Gating of the Mitochondrial Protein Import Complex TIM23*

Sonia Martinez-Caballero†, Sergey M. Grigoriev‡, Johannes M. Herrmann§, María Luisa Campo¶, and Kathleen W. Kinnally†,1,2

From the †Department of Basic Sciences, New York University College of Dentistry, New York, New York 10010, ‡Departamento de Biología Molecu lar y Genética, Universidad de Extremadura, 10071 Cáceres, Spain, and §Institute of Cell Biology, Erwin-Schroedinger-Strasse, 67663 Kaiserslautern, Germany

The TIM23 complex mediates import of preproteins into mitochondria, but little is known of the mechanistic properties of this translocase. Here patch clamping reconstituted inner membranes allowed for first time insights into the structure and function of the preprotein translocase. Our findings indicate that the TIM23 channel has “twin pores” (two equal sized pores that cooperatively gate) thereby strikingly resembling TOM, the translocase of the outer membrane. Tim17p and Tim23p are homologues, but their functions differ. Tim23p acts as receptor for preproteins and may largely constitute the preprotein-conducting passageway. Conversely depletion of Tim17p induces a collapse of the twin pores into a single pore, whereas N terminus deletion or C terminus truncation results in variable sized pores that cooperatively gate. Further analysis of Tim17p mutants indicates that the N terminus is vital for both voltage sensing and protein sorting. These results suggest that although Tim23p is the main structural unit of the pore Tim17p is required for twin pore structure and provides the voltage gate for the TIM23 channel.

Because more than 95% of the ~700 yeast mitochondrial proteins are encoded in the nucleus, newly synthesized proteins need to be translocated to their final destinations in the outer and inner membranes, the matrix, or the intermembrane space (1). Three multisubunit complexes or translocases mediate this routing of preproteins. All precursor proteins cross the outer membrane through the translocase of the outer membrane (TOM). The TIM22 and TIM23 complexes are two translocases in the inner membrane (for reviews, see Refs. 2–7). Multiple pass membrane proteins carrying internal targeting signals, e.g. phosphate carrier, are inserted into the inner membrane by the TIM22 complex. Those preproteins with cleavable N-terminal presequences that are destined for the matrix or the inner membrane are transported by the TIM23 translocase.

The TIM23 complex is formed by at least three integral membrane proteins including Tim23p, Tim17p, and Tim50p. Preproteins are recognized in the intermembrane space by the large C-terminal domain of Tim50p. This domain interacts with Tim23p and guides the precursor protein to the pore of the complex (8–10). Tim23p is embedded in the inner membrane and putatively forms the translocation pore of the complex. Tim23p also contains a hydrophilic domain of about 100 amino acids exposed to the intermembrane space that has receptor-like properties for the recognition of preproteins (11–14). For complete translocation, preproteins need the driving force of the presequence translocase-associated motor or PAM complex, which contains mtHsp70 (matrix heat shock protein), Mge1, Pam16p/Tim16p, Pam18p/Tim14p, and Tim44p (15–20).

Until recently, little was known of the function of the integral membrane protein Tim17p. The high degree of homology of Tim17p with Tim23p led to speculation that these two proteins form the protein-translocating channel (21–23). It has also been hypothesized that Tim17p might form a Tim23p-independent channel that mediates incorporation of proteins into the inner membrane by a stop-transfer mechanism (24). Recently Tim17p was shown to be essential for both sorting of proteins into the inner membrane and translocation of precursor proteins into the matrix where Tim17p may provide a link between the TIM23 and PAM complexes (2). Moreover Tim17p specifically interacts with the purified N-terminal domain of Pam18p/Tim14p, which is exposed to the intermembrane space (2).

At the core of each of the translocases is a channel, or pore, that provides the aqueous pathway for the transit of unfolded proteins. The TOM and TIM22 complexes were found to have twin pore structures by single particle analysis (25, 26); this approach has not yet been successfully applied to the TIM23 complex. In previous electrophysiological studies, the channel activities associated with the TOM and TIM23 complexes were found to be remarkably similar (13, 27). In this study, we provide evidence that the TIM23 channel has a twin pore structure, like the TOM and TIM22 channels, and that Tim17p is vital to maintaining this structure. Analysis of several mutants revealed...
that the N terminus of Tim17p acts as the voltage sensor for the TIM23 complex.

EXPERIMENTAL PROCEDURES

Isolation of Mitochondria and Preparation of Proteoliposomes—A mutant strain of Saccharomyces cerevisiae, Tim17(Gal10), in which the expression of TIM17 gene is controlled by a Gal10 promoter was used (28). Cells were cultivated at 30 °C on standard defined lactate medium with 2% glucose in the presence or absence of 1% galactose for 24 h as described by Meier et al. (28). Three additional Tim17 mutants were analyzed and include versions lacking the C-terminal 24 (Tim17∆C) or N-terminal 11 (Tim17∆N) amino acids, double point mutant D4R/D8K (Tim17DD→RK), and a four-point mutant with an additional replacement at amino acids 80 and 83 (Tim17DD→RK/RK→DD). Cells were grown at 30 °C on semisynthetic lactate medium as described by Meier et al. (29).

Mitochondria were isolated from logarithmically growing cells as described previously (13). Homogenization buffer was 0.6 M sorbitol, 10 mM Tris, 1 mM EDTA, 0.2% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride (pH 7.4) containing protease inhibitor mixture (Sigma catalog number P 8215). Mitoplasts were prepared from isolated mitochondria by the French press method (30), and the inner membranes were further purified according to Mannella (31) as described previously (32). Membrane purity was routinely assessed, and cross-contamination was typically less than 5%. Inner membranes were reconstituted into giant proteoliposomes by dehydration-rehydration as described previously (13, 33, 34) using soybean l-α-phosphatidylcholine (Sigma Type IV-S).

