Awaking TIM22, a Dynamic Ligand-gated Channel for Protein Insertion in the Mitochondrial Inner Membrane

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Aqueous channels are at the core of the translocase of the outer membrane (TOM) and the translocase of the inner membrane for the transport of preproteins (TIM23), the translocases mediating the transport of proteins across the outer and inner mitochondrial membranes. Yet, the existence of a channel associated to the translocase of the inner membrane for the insertion of multitiopic protein (TIM22) complex has been argued, as its function relates to the insertion of multispansing proteins into the inner membrane. For the first time, we report conditions for detecting a channel activity associated to the TIM22 translocase in organelle, i.e. intact mitoplasts. An internal signal peptide in the intermembrane space of mitochondria is a requisite to inducing this channel, which is otherwise silent. The channel showed slightly cationic and high conductance activity of 1000 pS with a predominant half-open state. Despite their different composition, the channels of the three mitochondrial translocoses were thus remarkably similar, in agreement with their common task as pores transiently trapping proteins en route to their final destination. The opening of the TIM22 channel was a step-up process depending on the signal peptide concentration. Interestingly, low membrane potentials kept the channel fully open, providing a threshold level of the peptide is present. Our results portray TIM22 as a dynamic channel solely active in the presence of its cargo proteins. In its fully open conformation, favored by the combined action of internal signal peptide and low membrane potential, the channel could embrace the in-transit protein. As insertion progressed and initial interaction with the signal peptide faded, the channel would close, sustaining its role as a shunt that places trapped proteins into the membrane.

Most cells depend upon mitochondria to accomplish their programmed role. Along with mitochondria’s own biogenesis, functioning and death entirely rely on protein translocation. The elevated protein content of mitochondria (~700 different proteins in yeast (1)) and the four different compartments enclosed by their two membranes add to the complexity of protein trafficking pathways in this organelle. In addition, the need to maintain a low permeability of the inner membrane for coupling oxidative phosphorylation must be balanced with the passage of large molecules as proteins through water-filled channels. Focused on the route to their final destination inside mitochondria, three multisubunit complexes or translocases are depicted as the main machineries for specific recognition, import, and sorting of precursor proteins synthesized in the cytoplasm (for review, see Refs. 2–7).

The translocase of the outer membrane (TOM)3 is the common gate for the transport of every mitochondrial protein across the outer membrane (for review, see Ref. 8), and the TIM23 translocase imports matrix-targeted preproteins with cleavable amino-terminal presequences. Few proteins of the inner membrane (IM) containing a single transmembrane segment are dependent on the TIM23 machinery (9).

The TIM22 translocase mediates the insertion in the IM of multispansing membrane proteins carrying internal targeting signals (5, 10). When this type of precursor reaches the intermembrane space, it is met by small soluble Tim proteins arranged in subcomplexes (Tim9p-Tim10p or Tim8p-Tim13p) (11–13) to be transferred to the TIM22 complex (14, 15). This insertion complex contains the integral proteins Tim22p, Tim18p, Tim54p, and the peripheral membrane protein Tim12p (14, 16, 17).

With the direct application of patch clamping to mitochondrial membranes, we have shown that channels are essential to the TOM and TIM23 translocases. The two channel activities of these translocases are remarkably similar and highly conserved among mammals and yeast, in accordance with their analogous function in protein translocation (6, 18). Slightly cation-selective and highly conductive channels of ~1000 pS with a predominant half-open state of ~500 pS have been described in proteoliposomes containing purified outer or inner membranes. The versatility of patch clamping combined with biochemical and

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§ The abbreviations used are: TOM, translocase of the outer membrane; TIM23, translocase of the inner membrane for the transport of preproteins; TIM22, translocase of the inner membrane for the insertion of multitiopic proteins; IM, inner membrane; pS, picosiemen(s).
genetic alterations of these two translocases has been essential to tackling their structure-function relationships and to pin down their subtle differences (6, 7, 19).

Yet, the existence of a channel associated to the TIM22 translocase has been elusive and remains more arguable, as its function relates to the insertion of multispanning proteins into the inner membrane of mitochondria. Importantly, recombinant Tim22p has been reported to form channels when the protein is heterologously overexpressed, detergent-purified, renatured in proteoliposomes, and inserted into bilayers (20). The activity reported somehow diverges from that of the detergent-solubilized translocase when reconstituted in proteoliposomes and fused with planar bilayers (21).

