Tim23, a Protein Import Component of the Mitochondrial Inner Membrane, Is Required for Normal Activity of the Multiple Conductance Channel, MCC

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Abstract. We previously showed that the conductance of a mitochondrial inner membrane channel, called MCC, was specifically blocked by peptides corresponding to mitochondrial import signals. To determine if MCC plays a role in protein import, we examined the relationship between MCC and Tim23p, a component of the protein import complex of the mitochondrial inner membrane. We find that antibodies against Tim23p, previously shown to inhibit mitochondrial protein import, inhibit MCC activity. We also find that MCC activity is altered in mitochondria isolated from yeast carrying the tim23-1 mutation. In contrast to wild-type MCC, we find that the conductance of MCC from the tim23-1 mutant is not significantly blocked by mitochondrial presequence peptides. Tim23 antibodies and the tim23-1 mutation do not, however, alter the activity of PSC, a presequence-peptide sensitive channel in the mitochondrial outer membrane. Our results show that Tim23p is required for normal MCC activity and raise the possibility that precursors are translocated across the inner membrane through the pore of MCC.

In eukaryotic cells, a key step in the sorting of proteins to intracellular compartments is the translocation of polypeptides across organelle membranes. Although the mechanisms of these processes are not well understood, it has been suggested that proteins may cross membranes through pores or channels (Blobel and Dobberstein, 1975). Recently, Simon and Blobel (Simon and Blobel, 1991, Simon and Blobel, 1992) used electrophysiological techniques to identify potential protein-translocating channels in the endoplasmic reticulum and in the bacterial plasma membrane. In mitochondria, proteins imported from the cytosol utilize import complexes in both the inner and outer membranes (Pfanner et al., 1994; Ryan and Jensen, 1995; Pfanner and Meijer, 1995; Lithgow et al., 1995). However, the mechanism by which imported proteins cross either mitochondrial membrane is unclear.

Most mitochondrial proteins are synthesized in the cytosol as precursor proteins, carrying amino-terminal extensions called presequences. Presequences carry the information that targets proteins to the mitochondrion and are removed during or after import into the organelle. Precursor proteins are imported via a multi-step process that includes binding to outer membrane receptors, and translocation across one or both mitochondrial membranes. Translocation of the precursor across the mitochondrial inner membrane requires an electrochemical potential which is set up by the electron transport chain (Gasser et al., 1982; Schleyer et al., 1982). In addition, a matrix-localized member of the hsp70 family (mt-hsp70) plays an important role in the translocation of precursors across the inner membrane (Kang et al., 1990; Ungermann et al., 1994, 1996). The Tim44 protein is associated with the matrix face of the inner membrane (Blom et al., 1993; Horst et al., 1993; Maarse et al., 1992; Scherer et al., 1992) and interacts with mt-hsp70 during the translocation reaction (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994).

Tim23 is an integral protein of the inner membrane essential for import (Emtage and Jensen, 1993; Dekker et al., 1993). Mitochondria isolated from yeast strains carrying the tim23-1 mutation are defective in the import of at least five different precursor proteins (Emtage and Jensen, 1993). Furthermore, antibodies to Tim23p inhibit import across the inner membrane (Emtage and Jensen, 1993). Tim23p can be chemically cross-linked to a precursor arrested in transit across the inner membrane (Ryan and Jensen, 1993; Kübrich et al., 1994), and depletion of Tim23p from cells results in a defect in import (Emtage and Jensen, 1993). Tim17p is another essential inner membrane import component (Maarse et al., 1994; Ryan et al., 1994) that associates with Tim23p (Blom et al., 1995; Berthold et al., 1996; Ryan, K.R., R. Leung, and R.E. Jensen, manus-
script submitted for publication). While the exact function of Tim23p and Tim17p in import is not known, it has been suggested that both proteins form part of a channel in the inner membrane through which precursors are translocated into the matrix.