Patch Clamping Techniques—Patch clamp experiments were carried out on reconstituted TIM23 channels of proteoliposomes containing purified mitochondrial inner membranes (13, 32). Briefly membrane patches were excised from giant proteoliposomes after formation of a gigaseal using microelectrodes with ~0.4-μm diameter tips and resistances of 10–30 megaohms. Unless otherwise indicated, the solution in the microelectrode and bath was 150 mM KCl, 5 mM HEPES, pH 7.4, at ~23 °C. Voltage clamp was established with the inside-out excised configuration (35) using a Dagan 3900 patch clamp amplifier in the inside-out mode. Voltages across excised patches were reported as bath potentials. The open probability, $P_o$, was calculated as the fraction of the total time the channel spent in the open state from total amplitude histograms generated with WinEDR Software (courtesy of J. Dempster, University of Strathclyde, Glasgow, UK) from 20–40 s of current traces. $V_o$ is the voltage at which the channel spends half of the time open ($P_o$ is 0.5). Mean open time was measured by analyzing >1000 transition events per patch. Filtration was 2 kHz with 5-kHz sampling for all analysis and currents traces shown unless otherwise stated. Simulations were generated by WinEDR Software as described previously (27) by providing single channel parameters including transition amplitude, mean open and closed times, and designating five openings/burst for each data set. The distribution of time spent in each of the three states (O, two open ($P_{O1O2} = P_o^2$); S, one open and one closed ($P_{O1C2 or C1O2} = 2P_o(1 - P_o)$); and C, two closed ($P_{C1C2} = (1 - P_o)^2$)) was fit to the open state of two independent channels. Permeability ratios were calculated from the reversal potential in the presence of a 150:30 mM KCl gradient as described previously (13). Peptides were introduced by perfusion of the 0.5-ml bath with 3–5 ml of medium. Flicker rates were determined from 20–40 s of current traces as the number of transition events/s from the open to lower conductance states with a 50% threshold of the predominant event (~250 pS).

The pore size was estimated using the polymer exclusion method (36, 37). The transition size and peak conductance in the presence of a series of polyethylene glycols (PEGs; molecular mass, 200–8000 Da) was determined. PEG solutions were 15% (w/v) in 150 mM KCl, 5 mM HEPES, pH 7.4, and were added to the bath by perfusion. The radius of the pore of the TIM23 channel was also estimated from the peak conductance assuming a pore length of 7 nm (38). That is, $R_{pore} = (l + (πa)^2)(ρ/πa^2)$ where $R$ and $a$ are the resistivity of the pore and solution, $l$ is pore length, and $a$ is pore radius (39).

Immunoblotting—Mitochondrial proteins were separated by SDS-PAGE (40) and electrotransferred (32) onto polyvinylidene difluoride membranes. Indirect immunodetection used chemiluminescence (ECL by Amersham Biosciences) using horseradish peroxidase-coupled secondary antibodies. Membrane proteins (0.5–12 μg/line) were decorated with antibodies against Tim23p, Tim17p, and Tim44p (gift of M. Brunner and I. Milisav). Scion imaging and densitometry were used to semiquantify the amount of Tim17p and Tim23p from the signal intensities of bands on Western blots that were normalized relative to 1 μg of total protein of cells grown in the presence of galactose.

Peptides—Peptides were prepared by the New York State Department of Health Wadsworth Center Peptide Synthesis Core Facility (Albany, NY) using an Applied Biosystems 431A automated peptide synthesizer as described previously (13). The presequence peptides used were based on amino acids 1–13 and 1–22 from the N terminus of cytochrome oxidase subunit IV of S. cerevisiae (yCoxIV-(1–13) and yCoxIV-(1–22)) and a synthetic mitochondrial presequence, SymB2 (46). Peptides were subjected to mass spectroscopy to determine impurities and proper composition and were typically >90% pure.

RESULTS

Considerable evidence links TIM23 channel activity to protein import and the TIM23 complex. Antibodies against Tim23p specifically block TIM23 channel activity in patch clamp experiments and protein import in mitoplasts (13). A tim23.1 strain that is import-deficient displays altered TIM23 channel activity (13). TIM23 channel activity is reversibly regulated by synthetic presequence peptides (13, 33, 41, 42). The frequency of detecting TIM23 channels is directly coupled to the amount of Tim17p (supplemental Fig. S1) or Tim23p (47) present in the membrane. Moreover the single channel properties of TIM23 are surprisingly similar to those of TOM channel, the import channel of the outer membrane (27).

To determine whether TIM23 channels have a twin pore structure like that of TOM channels, the architecture of the TIM23 channel was investigated by characterizing normal and
TIM23 behaves like a twin pore channel. TIM23 has twin pores of equal size that cooperatively gate. Either channel 1 or 2, but not both, would be open in the substate. Model E has twin pores of equal size that cooperatively gate. Open and filled circles indicate open and closed pores, respectively. F–K, the pore size of the TIM23 channel was measured using the polymer exclusion method. The relative transition size (F and G) or peak conductance (H) of TIM23 channels from wild-type (F) and Tim17(Gal10) channels was measured in the presence (g) and absence (g₀) of various PEGs. The PEG molecular mass range (radius) was: 200 (0.5 nm), 400 (0.7 nm), 600 (0.8 nm), 1000 (0.94 nm), 1450 (1.05 nm), 3350 (1.44 nm), and 8000 (3.05 nm) Da. The line is the best fit of the data according to Bezrukov and Kasianowicz (36). The corresponding second derivatives of the data of F–H are directly below in I–K and reveal the restriction radii for small and large non-electrolytes. Data points are typically mean ± S.D. of a minimum of seven independent patches; some S.D. are smaller than the square symbol. L and M, total amplitude histograms obtained from the current traces of wild type and Tim17(Gal10) + Gal shown above in A and B are shown in black. Total amplitude histograms of simulations for two independent 500-pS channels are shown in gray. Simulations are fit to the probability of occupying the open state (1000 pS) assuming a binomial distribution and no cooperative gating. See “Experimental Procedures” for further details. Interval durations were binned at a resolution of 200–500 bins, and histograms are not leak-subtracted.