To approach the basic question related to the existence of a channel activity intrinsic to the TIM22 translocase, in this study, the native membranes of mitochondria just devoid of most of the outer membrane (i.e. mitoplasts) were directly patch-clamped. In addition, vesicles fused with inner membrane fragments were characterized with this method. For the first time, we report the conditions to detect the channel activity inherent to the TIM22 translocase. The presence of an internal signal peptide was a requisite to triggering this channel, otherwise silent regardless of voltage. The effect of this internal signal peptide was side-dependent. Increasing concentrations of the peptide in contact with the intermembrane space side of mitoplasts progressively and cooperatively opened the channel. The properties of the TIM22 channel were remarkably similar to those of TIM23, in agreement with their purportedly analogous function at early stages of protein translocation. Still, conditions to discern both inner membrane channels are detailed. Interestingly, a low membrane potential was required to keep the TIM22 channel in its fully open conformation. These findings have a major implication for our current understanding of protein trafficking into the inner membrane of mitochondria and its modulation by signal peptides, as they portray TIM22 as a dynamic channel solely active in the presence of its cargo proteins. In its fully open conformation, favored by the combined action of internal signal peptide and low membrane potential, the channel could embrace the in-transit protein segments. As the process advanced and initial interaction with the internal signal peptides faded, the channel closed, sustaining its role as a shunt for the insertion of trapped proteins into the inner membrane.

**EXPERIMENTAL PROCEDURES**

**Isolation of Mitochondria and Preparation of Mitoplasts and Proteoliposomes**—Two strains of *Saccharomyces cerevisiae*, Tim23(Gal10), and Tim22(Gal10), in which the expression of *tim23* or *tim22* genes is controlled by a Gal10 promoter, were used (22). Cells were cultivated at 30 °C on SDLac medium with 0.6 M sorbitol, 10 mM Tris, pH 7.4, 1 mM EDTA, 0.2% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride containing protease inhibitor mixture (P8215; Sigma). Mitochondria mostly devoid of the outer membrane, i.e. mitoplasts, were prepared by the French press method (23). For reconstitution experiments, inner membranes were further purified according to Mannella (24). Membrane purity was routinely assessed, and cross-contamination with the outer membrane was typically <10%. Inner membranes were reconstituted into giant proteoliposomes by dehydration-rehydration as previously described (18, 25) using soybean 1-α-phosphatidylcholine (Sigma Type IV-S).

**Patch Clamping Techniques**—Patch clamp experiments were carried out directly on mitoplasts and on proteoliposomes containing purified mitochondrial inner membranes. Briefly, membrane patches were excised after the formation of a gigaseal using microelectrodes with ~0.4-μm-diameter tips and resistances of 10–30 MΩ. Unless otherwise indicated, the solution in the microelectrode and bath was 150 mM KCl, 5 mM HEPES, pH 7.4. The voltage clamp was established with the inside-out excised configuration (26) using a List-Medical D-61100 patch clamp amplifier. Voltages across excised patches were reported as bath potentials. The open probability, *P*<sub>o</sub>, was calculated as the fraction of the total time the channel spent in the open state from total amplitude histograms generated from current traces (40–60 s duration) with WinEDR Software (courtesy of J. Dempster, University of Strathclyde, UK). *V*<sub>c</sub> is the voltage in which the channel spends half of the time open (*P*<sub>o</sub> is 0.5). Filtration was 2 kHz with 5 kHz sampling for all analysis and current traces shown, unless otherwise stated. Permeability ratios were calculated from the reversal potential in the presence of a 150:30 mM KCl gradient as previously described (18). Signal peptides were introduced in the pipette. Alternatively, the 0.5-ml bath was perfused with 3–5 ml of medium containing the desired peptides. Flicker rates were determined from current traces (40–60 s) as the number of transition events per second from the open to lower conductance states, with a 50% threshold (~250 pS) of the predominant event.

**Immunoblotting**—Mitochondrial proteins were separated by SDS-PAGE (27) and electrotransferred onto polyvinylidene difluoride membranes. Indirect immunodetection employed chemiluminescence (ECL by Amersham Biosciences) using horseradish peroxidase-coupled secondary antibodies. Membrane proteins (1–5 μg/line) were labeled with rabbit polyclonal antibodies against yeast Tim23p or Tim22p. Anti-yeast voltage-dependent anionic channel, mouse monoclonal antibody was purchased from Molecular Probes-Europe. ImageJ version 1.34s (courtesy of NIH) was used to estimate the amounts of Tim22p and Tim23p and to assess the cross-contamination of the inner membranes. These values were extrapolated from the linear correlations obtained with increasing amounts of membranes.

