The Role of the N and C Termini of Recombinant Neurospora Mitochondrial Porin in Channel Formation and Voltage-dependent Gating*

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To investigate the role of the N and C termini in channel function and voltage-dependent gating of mitochondrial porin, we expressed wild-type and mutant porins from Neurospora crassa as His-tag fusion products in Escherichia coli. Large quantities of the proteins were purified by chromatography across a nickenitrotriacetic acid-agarose column under denaturing conditions. The purified His-tagged wild-type protein could be functionally reconstituted in the presence of detergent and sterol and behaved in black lipid bilayer membranes indistinguishably from native porin isolated from Neurospora crassa mitochondria. Mutants of porin lacking part of the N terminus (ΔN2-12porin, ΔN3-20porin), part of the C terminus (ΔC269-283porin), or both (ΔN2-12/ΔC269-283porin) also showed channel forming activity. The mutant porin lacking the C terminus had a smaller single channel conductance than the wild-type protein, but its other biophysical properties were identical. ΔN2-12porin and ΔN3-20porin formed noisy channels with decreased channel stability. These channels were still voltage-dependent. ΔN2-12/ΔC269-283porin lost channel stability and had altered gating characteristics. These results are discussed with respect to different models that have been proposed in the literature for the structure of mitochondrial porin channels.

The mitochondrial outer membrane separates the internal compartments of the mitochondrion from the cytoplasm. To allow the exchange of the mostly anionic metabolites between cytosol and intermembrane space, it has to act as a molecular sieve for hydrophilic solutes (O'Brien and Brierly, 1965; Pfaff et al., 1968). An outer membrane protein, the mitochondrial porin (Zalman et al., 1980; Roos et al., 1982) or voltage-dependent anion-selective channel (Schein et al., 1976; Colombini, 1979), is responsible for small molecule permeability. Mitochondrial porins form water-filled channels that are slightly anion-selective in the open state and cation-selective in the voltage-induced ion-permeable "closed" states (Benz, 1994).

According to reconstitution experiments with planar lipid bilayers and liposomes (Colombini, 1980; Freitag et al., 1982; Lindén et al., 1982), the mitochondrial pore has a diameter of about 2-3 nm in the open state, which agrees well with the electron microscopic analysis of mitochondrial outer membranes (Mannella et al., 1986; Mannella et al., 1989; Guo et al., 1995). Reconstitution of water-soluble porin into artificial membranes requires a detergent, such as Triton X-100 (Pfaller et al., 1985), and cholesterol. In particular, sterols seem to be important for association of the channel unit in the aqueous phase before its insertion into the membrane, rather than for association with the membrane (Popp et al., 1995).

The primary sequences of many eukaryotic porins are known at present (Mihara and Sato, 1985; Kleene et al., 1987; Trolle et al., 1992; Hains et al., 1994; Kayser et al., 1989; Fischer et al., 1994; Blachly-Dyson et al., 1993, 1994; Ha et al., 1993). The channel properties such as single channel conductance, voltage dependence, and selectivity are highly conserved among them. However, the overall identity of their primary structures is relatively low. Nonetheless there exists a high homology in their predicted secondary structures. Notably, all porins contain stretches of alternating hydrophobic and hydrophilic amino acids, suggesting the formation of amphipathic β-strands (for review see Benz, 1994). Thus, the polypeptide chain of mitochondrial porins may be arranged in a β-barrel cylinder containing either 16 antiparallel, amphiphilic β-strands (De Pinto et al., 1991; Benz, 1994) or 12 β-strands and the amphiphilic N-terminal α-helix (Blachly-Dyson et al., 1990). As a result of these predictions, the models to explain the gating of the mitochondrial outer membrane channel differ to a great extent. The latter structural model suggests large scale rearrangements of the channel core during closing, including trans-membrane movement of the N terminus and/or β-strands (Peng et al., 1992; Thomas et al., 1993; Zizi et al., 1995). The former model assumes that the movement of loops not embedded in the membrane is responsible for channel gating, as is the case in bacterial porins (Benz, 1994). To investigate the channel structure in more detail, we created deletion mutants of Neurospora crassa porin and analyzed the biophysical properties of the mutant channels. Specifically, we studied the role of the N terminus in channel formation and voltage dependence. In addition, we asked whether the C terminus contributes to the β-barrel structure.