Tim17 mutant strains of yeast with patch clamp techniques. Mitochondrial inner membranes were purified and fused with small liposomes to form giant proteoliposomes. Membrane patches were excised from proteoliposomes with a micropipette, and the conductance (which is proportional to the resistance to the current flow of ions and the pore size) was measured at various voltages in the presence and absence of molecules that can size the channel pore. Single channel properties were routinely measured to verify TIM23 channel identity. Like TOM, normal TIM23 channels have an open state conductance of 1000 pS and a half-open substate of 500 pS; most transitions in the current traces are 500 pS (Fig. 1, A and B, and Table 1) (13, 27).

The Putative Twin Pore Structure of the TIM23 Channel—The single channel behavior can shed light on the pore structure of the TIM23 channel. TIM23 behavior could be described by any of the three models in Fig. 1, C–E. Changes in the radius of the “iris-like” pore of Fig. 1C could account for the open (1000 pS), half-open (500 pS), and closed (0 pS) states. This type of behavior is typified by voltage-dependent anion-selective channel where large conformational changes reduce the radius of the open pore to one that is half-open or closed (43). Alternatiely the TIM23 channel may have two pores of equal size (Fig. 1, D and E). In both cases, the two pores are open when the conductance is ~1000 pS, one pore is open and one is closed when the conductance is 500 pS, and both are closed when the conductance is 0 pS. The transition size corresponding to opening or closing of one pore in current traces is typically 500 pS (Fig. 1, A and B). The difference between the models of Fig. 1, D and E, is that gating of the twin-sized pores of Fig. 1D is independent and that of Fig. 1E is cooperative.

The model with a single pore that changes diameter (iris-like) can be distinguished experimentally from the twin pore models by differences...
in the pore size of the open state. The iris-like model predicts a significant change in pore radius as the channel transits between the open and half-open states. Both twin pore models predict that the open and half-open states have the same pore size. The conductance of TIM23 channels was used to estimate the size of large pores by the method of Hille (39) (see "Experimental Procedures" for formula) from two different control strains. (The single channel behavior of TIM23 channels from both strains was identical (Fig. 1 and Table 1) (13, 27).) Assuming a pore length of 7 nm (38), this approach indicates that the iris-like single pore would have an open state radius of 1.43 ± 0.08 nm and half-open radius of 0.93 ± 0.03 nm (Table 2). Both twin pores are predicted to have a radius of 0.93 ± 0.03 nm. The polymer exclusion method was then used to measure the pore size to determine whether the radius actually changed during transitions between the open and half-open states.

The polymer exclusion method is a common means of measuring pore sizes and is based on an observed decrease in conductance when non-electrolytes, e.g. PEG of various molecular weights and known radii, enter the pore of the channel (3, 36, 37, 44). The presence of non-electrolytes in the pore reduces the "room" available for the current-carrying electrolytes K⁺ and Cl⁻, reducing the conductance. Impermeable non-electrolytes have no effect on the conductance once corrected for differences in the conductivity of media.

Both the transition size and the peak conductance of TIM23 channels decreased in the presence of 200–600-Da PEGs (0.5–0.8 nm) indicating that these PEG were permeable (Fig. 1, F–H). Neither were affected by the 1000–8000 molecular weight PEG (0.94–3.05 nm) indicating that these PEGs were not permeable in either the half-open or open state. A pore radius of 0.81–0.94 nm was calculated from the transition size (Fig. 1, J and F), and 0.90–0.94 nm was calculated from the peak conductance data (Fig. 1K and Table 2). Both values are similar to the 0.93 ± 0.03-nm radius estimated from the transition size by the method of Hille (39). However, these values are significantly different from 1.43 ± 0.08 nm predicted from the peak conductance (Table 2). Hence the TIM23 channel is not an iris-like single pore (Fig. 1C) because the fully open and half-open states have the same permeability for various sized PEGs, i.e. their radii are the same. Further investigations were needed to distinguish between the two twin pore models.

Do the twin pores open and close independently (Fig. 1D) or cooperatively (Fig. 1E)? If multiple, independent channels are in a membrane patch, the total amplitude histograms should fit a binomial distribution. This is not the case for the TIM23 channel. That is, distributions simulated to fit the occupancy of two open and independent 500 pS channels (O) poorly fit the observed occupancy for the two closed (C) or one open/one closed (S) states (Fig. 1, L and M). The closed state is almost never occupied in Fig. 1L, and the substrate is occupied much less in Fig. 1M than that predicted by a binomial distribution. Because the distribution of occupancy of the three states in the total amplitude histogram is not binomial at voltages ±30 mV, the twin pores are not independent. Instead they cooperatively gate. (Another example of cooperative gating is in the amplitude histogram of Fig. 5A, upper panel.) Hence the twin pore model of Fig. 1E accounts for the observed cooperative gating and the pore size determinations for the TIM23 channel.

