We present evidence that the mitochondrial channel, VDAC, when reconstituted into a phospholipid membrane, can catalyze the insertion of other VDAC channels. This property called “auto-directed insertion” was first proposed by Zizi and co-workers to explain observations on asymmetric VDAC channels. We found that 2 M urea or guanidinium chloride (GdmCl) caused a burst of insertions of VDAC channels when added to the same side as VDAC addition. More strikingly, when added to the opposite side they caused a 10–60-fold sustained yet reversible increase in insertion rate. Protein stabilization by sarcosine eliminated the effect of urea and GdmCl on VDAC insertion. Control experiments showed that water flow, ionic strength, osmotic force, phospholipid type, and membrane potential were not involved. Therefore, although both urea and GdmCl affect the properties of phospholipid membranes, it is more likely that these agents act either by changing the structure of the pre-inserted channels, allowing them to be more effective catalysts for VDAC insertion, or by flowing through the channels and acting on nearby VDAC channels inducing them to insert. Either way, insertion must be occurring next to pre-inserted channels. Urea and GdmCl may mimic chaperones by partially unfolding VDAC and keeping it in an insertion-competent state. “Auto-directed insertion” may ensure both correct targeting and orientation of nascent proteins in vivo.

The insertion of intrinsic membrane proteins into biological membranes is generally believed to be catalyzed by insertion machinery. Yet some proteins, such as toxins, insert spontaneously (Merrill and Cramer, 1990; Zhan et al., 1994). Indeed, there is evidence for large protein domains easily crossing membranes. Here we report experiments on a channel located in the mitochondrial outer membrane, called VDAC (mitochondrial porin), that has been reported not only to insert spontaneously into membranes but once inserted it also seems to catalyze the insertion of other VDAC channels and determine the direction of this insertion. This process, termed auto-directed insertion, was proposed by Zizi and co-workers to explain the voltage-dependent behavior of VDAC channels rendered asymmetric by a site-directed mutation (Zizi et al., 1995). Although the evidence presented was quite compelling, no direct demonstration of increased rate of channel insertion was reported. Here we report direct evidence that a VDAC channel in a membrane catalyzes the insertion of other channels.

A VDAC channel consists of one 30-kDa polypeptide and forms an aqueous pore approximately 3 nm in diameter (for review see Colombini (1994)). VDAC channels are highly conserved in mitochondria from all eukaryotic kingdoms. They exist in a high conductance, open, state at low membrane potentials (<20 mV) and enter closed states at elevated potentials using two separate gating processes. Thus channel insertion at low potentials exhibits a characteristic staircase appearance because channels rarely close. Treatments that increase or decrease the rate of insertion change the steepness of this staircase.

MATERIALS AND METHODS

All experiments were conducted using the planar phospholipid bilayer method of Montal and Mueller (1972) as revised by Schein et al. (1976) and Colombini (1987). Briefly, a Teflon chamber was divided into two compartments by a Saran partition with a 0.15-mm diameter hole. The partition was coated with 5% (w/v) asolectin or diphytanoyl phosphatidylcholine, 0.2% cholesterol solution in hexane and left to dry for 10–15 min. Aqueous solutions (1 M KCl, 1 mM CaCl₂, 1 mM MES, pH 5.8, or as indicated) were then layered in one compartment of the Teflon chamber, and 20–30 µl of a 1% (w/v) Neurospora crassa VDAC dissolved in 15% dimethyl sulfoxide, 2.5% Triton X-100, 50 mM KCl, 10 mM Tris, 1 mM EDTA, pH 7.0, solution was added to one side of the chamber (VDAC was purified as described previously, Freitag et al. (1983), as modified by Blachly-Dyson et al. (1990)). VDAC inserted spontaneously and was studied under voltage-clamp conditions.

The two compartments of the chamber were named cis and trans, respectively. The voltage in the trans side was maintained at virtual ground by the amplifier (Fig. 1). The desired voltage was applied to the cis side. Calomel electrodes were used to interface with the solution. In this paper the applied voltage for all data shown was –10 mV. The currents were converted to conductances for the convenience of the reader.