Both mitochondrial membranes contain a number of channel activities which have been identified using electrophysiological techniques (Kinnally et al., 1992; Sorgato and Moran, 1993). The multiple conductance channel (MCC) or mitochondrial megachannel; Kinnally et al., 1996; Zoratti and Szabó, 1994) is a channel activity found in the mitochondrial inner membrane of mammals and yeast. MCC has a large conductance and allows the passage of a variety of different ions across the membrane in patch-clamp studies (Lohret and Kinnally, 1995a). This channel is normally closed under metabolizing conditions unless activated (Kinnally et al., 1991, 1992, 1996). However, MCC activity is usually detected after its reconstitution into proteoliposomes, suggesting that regulatory components may be lost during the fractionation procedure (Lohret et al., 1996).

We have recently shown that the conductance of MCC is transiently blocked by synthetic peptides corresponding to mitochondrial presequences (Lohret and Kinnally, 1995b). Peptide sequences caused a momentary closure of MCC (a flicker blockade) that is reversible, voltage-, and dose-dependent. To determine whether MCC plays a role in mitochondrial protein import, we have examined the relationship between MCC and Tim23p, an inner membrane import component. Below we find that antibodies to Tim23p inhibit MCC activity. We also find the peptide sensitivity of MCC is altered in mitochondria isolated from the tim23-1 mutant. Our results indicate that Tim23p is required for normal MCC activity, and suggest that precursors are translocated across the inner membrane through the pore of the MCC.

Materials and Methods

**Isolation of Mitochondria and Preparation of Proteoliposomes**

Mitochondria were isolated from wild-type strain AH216 and the tim23-1 mutant as described (Emtage and Jensen, 1993; Daum et al., 1982). Mitochondrial membranes were prepared by the French press method (Decker and Greenewalt, 1977), and the outer membrane was separated from the inner membrane as described by Mannella (1982). The purity of the membrane fractions was assayed in two ways. First, immunoblotting showed, for the most part, that the outer membrane protein, voltage-dependent anion-selective channel (VDAC), was found only in the outer membrane preparations, and that the inner membrane protein Tim23 was found solely in the inner membrane preparation (see Fig. 1). Second, we found that VDAC activity detected by patch-clamp analysis was found only in outer membrane, but not in inner membrane preparations (Lohret and Kinnally, 1995a; Lohret et al., 1996). Inner and outer membranes were separately reconstituted into giant proteoliposomes (Sigma Type IV-S soybean 1-a-phosphatidylcholine) by dehydration-rehydration (Criado and Keller, 1987) as previously described (Lohret and Kinnally, 1995a; Lohret et al., 1996). To eliminate the contribution of VDAC to the channel activity of outer membrane preparations, strain M22-2 (Blachly-Dyson et al., 1990), which is disrupted for VDAC, was used as the source of outer

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1. Abbreviations used in this paper: MCC, multiple conductance channel; PSC, peptide-sensitive channel; VDAC, voltage-dependent anion-selective channel.

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**Figure 1.** Analysis of mitochondrial inner and outer membrane preparations. Immune blots indicate the presence of Tim23 in the inner and VDAC in the outer membrane preparations. Aliquots from inner (IM) and outer (OM) membrane preparations from wild-type mitochondria were subjected to SDS-PAGE and immune blots were decorated with antibodies to VDAC (A) and Tim23 (B) proteins. Immune complexes were visualized using AuroProbe BLplus secondary antibody reaction.
µg antibody/µg inner membrane protein) and incubated on ice for 60 min before the patch-clamp experiments.

Peptides

Peptides were prepared by the Wadsworth Center’s peptide synthesis core facility using a 431A automated peptide synthesizer as previously described (Applied Biosystems, Foster City, CA) (Lohret and Kinnally, 1995b). As shown in Table I, the presequence peptides used were based on amino acids 1-13 and 1-22 from the amino terminus of cytochrome oxidase subunit IV of *S. cerevisiae* (yCOX-IV<sub>1-13</sub> and yCOX-IV<sub>1-22</sub>), amino acids 3-22 of *N. crassa* subunit IV (ICOX-IV), and amino acids 1-20 from subunit VI of *S. cerevisiae* (yCOX-VI), Control peptides were the amino and carboxy termini and an internal segment of *N. crassa* VDAC (nVDAC, eVDAC, and iVDAC, respectively; Guo et al., 1995), the binding domain of antithrombin III (pAT-III; Smith and Knauer, 1987), and a synthetic mitochondrial presequence, synB2 (Allison and Schatz, 1986). Peptides were subjected to mass spectroscopy to determine impurities and proper composition.

