a minimum of four determinations are shown. D: The probability of occupying the substate (P_s) of the TIM23 and TOM channels was calculated as above. The proton IC_{50} s for increasing P_s are pH 6.4 and 5.9 for TIM23 and TOM, respectively. E: The probability of the TIM23 and TOM channels occupying the closed state (P_c) was calculated as above. The proton IC_{50} s are pH 5.25 and 3.2 for TIM23 and TOM, respectively.

nel resides only in the sub- and closed states, while TOM continues to return to the open state. This inhibition of TIM23 and TOM channels by protons may be related to H^+ binding sites on the channels that recognize the positively charged precursors of preproteins directed into mitochondria. Alternatively, the destabilization of the open states of TIM23 and TOM may be due to effects of pH on the lipid bilayer, protein-lipid interactions, or a direct effect on the channel proteins themselves.

Normally, the intermembrane space may be slightly acidic while the matrix space becomes more alkaline compared to the cytoplasm as the electron transport network pumps protons across the inner membrane. However, pH is not likely to impact protein import under normal conditions. In these studies, the pH dependences of peptidases [28,29] are not likely to significantly contribute to the pH dependence of protein import. While the protonmotive force may be affected (and increase at low pH), the pH of matrix space is unlikely to vary widely over the incubation time due to the low proton permeability of the inner membrane.

The proton IC_{50} s for the open probabilities of TIM23 and

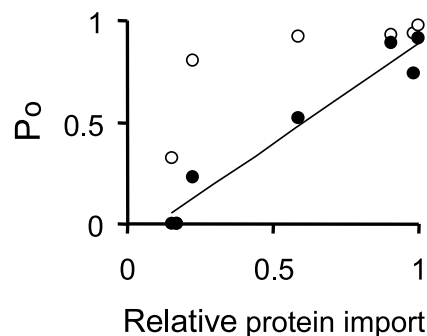


Fig. 4. Protein import capability is linked to TIM23 open probability. Media at various pH were used to modify the open probability of TIM23 and TOM as in Fig. 3. The relationship between the relative protein import function and the open probabilities of the TIM23 (●) and TOM (○) channels is shown. While a best fit line for the P_o of TIM23 and protein import has a correlation coefficient of 0.975, the data for the P_o of TOM do not have a linear relationship with protein import.

TOM and for protein import are low relative to pH 7, the normal cytoplasmic pH [30–32]. In this study, pH was used to probe the relationship between protein import and the channels associated with the import apparatus. Importantly, the IC_{50} for protein import into mitochondria is indistinguishable from that of the fully open state of the TIM23 channel. These data suggest that TIM23 open probability may limit import of preproteins into mitochondria at acidic pH. Furthermore, import decreases with the decline in TIM23 P_o (Fig. 4) and these data are consistent with the notion that both pores of TIM23 may need to be open in order for import to occur.

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