The conductance increase was indeed an increase in the number of channels. The stepwise insertion of channels is visible in most records, but the reduction in size required to display the figure makes individual insertions more difficult to see (see Fig. 4 for blown-up regions). Added urea, up to 5 M, had no significant effects on the gating of these channels.

Perfusions were performed in the cis compartment by displacing the solution present with a denser one without stirring. Usually 8 chamber volumes were used to ensure total replacement (probably overkill). A small Teflon tube linked to a separatory funnel delivered the new, denser solutions. A P-10 pipette tip was used to maintain the level of the aqueous solution by aspiration (Fig. 1). All chemicals were purchased from Sigma unless otherwise indicated.

RESULTS

The addition of triton-solubilized VDAC channels to the aqueous phase on one side of a planar phospholipid membrane results in the spontaneous insertion of some of these channels into the membrane. Typically, the addition of 2–10 µl of the VDAC-containing sample into the 4–5 ml aqueous phase re-
The calculated increase in conductance due to the addition of urea or GdmCl resulted in a marked acceleration of the rate of channel insertion. When the side to which VDAC had been added (cis side) was perfused with either GdmCl or urea (Fig. 2), a burst of channel insertions was observed. This leveled off shortly and the conductance remained steady.

The burst of insertions coincided with the time at which the perfusing solution reached the level of the channels that were already present in the membrane. This is indicated by the conductance changes immediately preceding the burst of insertions. In the case of GdmCl, the buffered 1 M KCl solution was replaced by one containing both 1 M KCl and 2 M GdmCl. In this solution (trans side unchanged), the conductance of the channels is 80% higher, based on single-channel observations (data not shown). The calculated increase in conductance due to the change in salt concentration is indicated by the horizontal arrow (Fig. 2). Note that the burst of insertions coincides with this conductance increase. This was the case for all experiments. In the case of urea perfusion of the cis compartment, the burst of insertions is preceded by a small dip because the single-channel conductance in the presence of urea is a bit smaller.

The mechanical process of perfusion was not responsible for the burst of insertions because perfusion with fresh, buffered KCl solution caused no burst of insertions. Insertion merely ceased as expected from a wash-out of the VDAC channels in solution by the perfusion process. The generation of an osmotic gradient was also not responsible for the burst of insertions because supplementing the perfusion solution with 1 M sucrose was without effect (Fig. 2).

If the concentration of urea in the perfusing solution was raised to 5 M, no burst of insertion was seen. Insertion ceased but the channels already in the membrane continued to conduct normally. Thus, high (perhaps denaturing) levels of urea inhibited the insertion of additional channels in the membrane.

A pronounced and sustained stimulation of the insertion rate was achieved if urea or GdmCl were introduced into the chamber on the opposite side of the membrane to which VDAC had been added (Fig. 3). VDAC was added to the trans side, and the cis side was perfused with 2 M GdmCl solution. As before, the increase in insertion rate was immediately preceded by a GdmCl-induced conductance increase. However, the rate of VDAC insertion did not increase transiently but increased (by a factor of 10–60) at a fairly steady rate until the membrane broke. Perfusion of the opposite side did not wash out VDAC from the trans compartment and thus allowed insertion to proceed indefinitely at the new, accelerated rate. Similar results with urea required the use of a 5 M solution (Fig. 3). When a 2 M urea solution was used, the rate of insertion was increased but by a smaller amount (2–5 fold) as compared with that observed when GdmCl was used (data not shown).

The ability of both urea and GdmCl to stimulate insertion from the opposite side of the membrane is best explained as acting through the channels already inserted into the membrane. However, these agents do change the properties of phospholipid membranes and thus could, in principle, facilitate VDAC insertion by altering the structure of the phospholipid portion of the membrane. This possibility was tested by using very different phospholipids for making the membrane and using substances that mimic the properties of either urea or GdmCl but without the ability to unfold proteins.