Immune Blotting

Mitochondrial proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose or Immobilon filters (Towbin et al., 1979). Filters were decorated with antibodies and visualized with an AuroProbe BLplus secondary antibody reaction (Amersham, Amersham, UK) or by chemiluminescence (ECL; Amersham).

Results

Conductance of MCC Is Transiently Blocked by Presequence Peptides

We previously showed that peptides based on two mitochondrial presequences transiently block the conductance of MCC (Lohret and Kinnally, 1995b). We have extended this investigation by examining the effect of additional peptides on MCC activity. Mitochondrial inner membranes were isolated from wild-type yeast strains and fused with small phosphatidylcholine liposomes to form large proteoliposomes. Membrane patches were excised from the proteoliposomes with a micropipette and the conductance was examined in the presence or absence of peptides. Peptides were either added to the bath solution, which represents the mitochondrial matrix, or added to the micropipette buffer, representing the intermembrane space.

As shown previously, MCC in the absence of peptide had a predominant transition size of ~500 pS, a peak conductance of ~1 nS, a mean open time of ~25 ms at 20 mV, and a cation selectivity (Lohret and Kinnally, 1995a; see also Figs. 2 and 3). When a peptide based on the first 13 residues of the *Saccharomyces cerevisiae* cytochrome oxidase subunit IV presequence (yCOX-IV<sub>1-13</sub>) was added to the bath solution, a transient blockade of MCC conductance was induced during perfusion of the chamber as indicated by the decrease in mean current (Fig. 2 A). While transitions to lower conductance levels were relatively infrequent in the absence of presequence peptide (seen as downward deflections in the current trace of Fig. 2 B), large amplitude, rapid flickering between the open and lower conductance states developed in the current trace during the introduction of yCOX-IV<sub>1-13</sub> (Fig. 2 C) and persisted after the perfusion was complete (Fig. 2 D).

The reduced occupancy of the open state of MCC and rapid flickering between open, sub-, and closed states brought on by presequence peptides is further illustrated by comparing the current amplitude diagrams and single channel current traces in the absence (control) and presence of yCOX-IV<sub>1-13</sub> in Fig. 3. The peptide-induced reduction in open probability from 0.9 (control) to 0.4 (yCOX-IV<sub>1-13</sub>) was seen as a decrease in the fraction of the total time spent in the open state peak of the amplitude diagrams. The transient blockade of MCC conductance by presequence peptide was seen from either side of the membrane, but the effect was voltage-dependent. When the presequence peptide was added to the bath, the rapid flickering was seen at bath positive potentials (negative in the micropipette), but not at bath negative potentials (Lohret and Kinnally, 1995b). When the peptide was included in the micropipette, the blockade of MCC conductance was seen only at bath negative potentials. Several additional presequence peptides induced rapid flickering and reduced open probability of MCC, including peptides based on the amino terminus of yeast cytochrome oxidase subunit IV (yCOX-IV<sub>1-22</sub>) and subunit VI (yCOX-VI), as
Table I. The Effects of Synthetic Peptides on MCC Activity