EXPERIMENTAL PROCEDURES

Materials—The plasmid pQE-9 and nickel-nitrotriacetic acid-agarose were obtained from Qiagen (Hilden, Germany). Ceramic HTP was bought from Bio-Rad (München, Germany) and ergosterol (cholest-5,7,22-trien-24-methyl-3β-ol) from Sigma. Genapol X-80 was obtained from Union Carbide (New York, NY). The abbreviations used are: HTP, hydroxyapatite; DiphPC, diphytanoyl-phosphatidylcholine; His-tag, hexa-histidyl tag; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; nS, nanosiemens.
from Fluka (Buchs, Switzerland). Media were purchased from Difco (Augsburg, Germany). All salts and buffers were of analytical grade and obtained from Merck (Darmstadt, Germany).

Purification of Native Porin—The standard laboratory strain of N. crassa, strain 74A, was grown and maintained as described (Davis and De Serres, 1970). Mitochondria were isolated from mycelia grown in liquid culture as previously outlined (Pfanner and Neupert, 1985), and porin was prepared from whole mitochondria and purified by chromatography over an HTP column essentially as described previously (De Pinto et al., 1987a, 1987b), except that Triton X-100 was exchanged by Genapol X-80 and normal HTP by ceramic HTP.

Construction of Porin Mutants—The porin constructs used in this study were generated from the cDNA clone obtained by Kleene et al. (1987). It was recloned in a pBluescript KS vector (Stratagene, La Jolla, CA). To produce the coding sequence for ΔN2–12porin, the BamHI-NarI fragment containing the first 229 base pairs of the porin coding sequence was replaced with a PCR product generated with primers encompassing the corresponding coding region but lacking the first 12 amino acids. The upstream primer (5′AAAGGATCCATGGCTTTCTACCACCTCGCTG) incorporates sites for BamHI and Ncol and an in-frame start codon into the 5′-end of the PCR product. The second oligonucleotide (5′CCTGAAATGGAGGTTGAACTTGGC) hybridized to a sequence just downstream of the endogenous NarI site. The resulting mutant genes in pBluescript were then cloned into pQE-9 as BamHI-EcoRI fragments encompassing the entire coding sequences. This strategy resulted in the addition of the amino acid sequence MRGSHHHHHHGS (single-letter code) upstream of the original initiation methionine (Fig. 8). The six histidine residues comprise a “His-tag” which allows purification of the protein as described below. In a similar manner, oligonucleotides 5′AAAGGATCCATGGCTGTTCGAGTCTCCTCCTACACACACTGCTG were used as upstream primers to generate the coding sequences for a His-tagged, full-length porin and for ΔN3–20porin, respectively. The ΔC269–283porin and ΔN2–12/ΔC269–283porin clones were produced by digesting either ΔN2–12porin or the His-tagged, wild-type DNA (in pQE-9) with HindIII and religation. This resulted in the deletion of amino acids 269–283 and the addition of a single glutamine residue at the C terminus (Fig. 8). The stop codon was encoded by the vector. All PCR-derived DNA fragments were sequenced using the Sequenase kit (U.S. Biochemical Corp.). No undesired alterations were found except for a silent mutation in codon 67 (GGA mutated to GGT) in ΔN2–12porin and its derivative ΔN2–12/ΔC269–283porin.

Expression and Purification of Recombinant Porins—An overnight culture in DYT medium (16 g of Bacto-tryptone, 10 g of yeast extract, and 5 g of NaCl dissolved in 1 liter of distilled water) of the E. coli DH5α strain (M15 (Villagero and Zabin, 1974) containing the respective vector was diluted and grown to an A600 of 0.7–0.9. Expression was induced by adding isopropylthio galactoside to a final concentration of 2 mM, and the culture was grown for an additional 4–5 h before harvest.

Native Porin

Hist6porin

![Fig. 1. Purified wild-type and mutant recombinant mitochondrial porins.Various forms of porins, as indicated, were expressed in E. coli and purified as described under “Experimental Procedures.” Aliquots of the resulting samples were analyzed by SDS-PAGE and staining with Coomassie Blue. Wild-type porin was run for comparison.](image)

![Fig. 2. Histogram of conductance fluctuations of native and recombinant Hist6porin.](image)
the stock solutions to the aqueous phase of the cis compartment (the compartment to which the voltage was applied) after the membranes had turned optically black in reflected light.