The stimulation of insertion by both urea and GdmCl was observed in membranes made with diphytanoyl phosphatidylcholine (DPyPC) instead of the mixed soybean phospholipids present in asolectin (Fig. 4). DPyPC has very little net charge at neutral pH as opposed to the highly negatively charged asolectin. Thus, GdmCl is probably not acting by simply screening the charges on the membrane. Being a pure lipid, DPyPC is unlikely to contain any special ingredient that would catalyze channel insertion. The possibility that GdmCl is acting by increasing ionic strength was also eliminated by perfusing with the buffered KCl solutions supplemented with 2 M NaCl (VDAC trans and NaCl cis). The NaCl had no effect, but subsequent reperfusion with 2 M GdmCl resulted in the normal acceleration of channel insertion (data not shown).

Because urea partitions significantly into the membrane, the possibility exists that it increases the volume of the membrane and thus stimulates the insertion of the channels. However, perfusion with solutions supplemented with 2–5 M glycerol...
(VDAC trans and glycerol cis) had no effect on the insertion rate (Fig. 5). The reperfusion of the compartment with 2 M GdmCl resulted in the expected acceleration of channel insertion. Because glycerol partitions as readily into a membrane as urea (Orbach and Finkelstein, 1980), it should increase the volume of the membrane to the same extent. Thus it seems unlikely that the urea acts by dissolving into the membrane.

In control experiments, both urea and GdmCl had no significant effect on membrane conductance at the concentrations used in these experiments (Fig. 4). The sign and magnitude of the applied potentials had no significant effect on the rate of insertion of VDAC channels (Table I).

To further demonstrate that the accelerated insertion was due to the action of either GdmCl or urea on VDAC protein structure, sarcosine, an osmolyte existing in some organisms, which can stabilize protein structure, was introduced into the perfusing solutions. Perfusing the opposite side (cis) of the membrane (VDAC in trans) with a buffered 1 M KCl solution supplemented with either 2 M GdmCl, 2 M urea, nothing, 1 M sucrose, or 5 M urea. The arrowhead indicates the conductance increase caused by the addition of the 2 M guanidinium chloride. Sucrose caused a large drop in conductance by changing the single-channel conductance.

FIG. 2. Records of experiments in which VDAC was added to the side of the membrane that would be perfused. The membranes were bathed in 1 M KCl, 1 mM CaCl₂, 1 mM MES, pH 5.8, the standard solution. A small aliquot of VDAC solution was added in cis side of the membrane made from soybean phospholipids. After channel insertion was proceeding at a steady rate, the membranes were perfused with about 40 ml of the above solution supplemented with either 2 M GdmCl, 2 M urea, nothing, 1 M sucrose, or 5 M urea. The arrowhead indicates the conductance increase caused by the addition of the 2 M guanidinium chloride. Sucrose caused a large drop in conductance by changing the single-channel conductance.

Fig. 3. Records of experiments in which VDAC was added to the compartment that would not be perfused (trans compartment). The experiments were performed as described in the legend to Fig. 2 except that the standard solution in the cis compartment was perfused with solution supplemented with either 2 M GdmCl, 5 M urea, or 2 M NaCl. The arrowhead in the top panel indicates the conductance increase caused by the addition of guanidinium ions. *2, the scale decreased by a factor of 2. *5, the scale decreased by a factor of 5. The decrease and increase in conductance shortly after the initiation of the perfusion in the middle and lower record, respectively, were due to changes in single-channel conductance. In these experiments, GdmCl increased the insertion rate from 5.3 to 111 channels/min, whereas urea increased it from 5 to 133 channels/min.

VDAC trans and glycerol cis) had no effect on the insertion rate (Fig. 5). The reperfusion of the compartment with 2 M GdmCl resulted in the expected acceleration of channel insertion. Because glycerol partitions as readily into a membrane as urea (Orbach and Finkelstein, 1980), it should increase the volume of the membrane to the same extent. Thus it seems unlikely that the urea acts by dissolving into the membrane.