| Peptide      | Sequence          | Source     | Net charge | Presequence | MCC block |
|--------------|-------------------|------------|------------|-------------|-----------|
| yCOX-IV<sub>1-13</sub> | 1MLSLRQSIRFFKY<sub>13</sub> | yeast      | +3         | +           | +         |
| yCOX-IV<sub>1-22</sub> | 1MLSLRQSIRFFKPAERTLCCS<sub>22</sub> | yeast      | +5         | +           | +         |
| yCOX-VI     | 1MLSLRQFLNQVINRTLLR<sub>22</sub> | yeast      | +5         | +           | +         |
| fCOX-IV     | 1RAPALRRSIATTYYVRCNAT<sub>22</sub> | N. crassa | +3         | +           | +         |
| SynB2       | 1MLSRQSQRSQRQSQRSQSR | synthetic  | +5         | −<sup>5</sup> | −         |
| pAT-III     | 1RNAVLKSSKNKRYLRCNLK<sub>52</sub> | antithrombin III binding domain | +7 | −<sup>*</sup> | +<sup>1</sup> |
| iVDAC       | 109RGAKFNLHFKQ<sub>19</sub> | N. crassa  | +3         | −<sup>1</sup> | −         |
| nVDAC       | 1MVPAPAFAKSANDLLN<sub>20</sub> | N. crassa  | −1         | −<sup>1</sup> | −         |
| cVDAC       | 272THKVGTSFIFES<sub>523</sub> | N. crassa  | 0          | −<sup>1</sup> | −         |

<sup>1</sup>Glasler, S.M., and M.G. Cumskey. 1990. J. Biol. Chem. 265:8817–8822.
<sup>2</sup>Prevented but not determined.
<sup>3</sup>Allison, D.S., and G. Schatz. 1986. Proc. Natl. Acad. Sci. USA. 83:9011–9015.
<sup>4</sup>pAT-III blocked from the bath side but not micropipette side.

Figure 3. Presequence peptides specifically induce a transient blockade of MCC conductance. The conductance of a patch excited from a proteoliposome-containing mitochondrial inner membranes from the wild-type strain was measured at 20 mV. The single channel current traces in the absence of peptide (control) and in the presence of 50 μM synB2 or yCOX-IV<sub>1-13</sub> peptide in the bath solution were band-width limited to 2 kHz. Total current amplitude diagrams and current traces show the occupancy of open (O), substate (S) and closed (C) conductance levels. The probability of occupying the open state was 0.9, 0.8, and 0.4 in the absence of peptide (control), in the presence of synB2, and in the presence of yCOX-IV<sub>1-13</sub>, respectively.

Table I. The Effects of Synthetic Peptides on MCC Activity

| Peptide      | Sequence          | Source     | Net charge | Presequence | MCC block |
|--------------|-------------------|------------|------------|-------------|-----------|
| yCOX-IV<sub>1-13</sub> | 1MLSLRQSIRFFKY<sub>13</sub> | yeast      | +3         | +           | +         |
| yCOX-IV<sub>1-22</sub> | 1MLSLRQSIRFFKPAERTLCCS<sub>22</sub> | yeast      | +5         | +           | +         |
| yCOX-VI     | 1MLSLRQFLNQVINRTLLR<sub>22</sub> | yeast      | +5         | +           | +         |
| fCOX-IV     | 1RAPALRRSIATTYYVRCNAT<sub>22</sub> | N. crassa | +3         | +           | +         |
| SynB2       | 1MLSRQSQRSQRQSQRSQSR | synthetic  | +5         | −<sup>5</sup> | −         |
| pAT-III     | 1RNAVLKSSKNKRYLRCNLK<sub>52</sub> | antithrombin III binding domain | +7 | −<sup>*</sup> | +<sup>1</sup> |
| iVDAC       | 109RGAKFNLHFKQ<sub>19</sub> | N. crassa  | +3         | −<sup>1</sup> | −         |
| nVDAC       | 1MVPAPAFAKSANDLLN<sub>20</sub> | N. crassa  | −1         | −<sup>1</sup> | −         |
| cVDAC       | 272THKVGTSFIFES<sub>523</sub> | N. crassa  | 0          | −<sup>1</sup> | −         |