**Peptides**—Peptides were prepared by the Molecular Resource Facility of New Jersey Medical School (Newark, NJ). The internal signal peptide P2 was composed, in part, of an intermembrane space loop and the beginning of the fifth transmembrane segment of the phosphate carrier (TSTTLNLSSLGLT) (28). P2 was dissolved in 0.2% Me<sub>2</sub>SO. The presequence signal peptide (yCoxIV1–13) was based on amino acids 1–13 (MLSLRQSIRFFK) from the amino terminus of the cyto-
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chrome oxidase subunit IV of *S. cerevisiae* and was dissolved in H$_2$O. Peptide composition and purity were assessed by high performance liquid chromatography and mass spectrometry and were typically >90% pure.

RESULTS

Decreasing Tim22p Does Not Affect the Electrical Behavior of the Mitochondrial Inner Membrane—Electrophysiological techniques play a key role in the study of mitochondrial protein import channels. The approach we undertook included direct patch clamping of mitochondria or mitoplasts and isolated membranes reconstituted into liposomes. With this methodology, the properties of TOM and TIM23 channels were depicted, and structure-function correlations were established between individual components of these translocases and their defined function (6, 18, 19, 25, 29). However, to date, no channel activity can be assigned to the TIM22 translocase existent in mitoplasts. Still, proteoliposomes containing isolated mitochondrial inner membranes have not revealed any clues for its occurrence. In the search for the pore of the TIM22 translocase in native membranes, we attempted two experimental strategies based upon the use of yeast strains in which the essential genes *tim22* or *tim23* were controlled by a Gal10 promoter. As shown in Fig. 1, the expression of either Tim22p or Tim23p was reduced below 8% by withdrawal of galactose from the growth medium. In addition, our inner membranes were basically free of contaminant voltage-dependent anionic channel, the most abundant pore-forming protein of the outer membrane.

First, we pursued whether reduction of Tim22p, the core component of the TIM22 translocase, modifies the electrophysiological behavior of the IM. Lipid vesicles containing isolated inner membranes from these mitochondria showed the typical channel activity we previously reported for TIM23 (18, 29), with a peak conductance of 1000 pS and a conductance of 500 pS (Fig. 2A). As expected, the channel responded to perfusion with micromolar amounts of amino-terminal signal peptides with a significant increase in the flickering rate or rapid transitions from open to substate or closed states. Importantly, regardless of the Tim22p levels present in the membranes, the detection frequency of this channel activity remained the same. Fig. 2C shows that ~50% of the total patches displayed single (occasionally double) channel behavior. Whether the protein subsisted (control) or was drastically reduced (↓ Tim22p) from the membranes. The behavior, frequency, and electrical properties of this channel activity were thus identical to that we previously described for TIM23 in the IM of several yeast strains (18, 19, 29–31). Significantly, Fig. 2A also shows that the TIM23 channel was insensitive to perfusion with P2, an internal signal peptide of the phosphate carrier (28).

Decreasing Tim23p Eliminates the Channel Activity of the Mitochondrial Inner Membrane—A different strategy consists of removal of Tim23p, the core component of the TIM23 translocase. Patches performed with these membranes were completely silent; i.e. no electrical activity could be detected at voltages up to ±150 mV. Fig. 2B contains representative current traces showing the lack of any channel activity. In addition, perfusion with either presequences or internal signal peptides was incapable of bringing about any electrical response on these membranes. Only in 8% of ~120 different patches was some channel activity recorded (Fig. 2C). Analysis of these patches revealed the typical TIM23-like activity, sensitive to perfusion with amino-terminal signal peptides and insensitive to internal signal peptides (not shown). Most likely, this activity was because of the residual levels of Tim23p expression through the leaky galactosidase promoter, as established for analogous yeast strains (29). In addition, the channel of TIM23 was entirely independent of Tim22p. These results confirm our previous work in which the only channel activity related to protein import in the inner membrane of mitochondria was that of TIM23 (6, 7, 29).