In summary, the single channel behaviors of the TIM23 and TOM channels are nearly identical and consistent with a twin pore structure that cooperatively gates as described above. Although the TOM pore is composed of only Tom40p, it is not known whether Tim23p forms the TIM23 pore alone or in conjunction with Tim17p. Several mutants were examined to

### Table 1
Comparison of the electrophysiological properties of TIM23 channels

|                     | Wild-type mitoplasts | Wild type | Tim17(Gal10)+Gal | Tim17(Gal10)−Gal |
|---------------------|----------------------|-----------|------------------|------------------|
| Peak conductance (pS) | 1200                 | 1160 ± 40  | 985 ± 74         | 679 ± 114        |
| Transition size (pS)  | 500                  | 490 ± 43 (n = 10) | 494 ± 39 (n = 10) | 0 (n = 15)       |
| Mean open time (ms) (+20 mV) | 7                  | 10.6 ± 4.3 (n = 13) | 11.14 ± 4.9 (n = 11) | 0 (n = 15)       |
| Voltage-dependent Gating charge | Yes          | Yes       | Yes              | No               |
| Voltage-dependent | −4.7 ± 1.1           | −4.2 ± 0.7 (n = 20) | −3.9 ± 0.7 (n = 7) | 0               |
| v_i (mV)             | 5–35                 | 50 ± 10 (n = 20) | 38.5 ± 7.7 (n = 7) | NA              |
| Permeability, K⁺/Cl⁻ | 6.0                  | 5.0 ± 0.3 (n = 20) | 5.8 ± 1.6 (n = 6) | 5.4 ± 1.9 (n = 10) |
| Cooperatively gate    | Yes                  | Yes       | Yes              | NA               |
| Peptide sensitivity   | ND                   | Twin      | Twin             | Single           |
| Pore structure        | Voltage-sensitive  | Twin      | Twin             | Single           |
|                      | Flicker              | Flicker   | Flicker          | Closure          |
|                      | Closure              | NA        | NA               | NA               |

### Table 2
Sizing the radius of the TIM23 channel

|                     | Wild type | Tim17(Gal10)+Gal | Tim17(Gal10)−Gal |
|---------------------|-----------|------------------|------------------|
| Polymer exclusion*  | 0.81–0.91 (n = 5) | 0.88–0.94 (n = 5) | NA               |
| Transition size (nm) | 0.90–0.94 (n = 5) | 0.95–1.14 (n = 7) | NA               |
| Pore resistance*    | 0.93 ± 0.03 (n = 10) | 0.94 ± 0.04 (n = 10) | NA               |
| Transition size (nm) | 1.33 ± 0.05 (n = 8) | 1.10 ± 0.09 (n = 15) | NA               |
| Peak conductance (nm) | 1.93 ± 0.08 (n = 10) | 1.33 ± 0.05 (n = 8) | 1.10 ± 0.09 (n = 15) |

* Calculated from the peak conductance assuming a pore length of 7 nm (38).
Twin Pore Structure and Voltage Gating of the TIM23 Channel

FIGURE 2. Behavior of TIM23 channels after depletion of Tim17p. A, Western blots are shown for mitochondrial inner membranes from Tim17(Gal10) yeast after 24 h of growth with (+gal) or without (−gal) galactose. B, radiolabeled preprotein Su9-(1–69)-DHFR was incubated for 5 min with mitochondria of Tim17(Gal10) strain grown with (+gal) or without (−gal) galactose. Preprotein (p) and mature protein (m) were separated by SDS-PAGE and detected by autoradiography as shown. Mature protein was quantified by densitometry. Plots show the amount of pSu9-(1–69)-DHFR imported as a function of time into mitochondria normalized to the amount imported after 10 min by mitochondria grown with galactose. C and D, single channel current traces are shown at +50 mV and were recorded from excised patches of proteoliposomes containing inner membranes from Tim17(Gal10) + Gal (C) and Tim17(Gal10) − Gal (D). E and F, the current-voltage plots between ±50 mV obtained from the total amplitude histograms of two single channels from Tim17(Gal10) + Gal (E) and from Tim17(Gal10) − Gal (F) are shown. The slope of linear regression indicates that the conductance levels for the open (O), substate (S), and closed (C) states were 1200, 710, and 210 pS, respectively, for the Tim17(Gal10) + Gal channel. The Tim17(Gal10) − Gal channel exhibits only one open state of 830 pS. Data are not leak-subtracted. G, current traces are shown of TIM23 channels from Tim17(Gal10) + Gal and Tim17(Gal10) − Gal at +20 mV. Current traces are shown before (Control) and after sequential perfusion of the bath with 20 μM SynB2 and then 20 μM yCoxIV(1–13). H, histograms of flicker rates (number of transition events/s) are shown in the absence (Control) and the presence of SynB2 or yCoxIV(1–13) for the TIM23 channels from Tim17(Gal10) + Gal and Tim17(Gal10) − Gal. Other conditions are as in Fig. 1.

determine the role of Tim17p in the twin pore structure and gating of the TIM23 channel.

Depletion of Tim17p Modifies the Architecture of the TIM23 Pore—Depletion of Tim17p by the removal of galactose from the growth medium of Tim17(Gal10) yeast did not significantly modify the expression levels of some other components of the TIM23 complex as shown in the immunoblot of Fig. 2A. But these −Gal mitochondria were protein import-incompetent as they imported significantly less Su9-(1–69)-DHFR than mitochondria of the same strain grown in the presence of galactose (Fig. 2B) (28).

The effects of depleting this protein on TIM23 channel activity were then determined by patch clamping proteoliposomes containing inner membranes from mitochondria with normal or depleted levels of Tim17p. As described above, the TIM23 channel activity from this strain grown with galactose is identical to that of wild-type mitochondria (Figs. 1, A and B, and 2C and Table 1) (13, 27). However, striking differences were seen in the TIM23 channels after depletion of Tim17p. (Note that a few normal TIM23 channels were detected after depletion of Tim17p, but as shown in supplemental Fig. S1, a low level of Tim17p expression through the leaky Gal promoter could account for these channels in Tim17gal10-Gal mitochondria.) Tim17p-depleted channels had a conductance of ~700 pS (Fig. 2D). The usual flickering between open, sub-, and closed states was eliminated, and the mean open time was dramatically longer than wild type (Table 1). This activity showed no voltage dependence as the channel remained open regardless of voltage (Fig. 2F). Thus, the gating properties of TIM23 channels were abolished by depletion of Tim17p.