<sup>1</sup>Glasler, S.M., and M.G. Cumskey. 1990. J. Biol. Chem. 265:8817–8822.
<sup>2</sup>Prevented but not determined.
<sup>3</sup>Allison, D.S., and G. Schatz. 1986. Proc. Natl. Acad. Sci. USA. 83:9011–9015.
<sup>4</sup>pAT-III blocked from the bath side but not micropipette side.
We preincubated proteoliposomes prepared with mitochondrial inner membranes with antibodies to Tim23p and then examined the conductance of patches excised from the treated vesicles. As shown in the current traces of Fig. 4 A, antibodies to Tim23p blocked MCC activity. When the conductance of patches from proteoliposomes preincubated with Tim23 IgG was measured, MCC was virtually absent. Tim23 IgG blocked essentially all conductance through MCC (Fig. 4 A) and blocked the effect of yCOX-IV1,13 peptide. In contrast, equivalent amounts of preimmune IgG did not affect MCC activity (Fig. 4 A). To quantify the effect of Tim23 antibodies, several patches were taken from proteoliposomes treated with Tim23 IgG and their conductance was measured. As controls, proteoliposomes were also incubated with IgG from preimmune serum, antibodies to the outer membrane VDAC channel (Stanley et al., 1995), or IgG to the Rieske iron-sulfur protein of the inner membrane electron transport chain (Beckmann et al., 1987) (Fig. 4 B). In a total of 28 patches from proteoliposomes treated with Tim23 IgG, no MCC activity was observed in 23 of the patches, whereas five patches had detectable MCC (Fig. 4 B). In all of the controls (90 total patches), MCC activity was found in normal amounts (Fig. 4 B).

To test the specificity of the inhibition of MCC by Tim23 antibodies, we examined the activity of an outer mem-

Figure 4. Tim23 antibodies specifically inhibit MCC activity. (A) Typical current traces at 40 mV recorded from proteoliposomes prepared from wild-type inner membranes that were preincubated with Tim23 IgG or preimmune IgG as detailed in Materials and Methods. (B) The fraction of patches in which MCC was detected (at voltages between ±60 mV) in a blind study after incubation of proteoliposomes containing ∼1 μg inner membrane protein and 25 μg of the indicated antibody. All studies were normalized to untreated proteoliposomes. n is the number of patches examined for each condition. (C) The fraction of patches in which the outer membrane channel activity PSC was detected (at voltages between ±60 mV) after incubation of proteoliposomes containing ∼1 μg outer membrane protein and 25 μg of the indicated antibody was normalized to untreated proteoliposomes.
brane channel activity, PSC (Fig. 4 C). Proteoliposomes prepared using outer membranes were incubated with equivalent amounts of Tim23 IgG or antibody from preimmune serum. Neither IgG had any effect on the detection level of PSC, and activity similar to untreated proteoliposomes was found in the preparations preincubated with either preimmune or Tim23 IgG (Fig. 4 C). Thus, antibodies to Tim23p appear to specifically inhibit the inner membrane channel MCC, and further support the possibility that MCC plays a role in mitochondrial protein import.

Conductance of MCC Isolated from the tim23-1 Mutant Is Not Transiently Blocked by Presequence Peptides

To further test the connection between MCC and protein import, we examined MCC activity in mitochondria isolated from the tim23-1 mutant. The tim23-1 mutation results in a substitution of aspartate for glycine at position 186 in Tim23p (Emtage, 1994). Mitochondria isolated from tim23-1 mutants are defective in the import of several different precursor proteins, including subunit IV of yeast cytochrome oxidase (Emtage and Jensen, 1993). We prepared mitochondrial inner membranes from either wild-type or the tim23-1 mutant, reconstituted the membranes into proteoliposomes, and then measured the conductance of patches excised from the vesicles.

We found that the electrical properties of MCC isolated from wild-type and tim23-1 strains were virtually identical (compare control current traces of Fig. 5 A with 5 B and Fig. 6 A with 6 B). In particular, MCC from both strains had the same peak conductance, predominant transition size, mean open time, and cation selectivity. Furthermore, permeability ratios for K+/Cl− were ~6 for MCC from wild-type and tim23-1 patches with a 150:30 mM KCl gradient. Conductance through MCC from both strains was voltage dependent, i.e., MCC is predominantly open at low (e.g., 20 mV) but not high potentials of either polarity. The V0 (voltage where the probability of opening and closing are the same) at positive potentials was less than V0 at negative potentials for MCC from both wild-type and tim23-1 strains.