Internal Signal Peptides Induce the Channel Activity of the TIM22 Translocase—The fact that silent patches were obtained in the absence of Tim23p presents the advantage of a system in which it is feasible to seek potential channel inducers. Internal signal peptides of carriers and other precursor proteins that use the TIM22 complex are among the possible channel triggers. P2 is a peptide present in the phosphate carrier that is specifically recognized by receptors of the TOM complex. Patches excised...
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from proteoliposomes lacking Tim23p were perfused with increasing concentrations of P2 from the nanomolar to the micromolar range. This treatment had no effect whatsoever, and the membranes remained electrically silent regardless of the voltage (Figs. 2B and 4B). Startlingly, a novel channel activity became plainly evident when the peptide was instead included inside the patch microelectrode rather than the bath. Fig. 3 illustrates the progressive opening of a novel channel activity induced in the presence of increasing concentrations of P2 in the microelectrode. The detection frequency and the top conductance of this activity followed a hyperbolic correlation with the increase in P2. Maximum levels of both parameters were reached at concentrations ~1 μM, and the AC50 values estimated were 0.3 μM. At least 100 patches were tested for each P2 concentration. The peak conductance calculated from current-voltage relationships reached 1100 ± 59 pS. Strikingly, this activity was not sensitive to amino-terminal presequences such as yCoxIV5–13, as revealed by the current traces and analysis of the flickering events per second (Fig. 3, A and C). Fig. 3C also compares the lack of effect of amino-terminal presequences on this novel channel activity with the well established increase in rapid flickering recorded on the TIM23 channel whether Tim22p was present (not shown) (18) or decreased (↓ Tim22p) in the membranes. In addition, when the same conditions (i.e. P2 included in the patch microelectrode) were reproduced using the Tim22p-deficient strain, no electrical activity such as the one outlined in Fig. 3A could be recorded in >200 different patches. Even more, induction of channel activity by P2 was specific, as in >50 different experiments, only silent patches were observed at all voltages when 20 μM yCoxIV1–13 was included in the patch pipette. Altogether, these data indicate that the activity induced by the internal signal peptide does not correspond to that of TIM23. In addition, they point to TIM22 as the complex directly involved in this ligand-gated channel activity. The frequency of TIM22 channel in inner membrane lipid vesicles was approximately four times lower than that of the TIM23 channel, in agreement with the stoichiometry ratio reported for Tim22p and Tim23p (32). We could observe no correlation between P2 concentration and a possible voltage activation of the channel (data not shown). Interestingly, low membrane poten-
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A. Current traces are shown to illustrate the progressive induction of a channel activity evoked by increasing concentrations (0, 0.5, and 5 μM) of internal signal peptide P2 included in the patch microelectrode. Recordings taken at +20 mV (upper traces) and −20 mV (lower traces) correspond to excised patches from proteoliposomes containing mitochondrial inner membranes with reduced levels of Tim23p (Control) and immediately after perfusion with 20 μM yCoxIV (1–13). Dotted lines correspond to zero current levels. The total amplitude histograms on the sides recall data taken from 30–60 s duration, B, the top conductance and the relative detection frequency of this channel activity increase with P2 concentration. The dose-response relationship in respect to the inducer P2 presents an estimated AC50 value of 0.33 μM. At least 100 patches were recorded at each P2 concentration. C, histograms of flicker rates in the presence of 5 μM P2 in the patch microelectrode are shown at several voltages in the absence (□) and presence (○) of 20 μM yCoxIV (1–13) in the perfusion chamber for active patches with reduced levels of Tim23p ( ▼ Tim23p). Rates are compared with those obtained under the same conditions ( ▼ Tim23p) when the Tim22p levels in the membranes are reduced ( ▼ Tim22p) (see Fig. 2, left current traces). The number of >250 pS transitions per second from the open state was determined using WinEDR version 2.5.9 software. Data points ± S.D. correspond to five independent patches. Other details are as described in the legend to Fig. 2.

**FIGURE 3. A new channel activity induced in the inner membrane by internal signal peptide.** A, current traces are shown to illustrate the progressive induction of a channel activity evoked by increasing concentrations (0, 0.5, and 5 μM) of internal signal peptide P2 included in the patch microelectrode. Recordings taken at +20 mV (upper traces) and −20 mV (lower traces) correspond to excised patches from proteoliposomes containing mitochondrial inner membranes with reduced levels of Tim23p (Control) and immediately after perfusion with 20 μM yCoxIV (1–13). Dotted lines correspond to zero current levels. The total amplitude histograms on the sides recall data taken from 30–60 s duration. B, the top conductance and the relative detection frequency of this channel activity increase with P2 concentration. The dose-response relationship in respect to the inducer P2 presents an estimated AC50 value of 0.33 μM. At least 100 patches were recorded at each P2 concentration. C, histograms of flicker rates in the presence of 5 μM P2 in the patch microelectrode are shown at several voltages in the absence (□) and presence (○) of 20 μM yCoxIV (1–13) in the perfusion chamber for active patches with reduced levels of Tim23p ( ▼ Tim23p). Rates are compared with those obtained under the same conditions ( ▼ Tim23p) when the Tim22p levels in the membranes are reduced ( ▼ Tim22p) (see Fig. 2, left current traces). The number of >250 pS transitions per second from the open state was determined using WinEDR version 2.5.9 software. Data points ± S.D. correspond to five independent patches. Other details are as described in the legend to Fig. 2.