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To further demonstrate that the accelerated insertion was due to the action of either GdmCl or urea on VDAC protein structure, sarcosine, an osmolyte existing in some organisms, which can stabilize protein structure, was introduced into the perfusing solutions. Perfusing the opposite side (cis) of the membrane (VDAC in trans) with a buffered 1 M KCl solution supplemented with 2 M GdmCl and 2 M sarcosine caused a delayed increase in VDAC insertion rate, indicating the opposite function of sarcosine to that of GdmCl on VDAC structure. If the concentration of sarcosine in the perfusing solution was raised to 4 M, no increase in VDAC insertion rate was seen. 4 M sarcosine might have completely offset the unfolding effect of 2 M GdmCl (Fig. 6). The same result could be seen when perfusing the membrane with the KCl solution supplemented with a mixture of 5 M urea and 2.5 M sarcosine, showing the similar effect of sarcosine on urea (Fig. 6). If the membrane was perfused with 1 M KCl solution supplemented with 4 M sarcosine alone, VDAC insertion was completely stopped (data not shown). All these were done on the opposite side of membrane as VDAC, indicating these agents are likely to be functioning on pre-inserted VDAC channels in the membrane.

The opposite effects of the chaotropic agents and sarcosine on VDAC insertion could also be tested by adding VDAC directly to the solutions containing these agents. Planar phospholipid membranes were made with one side bathed in buffered salt solution, the other side bathed in these agents. When a large amount of VDAC protein was added directly to solutions supplemented with 2 M GdmCl or 2–5 M urea bathing the membrane, no insertion was detected. This indicates the importance of having pre-inserted channels in the membrane to act as catalysts. Subsequent addition of VDAC to the other side of the same membrane (salt solution) resulted in fast insertion at a high rate. When VDAC was stirred into KCl solutions supplemented with 2–4 M sarcosine bathing the membrane, no inser-
tion occurred either, probably because the structure of the protein in these solutions was not flexible and thus not insertion-competent. Insertion did occur with subsequent addition of VDAC to the opposite side (salt solution) but at a much slower rate compared with the normal insertion process. Further, replacing the sarcosine solution with a solution containing 2M GdmCl resulted in the expected accelerated insertion. Moreover, when VDAC was added into a solution supplemented with a mixture of 5 M urea and 1–2 M sarcosine, the normal insertion was observed. (The best results were obtained at 1.5 M sarcosine.) If the sarcosine concentration in the mixture was higher (2.5–4 M), again, no insertion could take place, probably because the effect of sarcosine was dominating (data not shown). These observations suggest that the change in the ability of VDAC to insert is due to the opposite effects of these agents on VDAC structure and those two opposite effects can be offset by an increase in one or the other reagent. All these results are summarized in Table I.

If urea and GdmCl act by unfolding a domain or increasing the flexibility of VDAC in the membrane and thus allow VDAC to act as a better catalyst for protein insertion, the process must be cooperative. The acceleration of protein insertion varies with the urea concentration on the cis side in a highly nonlinear manner (Fig. 7). Although the variation in the amount of acceleration achieved limits the information that can be gleaned, the data can be fitted to a theoretical line if the urea concentration dependence is raised to the 17th power. While not putting too much credence in this number, its value not only indicates a high cooperativity as expected from many sites of urea interaction with the protein but also is quite small (compared with the expected number of H-bonds) indicating a small domain being affected.

Finally, the actions of urea and GdmCl are immediately reversible. Perfusing away these reagents during the accelerated insertion of VDAC reduced the insertion to normal rates just as soon as the new solution reached the membrane. The rate could once again be accelerated by restoring the urea or GdmCl (Fig. 8).

**DISCUSSION**

Urea and GdmCl are believed to act by hydrogen bonding with biological molecules more strongly than water. In this way they destabilize intramolecular hydrogen bonding and cause unfolding of proteins (Makhatadze and Privalov, 1992). By using relatively low concentrations in these experiments, an acceleration in VDAC insertion has been achieved without damaging these channel-forming proteins.
The benigneffect of these agents is demonstrated by the fact that VDAC can insert into the membranes bathed in these agents from the opposite side and form functional channels with normal voltage-dependent gating processes (data not shown). Thus the VDAC protein in the membrane is not denatured by these agents. In addition, the fact that the action of these agents is immediately reversible indicates that their effects are likely to be limited to small portions of the protein that can almost immediately refold to their previous conformation.