In the presence of presequence peptides, however, MCC activity from wild-type and tim23-1 strains differed dramatically. The conductance of MCC isolated from wild-type strains is transiently blocked by presequence peptides (Fig. 5 A, see also Figs. 2 and 3, and Table I). The flicker rate of MCC from the wild-type strain increased ~10-fold upon addition of the yCOX-IV1-13 peptide to the bath solution at positive potentials (Fig. 5 A). Similarly, the flicker rate of wild-type MCC was ~10-fold higher at negative potentials if yCOX-IV1-13 was included in the micropipette buffer (Fig. 6 A). The flicker rate of MCC isolated from wild-type cells increased with yCOX-IV1-13 concentration in the bath and saturated by ~25 μM (Fig. 7). This dose-dependent inhibition of MCC is similar to that required for the inhibition of protein import by presequence peptides (Glaser and Cumsby, 1990). In marked contrast, the activity of MCC isolated from the tim23-1 mutant was only slightly affected by the addition of yCOX-IV1-13 peptide (Figs. 5 B and 6 B). Quantification showed that the tim23-1 mutation caused at least an 85%
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reduction in the transient conductance blockade (or increased flicker rate) induced by yCOX-IV 1-13 and fCOX-IV in the bath (Fig. 5C). Similar results were also found with yet another presequence peptide yCOX-VI at 2 μM concentrations (data not shown). Furthermore, we found that peptides added to the micropipette, instead of the bath, failed to induce flickering of MCC isolated from the tim23-1 mutant (Fig. 6). The flicker rate of wild-type MCC, but not tim23-1 MCC, was ~10-fold higher at negative potentials when the yCOX-IV 1-13 peptide was present in the micropipette solution. At the same time, the lower open probability for wild-type MCC that is associated with the peptide-induced rapid flickering was not seen with MCC from the tim23-1 strain (Fig. 6).

tim23-1 mutants are temperature-sensitive for growth and mitochondrial protein import, but mitochondria isolated from tim23-1 cells are defective in protein import in vitro at all temperatures (Emtage and Jensen, 1993). We have recently found that the Tim23 protein is rapidly degraded during mitochondrial isolation from tim23-1 strains (Ryan, K.R., R. Leung, and R.E. Jensen, manuscript submitted for publication). Similarly, we find that proteoliposomes prepared from tim23-1 inner membranes contain greatly reduced levels of the Tim23 protein (Fig. 8). Equal amounts of proteoliposomes prepared from wild-type and tim23-1 mitochondrial inner membranes were analyzed by immune blotting. While similar levels of cytochrome oxidase subunit IV (Cox4p; Fig. 8) and the β-subunit of the F1-ATPase (not shown) were seen in the two preparations, the level of the Tim23 protein was reduced at least 10-fold in the tim23-1 proteoliposomes. Importantly, the frequency of detecting MCC in proteoliposomes prepared from similar quantities of wild-type and tim23-1 inner membrane protein was virtually the same. Twelve MCC were detected in thirteen patches from proteoliposomes prepared with 16 μg tim23-1 inner membrane protein/mg lipid, while 0, 7, and 32 MCC were recorded from fifteen patches each from proteoliposomes prepared with 3, 27, and 133 μg wild-type inner membrane protein/mg lipid, respectively. Hence, the reduced level of Tim23 protein in the inner membrane of tim23-1 mitochondria may explain their defect in protein import, as well as the alteration (but not loss) of MCC activity.

tim23-1 Mutants Are Not Altered in the Activity of PSC, an Outer Membrane Peptide-sensitive Channel