Wild-type TIM23 channels flicker (downward deflections in the current traces at positive voltages) more rapidly between conductance levels in the presence of synthetic signal peptides whose sequences mimic that of the targeting region of many mitochondrial preproteins, e.g. yCoxIV(1–13) and yCoxIV(1–22) (45). This increase in flickering is reversible and voltage-dependent (45) and likely corresponds to peptide translocation (3). SynB2 is a control peptide whose charge and secondary structure is similar to that of signal peptides. However, SynB2 does not modify TIM23

FIGURE 2A. Western blots are shown for mitochondrial inner membranes from Tim17(Gal10) yeast after 24 h of growth with (+gal) or without (−gal) galactose. B, radiolabeled preprotein Su9-(1–69)-DHFR was incubated for 5 min with mitochondria of Tim17(Gal10) strain grown with (+gal) or without (−gal) galactose. Preprotein (p) and mature protein (m) were separated by SDS-PAGE and detected by autoradiography as shown. Mature protein was quantified by densitometry. Plots show the amount of pSu9-(1–69)-DHFR imported as a function of time into mitochondria normalized to the amount imported after 10 min by mitochondria grown with galactose. C and D, single channel current traces are shown at +50 mV and were recorded from excised patches of proteoliposomes containing inner membranes from Tim17(Gal10) + Gal (C) and Tim17(Gal10) − Gal (D). E and F, the current-voltage plots between ±50 mV obtained from the total amplitude histograms of two single channels from Tim17(Gal10) + Gal (E) and from Tim17(Gal10) − Gal (F) are shown. The slope of linear regression indicates that the conductance levels for the open (O), substate (S), and closed (C) states were 1200, 710, and 210 pS, respectively, for the Tim17(Gal10) + Gal channel. The Tim17(Gal10) − Gal channel exhibits only one open state of 830 pS. Data are not leak-subtracted. G, current traces are shown of TIM23 channels from Tim17(Gal10) + Gal and Tim17(Gal10) − Gal at +20 mV. Current traces are shown before (Control) and after sequential perfusion of the bath with 20 μM SynB2 and then 20 μM yCoxIV(1–13). H, histograms of flicker rates (number of transition events/s) are shown in the absence (Control) and the presence of SynB2 or yCoxIV(1–13) for the TIM23 channels from Tim17(Gal10) + Gal and Tim17(Gal10) − Gal. Other conditions are as in Fig. 1.
channel activity, and the sequence does not target preproteins to mitochondria (45, 46).

Although the TIM23 channels depleted of Tim17p were still affected by signal peptides, the response was markedly different from that of wild-type and +Gal channels. The current traces and histograms of mitochondria of Tim17(Gal10) + Gal show the same rapid flickering as those of wild-type strains (Fig. 2, G and H) (45). In contrast, signal peptides induced a closure of Tim17p-depleted channels (Fig. 2, G and H) that was not reversible as repeated replacement of the bath solution to remove the peptides did not reopen the channel. Therefore, signal peptides were recognized, but the normal response of flickering was not induced. This finding indicates that Tim17p is not requisite for recognition of signal peptides, but this component is essential, either directly or indirectly, for the increase in flickering normally induced by signal peptides.

Depletion of Tim17p disrupted the twin pore structure of the TIM23 channel. We estimated the pore size using the polymer exclusion method from the peak conductance because Tim17p-depleted channels showed no transitions in current traces regardless of voltage (Fig. 2D). The peak conductance of Tim17p-depleted channels decreased in the presence of 200–1450-Da PEGs indicating that these PEGs were permeable (Fig. 3A); normal TIM23 channels were permeable to 200–600-Da PEG (Fig. 1, F–H). These data show that the pores of the Tim17p-depleted channels were slightly larger than normal as they also allowed permeation of 1000- and 1450-Da PEGs, whereas the wild-type channels did not. Plots of the second derivative of the relative conductance and polymer radius predicted a pore size of 0.81–0.94 nm for normal TIM23 channels (Fig. 1, I–K) and a slightly larger 0.95–1.14-nm pore for those depleted of Tim17p (Fig. 3B and Table 2). Interestingly these channels maintained their poor ion selectivity (Table 1). These data indicate that TIM23 channels depleted of Tim17p are single, not twin, pores because no transitions to half-open states were seen in the current traces, and the pores sizes estimated by the methods of Hille (39) and polymer exclusion agree (Fig. 3E and Table 2). In summary, Tim17p is essential to the twin pore structure of TIM23 channels as depletion of this protein resulted in channels that were single pores and voltage-independent.

**Twin Pore Structure and Voltage Gating of the TIM23 Channel**

**FIGURE 3.** Depletion of Tim17p modifies the size of the pore and the cooperative gating characteristics of TIM23 channels. A and B, estimation of the pore sizes of the TIM23 channels of the Tim17p1 grown without galactose (–gal) was done by the polymer exclusion method. A, the relative peak conductance is shown in the presence (g) and absence (g0) of various PEGs as described in Fig. 1. B, the second derivative of the data of A reveals the restriction radii for small and large non-electrolytes. C, a total amplitude histogram is shown for 30 s of the current trace at +30 mV shown in D. D, single channel current traces recorded at +30 mV from proteoliposomes containing inner membranes from Tim17p1 grown without Gal. The dotted line corresponds to f = 0 pA. Other conditions are as in Fig. 1. E, model of TIM23 channel shows that the “twin pore” structure that cooperatively gates becomes a “single pore” after depletion of Tim17p. O corresponds to the open state.