Many control experiments were done to test the possibility that urea or GdmCl caused the accelerated insertion by changing the phospholipid bilayer membrane. Neither these agents nor sarcosine (data not shown) changed the membrane conductance, indicating no membrane disruption. Actually, it has been reported that the lamellar phase of phospholipids such as phosphotidylethanolamine is stabilized by urea and GdmCl and is prevented from undergoing a transition to the hexagonal II phase (Yeagle and Sen, 1986). The hexagonal phase is more likely to be involved in such dynamic membrane processes as fusion, so the action of these agents on phospholipid membranes seems to be in the wrong direction. At room temperature, urea has no detectable perturbing effect on the permeability of phospholipid vesicles (Anchordoguy et al., 1990).

However, these agents do affect some properties of phospholipid membranes such as phase transition temperature. However, so does the nature of the phospholipids. By using two very different types of phospholipids to make the membranes used in these experiments and finding essentially the same results, the likelihood that lipid effects are responsible for the accelerated insertion is diminished but not eliminated. The observations that no insertion could occur if VDAC was added directly into 2 M GdmCl or 2–5 M urea also indicate that the importance of having pre-inserted channels in the membrane to catalyze the insertion process.

The ability of urea and GdmCl to accelerate insertion when added from the opposite side of the membrane makes it less desirable.
likely that any disruption of hydrogen bonding at the head groups of the phospholipids on one side of the membrane will somehow affect insertion from the other side, although structural changes could, in principle, be transmitted to the other side of the membrane. In addition, properties such as charge and lipid solubility of urea and GdmCl are quite different, and mimicking these properties with NaCl or glycerol was without effect. It is clearly the chaotropic properties of these molecules that are acting on the insertion process as supported by the antagonistic effects of sarcosine.

The effects of urea and GdmCl on VDAC can be offset by sarcosine, an osmolyte that is a protein stabilizer (Yancey et al., 1982; Santoro et al., 1992). Because urea and GdmCl are thought to change protein structure by weakening hydrogen bonds (Makhatadze and Privalov, 1992), sarcosine may do the reverse as it favors the preferential hydration of proteins (Arakawa and Timasheff, 1985). Combinations of urea and methylamines (such as trimethylamine-N-oxide, betaine, and sarcosine) are used in some organisms to increase cytoplasmic osmotic pressure without altering the flexibility of cytoplasmic proteins (Yancey et al., 1982). The opposing effects of urea and methylamines were reported to balance when the ratio of the concentrations of urea and methylamines is about 2:1. We found the same thing. A mixture of 5 m urea and 2.5 m sarcosine resulted in no change in the rate of VDAC insertion. For 2 m GdmCl, 2 m sarcosine only caused a delay in the acceleration of the insertion process. 4 m sarcosine was required to balance the GdmCl, indicating that GdmCl has a much stronger unfolding effect on VDAC than urea.

It was suggested that the use of methylamines alone might be as deleterious as the use of urea alone because high concentrations of methylamines could make some proteins too rigid to function effectively (Yancey et al., 1982). This is supported by our experiments. When VDAC was added to 2–4 m sarcosine, no insertion could be detected. However, when VDAC is added into a mixture of 5 m urea and 1.5 m sarcosine, the normal insertion process was seen, probably due to the offset of the two opposite actions on VDAC. If the opposite side of the membrane as VDAC was perfused with 4 m sarcosine alone, the normal insertion process stopped completely, suggesting that sarcosine makes the VDAC protein in the membrane too rigid and thus slows down the auto-directed insertion.

The observations can best be explained by channel insertion being a cooperative process. Channels still in solution interact with already inserted channels, and these facilitate the insertion process. The ability of urea or GdmCl in the opposite compartment to stimulate insertion can be explained by these agents acting on inserted VDAC channels inducing structural changes that result in these channels becoming better insertion catalysts.