To show that the tim23-1 mutation specifically affected MCC activity, we examined the activity of the outer membrane peptide-sensitive channel, PSC. Like MCC in the inner membrane, the conductance of PSC is transiently blocked by presequence peptides (Henry et al., 1996; Fève et al., 1994; Theiffry et al., 1992; Juin et al., 1995). Outer membranes were isolated from mitochondria of wild-type cells and the tim23-1 mutant, and then reconstituted into proteoliposomes. When membrane patches were examined, we found that the PSC activities from both preparations were comparable in terms of conductance, selectivity, and voltage dependence. Furthermore, addition of the yCOX-IV 1-13 peptide increased the flicker rate of PSC from both wild-type and tim23-1 strains by ~10-

Figure 6. Rapid flickering induced by presequence peptides in the micropipette solution is reduced in MCC from the tim23-1 mutant. Patches were excised from proteoliposomes containing wild-type (A) or tim23-1 (B) mitochondrial inner membranes. Typical current traces of MCC activity at ~20 mV recorded from different patches in the presence and absence of 100 μM yCOX-IV 1-13 peptide in the micropipette are shown. P0 corresponds to probability of occupying the open state.

Figure 7. Dose dependence of blockade of MCC conductance by yCOX-IV 1-13 peptide. Flicker rates for typical MCC activity from wild-type (●) or tim23-1 (○) strains at different concentrations of the yCOX-IV 1-13 peptide in the bath were determined from current traces (5–15 s) at 20 mV in single channel patches.
fold as shown by the current traces and histograms of Fig. 9. This inhibition was both dose- and voltage-dependent (data not shown). In addition, we previously found deletion of VDAC, or the adenine nucleotide translocator had no effect on MCC activity in proteoliposomes (Lohret and Kinnally, 1995; Lohret et al., 1996). Our results indicate the tim23-1 mutation specifically alters the activity of the inner membrane MCC, and has no effect on the outer membrane PSC activity. The Tim23 antibody and the tim23-1 mutation allow discrimination between MCC and PSC activities.

Discussion

Our studies indicate a striking correlation between MCC activity and the translocation of precursors across the inner membrane. We find that Tim23p, an inner membrane import component, is required for peptide-sensitive activity of MCC. Antibodies to Tim23p block both protein import and the MCC channel. We find that MCC conductance is specifically blocked by presequence peptides. Furthermore, we find that the presequence peptide sensitivity of MCC isolated from the protein import deficient mutant tim23-1 is dramatically reduced. MCC has several properties expected for a protein-translocating channel in the mitochondrial inner membrane. For example, the pore size of MCC is estimated at 2–3 nm (Zoratti and Szabó, 1994), which is sufficiently large to allow the passage of unfolded precursor proteins. In addition, the predominant transition size of MCC (500 pS) is similar to potential protein-conducting channels observed both in the endoplasmic reticulum and the bacterial plasma membrane (Simon and Blobel, 1991, 1992).

Figure 8. Mitochondria isolated from the tim23-1 mutant contain reduced amounts of the Tim23-1 protein. Mitochondria were isolated from wild-type cells and the tim23-1 mutant, and proteoliposomes were prepared as described in Materials and Methods. Aliquots representing 30 μg of proteoliposomes were immune blotted and decorated with antibodies to the Tim23 protein (Tim23p) and to cytochrome oxidase subunit IV (COX4p).

Figure 9. The tim23-1 mutation does not affect the peptide-sensitive channel, PSC, of the mitochondrial outer membrane. (A) Sample current traces for PSC from proteoliposomes containing outer membranes from wild-type or tim23-1 strains are shown in the presence and absence (control) of 50 μM γCOX-IV_{1-13} peptide in the bath. (B) The flicker rates of PSC of wild-type and tim23-1 strains were determined from current traces represented by those in A above.
either side of the membrane in a potential-dependent wild-type MCC by presequence peptides could occur from either side of the membrane in a potential-dependent manner, i.e., at positive potentials (pipette negative) when the presequence peptide was added to the bath and at negative potentials (pipette positive) when peptide was included in the pipette. In addition, flicker rate increased with the magnitude of the voltage. Moreover, the reduction in open probability and increase in flickering induced by addition of presequence peptide to the bath was reversed by perfusion with fresh media, suggesting the presequence peptides are not tightly bound. The peptide-induced flickering, however, was specific for presequence peptides. For example, peptides shown not to function as presequences, such as synB2 (Allison and Schatz, 1986; Roise et al., 1988), or peptides that were not cationic, did not affect MCC activity.