**TABLE 3**

Characteristics of Tim17 mutant channels

| Mutant | Tim17ΔC | Tim17ΔN | Tim17DD→RK | Tim17DD→RK/RK→DD |
|--------|---------|---------|------------|------------------|
| Pore structure | Modified twin | Modified twin | Single | Modified twin |
| Frequency (% patches) | 100 (n = 8) | 27 (n = 7) | 73 (n = 19) | 87 (n = 26) |
| Peak conductance (pS) | 1170 ± 82 (n = 4) | 1040 ± 150 (n = 4) | 759 ± 112 (n = 18) | 1190 ± 160 (n = 5) |
| Permeability, K+/Cl− | 6.4 ± 0.9 (n = 3) | ND | 5.2 ± 1.1 (n = 5) | 7.7 ± 2.4 (n = 4) |
| Peptide sensitivity | Flicker | Flicker | Closure | Flicker |

n is the number of independent patches. ND, not determined.
Twin Pore Structure and Voltage Gating of the TIM23 Channel

FIGURE 4. Deletion of the N or C terminus of Tim17p modifies the voltage gating and transition sizes of TIM23 channels. Current traces (upper panels) and current-voltage plots between 0 to 50 mV (lower panels) are shown to illustrate the behavior of TIM23 channels of wild-type (WT) (A), Tim17ΔC (B), and Tim17ΔN strains (C and D). Traces from two different channels of the Tim17ΔN strain are shown because two different behaviors were observed. The lines in upper panels show variability in transition sizes. O, S, S*, and C correspond to the open, sub-, multiple sub-, and closed states, respectively.

FIGURE 5. Behavior of TIM23 channels after deletion of the N terminus of Tim17p. A and B, typical current traces and total amplitude histograms (30-s duration, +40 mV) are shown for modified twin- (A) and single (B) pore behaviors of the Tim17ΔN strain before (top) and after (bottom; yCoxIV-(1–13)) perfusion of the bath with 20 μM yCoxIV-(1–13). O, S, and C correspond to the open, multiple sub-, and closed states, respectively. C, the polymer exclusion method was used to estimate the size of the TIM23 channel single pore from the Tim17ΔN strain. The left panel shows the relative peak conductance in the presence (g) and absence (g0) of various PEGs. The right panel shows the second derivative of the data of C corresponding to the restriction radii for small and large non-electrolytes. Because of the multiplicity of substrates, the same approach could not be applied to channels showing the modified twin pore behavior.

Unlike the C terminus, the N terminus of Tim17p is highly conserved. Several mutants, including N-terminal deletions, severely diminish the import of preproteins that depend on the TIM23 complex for translocation (29). Deletion of the first 11 amino acids of Tim17p (Tim17ΔN) generated two different channel activities (Figs. 4, C and D, and 5). As shown in Table 3, most of the Tim17ΔN channels behaved like the Tim17p-depleted channels (Tim17(Gal10)–Gal, Fig. 3), suggesting that Tim17p may have failed to incorporate into the TIM23 complex. The single pore channels had no transitions and no voltage dependence (Figs. 4C and 5B). The Tim17p-depleted channels, the size of the single pores of the Tim17ΔN channels was slightly larger (0.92–1.21 nm (Fig. 5C) than the wild type (0.81–0.91 nm). The remaining Tim17ΔN channels more closely resembled the Tim17ΔC channels as the transition sizes were variable, and some other characteristics, i.e. peak conductance and effects of signal peptides, were not modified (Figs. 4 and 5). Like wild type, these Tim17ΔN channels displayed cooperative gating as the amplitude histograms were not easily fit by binomial distributions (Fig. 5A). However, in contrast to wild-type and Tim17ΔC channels, these Tim17ΔN channels were not voltage-dependent (Fig. 4). Hence the N terminus of Tim17p is necessary for normal voltage gating of TIM23 channels.

Further studies were undertaken to identify the voltage gate. Previously two highly conserved, negatively charged residues at positions 4 and 8 of Tim17p were changed to positively charged residues (29); this mutant is referred to as Tim17DD→RK (Fig. 6A). This mutation caused a severe growth defect and suppression of protein import. Both growth and protein import were improved by the additional replacement of two conserved positively charged residues between transmembrane domains 2 and 3 by aspartate residues (positions 80 and 83) in the Tim17DD→RK/KR→DD mutant (29). We characterized the TIM23 channel activity of these two mutants (Fig. 6). For the most part, Tim17DD→RK channels were modified twin pores with variable transition sizes and cooperative gating (Fig. 6B and Table 3). Importantly the voltage dependence of Tim17DD→RK channels was lost like that of the Tim17ΔN channels. Hence the two aspartates in the N terminus of Tim17p form, at least in part, the voltage sensor for the TIM23 channel. Despite the fact that Tim17DD→RK/KR→DD yeast grow normally and recover much of their protein import function (29), these channels do not behave normally. The Tim17DD→RK/KR→DD channels display modified twin behavior with transitions of variable sizes (Fig. 6C and Table 3). Unlike the previous mutants, these channels displayed an inverted voltage dependence (Fig. 6C), i.e. substates were occupied at low voltage, and the pores fully opened...
with high voltage of either polarity. Thus, reinstating two essential negative charges on the intermembrane space face of Tim17p restored some voltage dependence (although it is typically inverted) but does not cure the modified twin pore structure.