Alternatively, these agents may cross the membrane through the aqueous pores formed by the VDAC channels in the membrane, resulting in a high concentration of these substances at the mouth of the channel on the opposite side. This could change the structure of an uninserted channel that is interacting with the one inserted in the membrane. Assuming that urea and GdmCl are simply increasing the rate of the normal insertion process, a long-lived complex between the inserted and noninserted channel could be influenced by urea or GdmCl flowing through the pore. Due to the three-dimensional nature of the diffusion process away from the mouth of the channel, the concentration of urea or GdmCl drops precipitously with distance away from the mouth and thus only proteins very close to the mouth of the channel could be affected.

The possibility that urea and GdmCl act by disturbing the phospholipid bilayer is less likely especially in view of previous observations (Zizi et al., 1995) that point toward an auto-directed insertion mechanism. Fig. 9 summarizes our view of the VDAC insertion process. This is one possible way of interpreting the data, and there is no evidence for the conformational changes proposed. This cartoon is only useful in showing that the observations can be accounted for in a mechanistic way. VDAC channels become associated with the membrane, but their insertion into the membrane is a slow process. Insertion does occur spontaneously but is a rare event that may be accelerated just after VDAC addition due to detergent-dependent partial unfolding. Once one channel inserts it acts as a catalyst for further insertions. Urea or GdmCl accelerates this process by exposing a site that allows an inserted channel to bind to an uninserted channel. The binding causes the channels in solution to insert in a directed manner next to the inserted channel. The inserted channels may remain attached forming a two-dimensional array. In this way, insertion does not proceed in an exponential manner, rather the rate remains linear with time. Alternatively, the linear rate of insertion may reflect that the process is proceeding at a diffusion-limited rate. Calculations based on the infinite-sink equation (Hille, 1992) show that the observed rates of insertion are very close to this upper limit (target size 2.5 nm in radius). The higher rates, rarely observed, seemed to be multiples of the average rate.
Auto-directed Insertion

Fig. 9. Model for VDAC insertion. Each VDAC molecule is assumed to have a binding site at one end (illustrated as a notch) and a flexible arm at the other (black rod). The end of the flexible arm could bind to the aforementioned site but does not do so because they are far apart. Binding therefore occurs to other channels potentially forming a chain. The first VDAC channel can insert in the membrane in one of two directions (top left and right). Once the first VDAC inserts, its binding site and arm face opposite aqueous phases. The channel allows urea or GdmCl to cross the membrane, forming an elevated concentration at the mouth of the channel. The presence of urea or GdmCl allows the arm to be more accessible for binding to sites on nearby channels, thus facilitating the insertion process. Thus, catalyzed insertion can occur without urea or GdmCl, but it occurs at a slower rate because the arm is less accessible. Note that irrespective of the direction of orientation of the first channel, subsequent insertions occur in the same direction as that of the first event.

This model was designed to account for the previously published observations by Zizi et al. (1995) that lead these investigators to propose the auto-directed insertion process. They concluded that the first insertion should be in a random direction, either head first or tail first into the membrane. All subsequent insertions occurred in the same direction as the first. One can easily use the same model in the figure to obtain directed insertion from the other side of the membrane. The ability of VDAC to form two-dimensional crystals was shown as early as in 1982 (Mannella, 1982). Although neither the data in this paper nor that in the previous Zizi et al. paper required the formation of two-dimensional arrays during the insertion process, such formation might account for the preferred direction of the insertion. It also helps to explain the relatively constant rate of insertion because binding sites would become buried.

The properties of the protein insertion process observed under these simplified conditions may reflect inherent properties of VDAC channels and the protein insertion process occurring in vivo.