**DISCUSSION**

Electrophysiological techniques applied to native and reconstituted mitochondrial membranes have provided us with a new perspective to protein translocation into and out of the mitochondria, especially in relation to the channels involved in such processes. Indeed, the channels associated with TOM and TIM23 have been identified (18, 33–35). Two of the main lines of evidence connecting a particular mitochondrial channel with any of these translocon cases rely on the effect of amino-terminal signal sequences and the outcome of different mutations involving the core components of both complexes (18, 19, 29, 30). The channel associated with the TIM22 complex has remained more elusive. Important evidence for its existence came from the work of Pfanner and co-workers (20). These authors show that recombinant Tim22p reconstituted in bilayers is capable of forming voltage-activated, single-pore channels. In addition, they reconstituted a detergent-solubilized and purified TIM22 complex into liposomes and then fused it with planar bilayers. With this approach, the currents they have recorded show the characteristics of twin pores (21). In the present work, for the first time we have described the conditions required to detect the channel activity of the TIM22 translocase. The additional relevance relies on the fact that this activity could be established in the native membranes of mitoplasts. Except for contact sites, mitoplasts are mitochondria devoid of the outer membrane, representing the closest system to native inner membranes. For a long time, the functionality of mitoplasts has been well established both on electron transport and oxidative phosphorylation processes (36, 37) as well as on protein import across the inner membrane (23, 38).

To pursue whether there is a channel associated with TIM22, we adopted two strategies based on selective mutations affecting each of the core components of both inner membrane translocases. As expected for two independent complexes, the lack of Tim22p did not modify the properties or frequency of the channel existing in TIM23. Alternatively, the lack of Tim23p prevented the activity of TIM23, as verified when a core component of the channel is absent (29). The evidence that no other channel activity gets uncovered, even at high membrane potentials, could be interpreted as the result of the nonexistence of such activity. Alternatively, an electrically silent membrane could denote a latent chan-
nel requiring a specific signal to be triggered. Signal peptides are amenable candidates to inducing an activation response. In particular, an internal signal peptide termed P2 has been reported to serve as a specific binding site for the receptors Tom20p and Tom70p of the TOM complex (28). As anticipated, P2 launched the opening of a novel electrical activity both in lipid vesicles containing inner membrane fragments but most importantly in intact mitoplasts. The activation was specific and side-dependent. Only the intermembrane space side of mitochondria was sensitive to the presence of a peptide with internal targeting information. Amino-termini

nal signal peptides, at concentrations confirmed to affect both TOM and TIM23 translocases, did not exert any effect on this new activity. These data, along with the lack of interference of Tim23p in these membranes, argue against TIM23 being responsible for the activity recorded. Importantly, in hundreds of patches, in the absence of the internal signal peptide, no channel activity could be observed, even when Tim22p was present and regardless of the voltage. Accordingly, in the absence of Tim22p, the internal signal peptide at either side of the membrane was incapable of inducing any channel activity. This evidence led us to conclude that there is a direct connection between the stimulus caused by the internal signal peptide and the onset of the channel activity of TIM22. At the same time, the distinctive sensitivity to different types of signal peptides becomes a useful tool in discriminating the activities of both inner membrane protein translocases.

Despite of their different composition, the electrophysiological properties of the fully activated TIM22 channel are remarkably similar to those reported for TOM and TIM23 translocases (6, 18). This could be conceivable in the framework of their analogous function as pores transiently trapping proteins in route to their final destination. Yet, several features are noteworthy. The latency of the TIM22 channel is an aspect at first not shared by the other translocases. Presumably, the nature of the preparation is an important factor that may answer for this observation. Something similar occurs in the case of TIM23. Whereas both isolated Tim23p and purified inner membranes form an open pore when reconstituted into bilayers or proteoliposomes (18, 33), our studies with mitoplasts account for silent membranes that only reveal TIM23 channel activity in the presence of amino-terminal signal peptides (39). In the same sense, a recent work of Meinecke et al. concludes that the intermembrane space domain of Tim50p is responsible for maintaining TIM23 closed. Presequences overcome this effect and activate this channel (40). The lack of this particular domain of Tim50p in the two reconstituted systems, as oppose to its presence in organelle explains the experimental observations outlined.