When applied to Tim17 mutants, signal peptides induced rapid flickering of modified twin pore channels and irreversible closure of single pores channels (Table 3). A wild-type increase in flickering was induced by signal peptides (yCoxIV-(1–13)) in Tim17ΔN (Fig. 5A), Tim17DD→RK, Tim17DD→RK/KR→DD, and Tim17ΔC channels (not shown) displaying modified twin pore behavior. In contrast, signal peptides induced irreversible closure like that of the Tim17p-depleted channels (Fig. 2E) if the Tim17ΔN (Fig. 5B), Tim17DD→RK, and Tim17DD→RK/KR→DD channels exhibited single pore behavior (not shown). In agreement with the data obtained with Tim17p-depleted channels, the N terminus of Tim17p is not essential for signal peptide recognition, but this domain is intimately involved in the response of TIM23 channels to signal peptides.

In summary, the N terminus of Tim17p is vital to the twin pore structure. Two negative charges at positions 4 and 8 are essential to the normal voltage dependence of TIM23 channels. Truncation of the C terminus resulted in changes in pore structure that had little effect on import competence or growth. Interestingly signal peptides were recognized and modified the channel activities regardless of mutations in Tim17p.

**Twin Pore Structure and Voltage Gating of the TIM23 Channel**

DISCUSSION

The TIM23 complex is responsible for translocation across or insertion into the inner membrane for many mitochondrial preproteins. Here we showed that the TIM23 complex has a twin pore structure, i.e. two equal sized pores that cooperatively open and close. Previously single particle analysis showed that the TOM and TIM22 complexes also have twin pores (25, 26, 45) suggesting a common principle in the working mechanism of the mitochondrial translocases. An important issue in the field is to define the mechanism(s) that requires a regulated twin pore structure for efficient protein import for all three of these translocases. Studies of both native and reconstituted membranes have found that TOM and TIM23 channels have an open state of 1000 pS and a half-open state of 500 pS, but the pore structure for TIM23 had not been described previously (3, 4, 13, 27, 33, 34, 45). Measurements of the pore sizes of the open and half-open states were used to establish the twin pore structure of the TIM23 channel. The finding that the open and half-open states have the same permeability to various PEGs, and hence have the same radius, is strong evidence supporting this assignment. The twin pore structure was further probed in this study by examination of a variety of mutants, most of which were characterized previously using biochemical approaches (12, 28, 29). Patch clamp techniques allowed characterization of individual TIM23 channels and distinguished modified twin and single pore behaviors in various mutants (Fig. 4–6), behaviors that were not resolved by other techniques.

Most of TOM and TIM23 channel characteristics (like size and voltage dependence) are almost the same (3, 27). Both are presequence-sensitive and translocate preproteins. However, TOM channels have a β-barrel structure composed only of Tom40p, whereas TIM23 channels are composed of α-helices mostly due to Tim23p and perhaps in part due to Tim17p.

The role of Tim17p in the TIM23 complex is a subject of speculation. Previously Tim23p and Tim17p were thought to form the pore of the channel because of their high degree of sequence homology and other studies (11, 12, 23, 48). However, the involvement of Tim17p in the pore was largely dismissed after reports showing that recombinant Tim23p formed channels (14). It has also been suggested that Tim17p might form a second, independent channel (24). Unfortunately difficulties in producing other individual recombinant subunits of the TIM23 translocase like Tim17p have excluded their electrophysiological characterization. In this study, Tim17p was found to play a principal role in regulating pore structure and voltage gating of the TIM23 channel. Nevertheless no novel channel activity could be attributed to Tim17p in a Tim23-depleted strain suggesting that Tim17p did not form pores in the absence of Tim23p (47).

The functional status of Tim17p is crucial to the twin pore structure of the TIM23 channel. The Tim17ΔC mutants were viable but displayed a modified twin pore phenotype with wild-type voltage dependence. As this mutant is protein import-competent, the modified twin pore structure is functionally operational. Modified twin pore behavior was also seen in Tim17DD→RK, Tim17DD→RK/KR→DD, and some of the Tim17ΔN channels. Although a variety of substrates were
observed, transitions of 300 pS were common in channels expressing the modified twin pore structure. Transitions of 300 pS would correspond to a pore radius of around 0.7 nm calculated by the method of Hille (39) (see “Experimental Procedures”). Although a single α-helix (~0.6-nm radius) could slip through a 0.7-nm pore, the decrease in the pore size may compromise but not prohibit translocation. Previous studies indicate that Tim17p interacts with the hydrophobic C-domain of Tim23p (23). Integrity of both the N and C termini of Tim17p may be crucial to its stable interaction with Tim23p. In contrast, single pore behavior was typically observed in the absence of competent Tim17p for all of the Tim17p-depleted, most of the Tim17ΔN, and some of the Tim17DD→RK and Tim17DD→RK/KR→DD channels. Hence the N terminus of Tim17p may provide a “pivotal switch” necessary to maintain normal twin pore structure. The single pore structure would not preclude translocation as it is slightly larger than the normal pore. However, targeting peptides cause an irreversible closure of the single pores. These “plugged” channels would be incompetent to translocate proteins.

Are these mutant phenotypes the result of instability in Tim17p or assembly of the TIM23 complex? Reconstitution procedures may weaken the stability of the complex, which may result in a dissociation of some components. However, studies with native membranes showed that Tim17p-depleted complexes contain at least Tim50p, Tim44p, Tim23p, and Tim14p (19), whereas the Tim17ΔN complexes contain at least Tim50p, Tim44p, Tim23p, Tim17p, Tim16p, and Tim14p (29). Therefore, the two phenotypes may not be the result of a loss of associated proteins but rather may be attributable to the ability of Tim17p to stabilize the twin pore structure. Modified twin pore structure may be intermediate in the collapse to a single pore structure in the Tim17ΔN channels.