The protein machinery responsible for the import of most mitochondrial proteins in fungal cells (N. crassa and Saccharomyces cerevisiae) has been well studied (a review by Kiebler et al. (1990); Horst et al. (1995); Gratzer et al. (1995)). Most proteins destined for mitochondria are thought to be transported across the outer membrane by the general insertion pore (GIP) (Söllner et al., 1992; Pfaller et al., 1988). Many proteins involved in precursor protein transport have been identified. For N. crassa, seven proteins (MOM19, MOM72, MOM22, MOM7, MOM8, MOM30, and MOM38) in the mitochondrial outer membrane have been discovered so far. MOM19 and MOM72 function as receptors for precursor proteins (Harkness et al., 1994; Schlossmann et al., 1994). MOM22 can transfer the precursor proteins from MOM19 and MOM72 to the GIP (Kiebler et al., 1993), which is believed to consist of 4 MOM proteins (MOM7, MOM8, MOM30, MOM38) (Söllner et al., 1992). Precursors are transported into and across the outer membrane at the GIP. For those destined for the inner compartments of the mitochondria (inner membrane and matrix), GIP passes them to the transport machinery in the inner membrane.

VDAC insertion into the mitochondrial outer membrane in N. crassa was found to be dependent on protease-sensitive receptors in the mitochondrial outer membrane (Pfaller and Neupert, 1987; Kleene et al., 1987). But bypass of the receptors can happen. Protease-treated mitochondria can still import proteins, although with low efficiency, indicating other mechanisms of transport might exist (Pfaller et al., 1989). For N. crassa, the major receptor, MOM19 was reported to be responsible for recognition and binding of VDAC (Harkness et al., 1994). The MOM22 and the GIP also seem to be involved (Kiebler et al., 1993; Nargang et al., 1995; Pfaller et al., 1988). However, deletion of the MOM19 in mitochondria greatly inhibits VDAC insertion but does not eliminate the insertion process, and the level of VDAC protein in the mitochondria from the MOM19 gene-depleted cells does not change at all (Harkness et al., 1994). Similar results were reported for MOM22-depleted mitochondria (Nargang et al., 1995). Furthermore, antibodies against MOM22 reduce the rate of VDAC insertion but do not prevent insertion (Kiebler et al., 1993). All the evidence listed above points to an alternative VDAC import mechanism. Auto-directed insertion may be this alternative pathway. How this pathway would interact with the general insertion pathway is unclear.

Both MOM19 and VDAC might function as receptors for the VDAC precursor. MOM19 accelerates VDAC insertion, but it may not be essential and may not actually be responsible for inserting the channels because antibodies against MOM19 are less effective at blocking VDAC insertion as compared with other imported proteins. In addition, antibodies against VDAC also showed some inhibition of VDAC insertion (Mayer et al., 1993). Moreover, antibodies against VDAC can stop VDAC insertion into planar phospholipid membranes (Mannella and Colombini, 1984). Experiments done by Schatz’s group on yeast VDAC showed that VDAC was able to insert into isolated mitochondria in which the receptors had been destroyed by trypsin-pretreatment (Gasser and Schatz, 1983). Recently many exceptions have been found to deviate from the general receptor pathway, suggesting different proteins can use different mechanisms to ensure correct targeting. The precursor to cytochrome c, a protein in the mitochondrial inter-membrane space, does not appear to require a receptor on the outer membrane (Mayer et al., 1995). The import of the subunit Va of cytochrome c oxidase, an inner membrane protein, does not require receptors either (Gärtert et al., 1985). A matrix protein, the mitochondrial transcription factor MTF1, does not depend on receptors, membrane potential, or ATP for transport (Sanyal and Getz, 1995).

The import of most precursor proteins into mitochondria seems to require the action of chaperones. These unfold pre-
cursor proteins or keep them in a partially unfolded, insertion-competent state (Eilers and Schatz, 1988). The insertion of VDAC into mitochondria requires ATP, presumably used by chaperones for unfolding the precursor (Pfanner and Neupert, 1987; Kleene et al., 1987). If other methods can be introduced to keep VDAC in an unfolded state, then ATP (or chaperones) may not be necessary. This is indicated by the ability of a water-soluble form of VDAC to insert into isolated mitochondria (MOM19) on the outer membrane may function to recognize the appropriate protein and help in the removal of chaperones that are associated with the newly synthesized protein. The partly unfolded protein then binds to channels already in the membrane, and these physically insert it into and through the membrane. This would ensure both proper direction of insertion and targeting to the right membrane.

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