The presequence peptide-induced flickering between open and subconductance states seen in wild-type MCC may be due to the momentary occlusion of the channel during translocation of the peptide from one side of the membrane to the other. Although direct demonstration of peptide translocation through MCC will likely require reconstitution of MCC using purified components, a similar peptide-induced flickering seen in studies with the outer membrane channel, PSC, was shown to be associated with the movement of peptide across the membrane (Thieffry et al., 1992; Juin et al., 1994; Henry et al., 1996). Alternatively, the rapid flickering of MCC may result from a destabilization of the open state upon binding of the peptide to one or more sites on MCC. Since MCC isolated from the tim23-1 mutant is not blocked by presequence peptides added to either side of the membrane, we suggest that the tim23-1 mutation may either hinder translocation of presequence peptides through MCC or alter presequence peptide recognition by MCC.

Mitochondria must maintain an electrochemical gradient for ATP synthesis. Therefore, any protein-translocating channel in the inner membrane must be tightly regulated. While MCC is normally closed in mitochondria under metabolizing conditions, it is usually detected after permeabilization of the inner membrane to the other. Although direct demonstration of peptide translocation through MCC will likely require reconstitution of MCC using purified components, a similar peptide-induced flickering seen in studies with the outer membrane channel, PSC, was shown to be associated with the movement of peptide across the membrane (Thieffry et al., 1992; Juin et al., 1994; Henry et al., 1996). Alternatively, the rapid flickering of MCC may result from a destabilization of the open state upon binding of the peptide to one or more sites on MCC. Since MCC isolated from the tim23-1 mutant is not blocked by presequence peptides added to either side of the membrane, we suggest that the tim23-1 mutation may either hinder translocation of presequence peptides through MCC or alter presequence peptide recognition by MCC.

The tim23-1 mutation results in a substantial reduction in the import of several different precursors into isolated mitochondria (Emtage and Jensen, 1993). Although tim23-1 strains are temperature-sensitive for viability, the protein import defect in vitro was seen at the permissive temperature. Consistent with previous results (Ryan, K.R., R. Leung, and R.E. Jensen, manuscript submitted for publication), we found the Tim23 protein was reduced 10–20-fold in mitochondria isolated from the tim23-1 mutant as compared to the wild-type strain. Associated with loss of Tim23 protein and a defect in protein import, we found that the activity of MCC from tim23-1 mitochondria was markedly insensitive to presequence peptides. In the absence of peptide, however, MCC activity from wild-type strains and the tim23-1 mutant was similar. For example, no significant differences in pore size (as reflected by peak conductance), predominant transition size, voltage dependence, or ion selectivity were seen. Furthermore, the number of channels detected in the wild-type and tim23-1 membrane preparations was virtually the same. Our findings that the Tim23 protein is reduced in tim23-1 mitochondria suggests that Tim23p is not a structural component of the pore of MCC. Instead, we argue that Tim23p plays a regulatory function in MCC activity.

The Tim23 protein contains a 9-kD hydrophilic amino-terminal domain facing the intermembrane space, and four potential carboxyl-terminal membrane-spanning segments (Bauer et al., 1996; Emtage, J.L.T., and R.E. Jensen, manuscript submitted for publication). The amino terminus of Tim23p appears to function as a receptor for presequences as they are translocated through the outer membrane import machinery (Emtage et al., 1993; Bauer et al., 1996). In addition, Tim23p has been shown to form dimers in mitochondria in a potential-dependent manner (Bauer et al., 1996). Dimerization of Tim23p is disrupted upon the binding of precursors. The role of Tim23, therefore, may be to bind the presequence and pass the precursor to the inner membrane import channel. In this view, tim23-1 mutants would be defective in the recognition of the presequence, but MCC activity would not otherwise be affected. To further test the relationship between MCC and mitochondrial protein import, we are currently analyzing MCC defective in or lacking different import components.

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