Patch clamping mitoplasts revealed the in situ conductance and voltage dependence of the TIM23 channel that is, for the most part, maintained after reconstitution into proteoliposomes by dehydration-rehydration (Figs. 2 and 4 and Refs. 13, 33, 34, and 45). The voltage gating of the TIM23 channel is an essential characteristic and is responsible for maintaining a closed state in the absence of import. Opening TIM23 channels in the absence of import would depolarize the potential and uncouple oxidative phosphorylation. Binding a presequence to the complex shifts the voltage dependence and opens the TIM23 channel (41, 42). Similarly calcium-activated K⁺ channels shift their \( V_0 \) (voltage at which the channel spends half of the time open) upon ligand binding so that the open probability changes and the channels, which are always closed, now open (49). Hence binding of a ligand, like signal peptide, would enable a TIM23 channel, which is normally closed at physiological potentials, to briefly open while the presequence is inserted into the pore (41, 42).

Conversely, depletion of Tim17p or deletion of its N terminus, which eliminated or severely compromised protein import, usually rendered the complex a single open pore with essentially no voltage dependence at ±60 mV (Figs. 4 and 5). This finding is consistent with the reduced membrane potential estimates made by Meier et al. (29) as open channels would cause depolarization. Furthermore the modified twin pore channels of the Tim17DD→RK strain did not show voltage dependence (Fig. 6B). These findings suggest that Tim17p, in particular the aspartates at positions 4 and 8, are vital to the normal voltage sensing of TIM23 channels. Although inverted, a voltage dependence was observed when two negatives charges were replaced at positions 80 and 83, which is similar to the voltage dependence of recombinant Tim23p (see below). This inverted voltage dependence would render the channel open at physiological potentials, which would depolarize the mitochondria. Interestingly this strain inserted inner membrane proteins as well as wild type, although translocation remained reduced. This finding, although speculative, suggests there is yet another means of preventing stochastic opening of TIM23 channels. A recent report proposes that Tim50p maintains the permeability barrier of mitochondria by closing the TIM23 channel, which is only activated when presequences need to be translocated (50). Accordingly these essential negative charges may be vital to appropriate association of other translocon components, e.g. Tim50p, PAM18/Tim14p, and/or Tim21p (2). Thus, it appears that TIM23 channels require both a twin pore structure and normal gating properties for efficient import of proteins destined for the matrix. All of the Tim17 mutants examined in this study retained some peptide sensitivity; twin pore channels flickered, whereas single pores closed in the presence of signal peptides. These findings indicate that Tim17p is not integral to presequence recognition in agreement with studies assigning this role to the N terminus of Tim23p (14, 23).

The intimate role of Tim23p in the translocation pathway was shown in patch clamp experiments by the effects of a point mutation and antibodies on Tim23 channel activity (13, 14). Importantly seminal studies found that recombinant Tim23p formed channels in planar bilayers (14). Nevertheless significant differences exist between the channel activities of recombinant Tim23p channels or purified TIM23 complexes and the channel activities recorded from the native inner membrane by patch clamping mitoplasts or proteoliposomes. These differences include significantly smaller peak conductance and transition sizes, longer mean open times, and lack of voltage dependence for recombinant Tim23p (14, 50). Furthermore Truscott et al. (14) reported no significant changes in channel activity in planar bilayers after loss of Tim17p by destabilization of the complex in the Tim23.2 mutant, results strikingly different from the results presented here (Figs. 2–6). These marked differences in channel behavior are likely due to reconstitution and/or the influence of other components, almost certainly Tim17p and possibly Tim50p whose role in closing TIM23 was recently reported (50). Differences in membrane curvature and/or fluidity may also be contributing factors as reported in other systems (51, 52). For example, the conductance of colicin E1 channels changes from 60 to 600 pS depending on the lipid environment (51).

Both genetic and biochemical data indicate that equimolar amounts of Tim23p and Tim17p are organized in a subcomplex (22). Modifying this stoichiometry by depletion of either protein abolishes protein import (28). Tim17p and Tim23p are closely related homologues, but the function of these subunits differs. Tim23p is crucial for pore formation and largely consti-
tutes the preprotein-conducting channel in the inner membrane. Depletion of Tim17p induces a collapse of the normal twin pore structure into a single pore. Analysis of Tim17 mutants identified the N terminus as vital for normal gating and the twin pore behavior. These results show that the TIM23 channel is formed by a mainly structural subunit, Tim23p, with its voltage gate Tim17p, which together in a concerted action, mediate presequence-gated protein translocation into mitochondria through a twin pore structure.

Acknowledgments—We thank M. Brunner and I. Milisav (University of Heidelberg) for the Tim17(Gal10) yeast strain. We thank L. Dejean (New York University College of Dentistry), S. Meier (Universität Munchen), and J. Kasianowicz (New York University College of Dentistry), P. Veras-Peixoto (Universität Heidelberg) for the Tim17(Gal10) yeast strain. We thank L. Dejean (New York University College of Dentistry), S. Meier (Universität Munchen), and J. Kasianowicz (New York University College of Dentistry), P. Veras-Peixoto (Universität Heidelberg) for the Tim17(Gal10) yeast strain. We thank L. Dejean (New York University College of Dentistry), S. Meier (Universität Munchen), and J. Kasianowicz (New York University College of Dentistry), P. Veras-Peixoto (Universität Heidelberg) for the Tim17(Gal10) yeast strain. We thank L. Dejean (New York University College of Dentistry), S. Meier (Universität Munchen), and J. Kasianowicz (New York University College of Dentistry), P. Veras-Peixoto (Universität Heidelberg) for the Tim17(Gal10) yeast strain.