The progressive activation of TIM22 is another distinctive characteristic of this channel. Along

TABLE 1

| Property                  | Mitoplasts (n = 4) | Inner membrane vesicles (n = 4) |
|---------------------------|--------------------|-------------------------------|
| Peak conductance (pS)     | 1082 ± 111         | 1024 ± 154                    |
| Mean substates (pS)       | 505 ± 104          | 561 ± 67                      |
| Number of substates       | >9                 | >11                           |
| P9 (−40 mV → +40 mV)      | High (0.2 → 1)     | High (0.5 → 1)                |
| Gating charge (±)         | −2.1/2.0           | −1.9/1.8                      |
| Vg (mV) (±)               | 23.8/−41.8         | 47.1/−44.8                    |
| Permeability K+/-Cl−      | 8.4 ± 6.3          | 7.2 ± 1.6                     |
| N-terminal signal peptide| No effect          | No effect                     |

From Muro et al. (18).

ND, not determined.

FIGURE 4. The TIM22 channel activity of mitoplasts. A, current recordings at different voltages and corresponding amplitude histograms of patches from mitoplasts with reduced levels of Tim23p (Control) and immediately after perfusion with 10 μM yCoxIV (1–13) (right traces). Measurements were performed in symmetrical 150 mM KCl, 5 mM HEPES, pH 7.4, with 5 μM yP2 inside the patch pipette. Open (O), closed (C), and substate (S) levels are indicated. B, voltage dependence of TIM22 channel activity induced by 5 μM yP2 in the patch microelectrode and recorded in mitoplasts O or in mitochondrial inner membrane vesicles (C). Alternatively, the internal signal peptide yP2 was introduced in the perfusion medium (A). Voltage dependence is represented as the occupation of the 1000 pS open state (P9) obtained from 30–60 s total amplitude histograms versus the membrane potential (mV). Data points are the average ± S.E. of at least five independent patches. C, gating charge and Vg (voltage at P9 = 0.5) are proportional to the slope of Ln[P9/(1−P9)] versus voltage plots (95% confidence) and show that the gating charge is −2.15 and 2.03 at positive and negative potentials, respectively. Vg values are ±23 mV and −42 mV.
with the observation that transitions between noncontiguous current levels are recurrent and become increasingly frequent as the channel grows to be open4 these data stand for a large, single dynamic channel with a cooperative behavior.

Differences in the preparation may also account for the not trivial differences observed in the channel properties of TIM22. Thus, the studies with isolated Tim22p or purified TIM22 complex, after several biochemical procedures prior to reconstitution into bilayers, depict the channel-forming capabilities of these two preparations (20, 21). Nevertheless, the electrophysiological characteristics reported diverge considerably from those here described. Properties such as size, effect of internal signal peptide, and membrane voltage dependence are significantly different. According to these authors, the conductance of one fully open channel in 150 mM KCl is barely 324 pS for the isolated protein and twice as large (720 pS) for the isolated complex. Our results set up the peak conductance of >1000 pS. Given that conductance is a reflection of the pore size of the channel (41), a different structure should be behind these differences. The effect of the internal signal peptide sets another point of divergence, as it is described as an increase in rapid flickering only apparent at elevated voltages. A membrane potential as high as 140 mV is required to sense the effect of P2 on isolated Tim22p.

The combined action of membrane potential and allowing the reestablishment of the electrochemical gradient. The characteristic gradient of TIM22 would cooperate in the progressive opening along with the small aperture of the channel. This may cause a slight and brief depolarization of the inner membrane that, together with the persistent interaction of the protein in transit, would cooperate in the progressive opening along with the clamping of this protein inside the channel. The transient and most likely local depolarization, given the compartmentalization of the mitochondrial inter membrane space (42–44), would not compromise the overall permeability barrier of the inner membrane. As the internal signal sequences get inserted and initial interaction with the intermembrane space fades, the channel would close and shunt off the way, leaving the protein arrested in the membrane and allowing the reestablishment of the electrochemical gradient. The combined action of membrane potential and cargo proteins is thus responsible for the coordinated trigger and halt of the TIM22 translocase.

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