RESULTS

Expression, Purification, and Reconstitution of the Recombinant Porins—Previous studies of mutant mitochondrial porins utilized proteins that had been expressed in yeast (Blachly-Dyson et al., 1994). These mutant proteins had to be isolated and purified from yeast mitochondria. We chose an bacterial expression system, which allows the rapid and simple production of large amounts of mitochondrial porin. To ease purification, recombinant mitochondrial porin was expressed with an N-terminal hexa-histidinyl tag (His-tag). Full-length, His-tagged recombinant mitochondrial porin was expressed with an expression system, which allows the rapid and simple production of large amounts of mitochondrial porin. To ease purification, recombinant mitochondrial porin was expressed with an N-terminal hexa-histidinyl tag (His-tag). Full-length, His-tagged recombinant mitochondrial porin (which will be referred to as His6porin) and different deletion mutants could be obtained in high amounts from the E. coli cells. The expression level for all proteins was around 50 μg of recombinant protein/ml of bacterial culture. The purified protein samples were free of contaminating bacterial protein as judged by SDS-PAGE (Fig. 1). The denatured protein could be functionally renatured by dilution into a buffer that contains 2% Genapol X-80 to prevent aggregation of the protein. This protein stock could be used after further dilution and preincubation with ergosterol (the major sterol from fungi) for reconstitution experiments.

The Recombinant His-tagged Mitochondrial Porin Behaves Like the Native One—We first compared the properties of wild-type porin carrying an N-terminal His-tag with those of “native” mitochondrial porin purified from N. crassa mitochondria. The recombinant His-tagged protein was added to the aqueous phase bathing a lipid bilayer membrane. Channel formation was not observed until ergosterol was added (see below). In similar experiments we added native porin (isolated from mitochondria) to the membranes. We did not observe any significant difference in the distribution of the single channel conductances (Fig. 2) of channels formed by the two types of porins. Furthermore, the voltage dependence, a characteristic feature of the mitochondrial porins, was identical for both porins (Fig. 3). Likewise, no difference was seen in the ion selectivity of the two preparations (see Table I). Thus, the purified His-tagged mitochondrial porin behaves indistinguishably from native porin isolated from mitochondria.

Channel Forming Activity of the Mutant Proteins and Influence of Sterol on Channel Insertion at Different Salt Concentrations—Purified mutant porins carrying an N-terminal His-tag were used to study the roles of the N and C termini in porin structure and function. ΔN2–12 porin and ΔN3–20 porin lack part or all of the potentially amphipathic α-helix at the N terminus of the protein. ΔC269–283 porin lacks the sequence predicted to form the final one or two β-strands (see Fig. 8), and ΔN2–12/ΔC269–283 porin contains a combination of the deletions found in ΔN2–12 porin and ΔC269–283 porin. The N-terminal sequences deleted in the first two mutants are predicted to cross the membrane (Blachly-Dyson et al., 1990) or to lie at the surface of the outer membrane facing either the cytoplasm (De Pinto et al., 1991) or the intermembrane space (Stanley et al., 1995). Channels were observed when each of the deletion mutant porins was added to the salt solution bathing lipid bilayer membranes (Fig. 4). However, the channel forming activity, i.e. the conductance increase for a given protein concentration, was about 10-fold lower for all deletion mutants in comparison to the His6porin (data not shown). The reason for this is unknown, but it is possible that imperfect folding of part of the protein caused the reduced channel forming activity of the deletion mutants. As found for His6porin, the channel forming activity of the deletion mutants was dependent on preincubation of the protein with ergosterol. At low salt concentrations (50 and 150 mM KCl) or in 1 M LiCl, no channels could be observed without addition of ergosterol. Only at higher salt concentrations we observed channel formation, albeit with lower activity (data not shown).

Table I

The single channel conductances represent the peaks of the single channel histograms. Numbers in parentheses represent a peak at a smaller conductance that was observed in addition to a peak at a larger conductance. The voltage dependence was derived from experiments similar to those shown in Figs. 3 and 6. The voltage dependence is characterized by the ratio G_{−50 mV}/G_{+50 mV} for the stationary membrane conductance at a voltage of ±50 mV. G_{+50 mV} is the stationary conductance at 10 mV. P_{i}/P_{a} is the ratio of the permeability for cations versus anions. ND means not determined. The applied membrane potential for the determination of the single channel conductances was +10 mV; T = 20 °C.

| N. crassa mitochondrial porin | Single channel conductance | Voltage dependence (G_{−50 mV}/G_{+50 mV}) | Ion selectivity (P_{i}/P_{a}) |
|-----------------------------|-----------------------------|---------------------------------|-------------------------------|
| Native | (2) | 4 | 1.5 | 0.5 | 1.3 ± 0.4 |
| Recombinant | (2) | 4 | 1.5 | 0.43 | 1.5 ± 0.2 |
| ΔN2–12 porin | 0.5–1.5 | 4 | 1.5 | 0.60 | 1.2 ± 0.3 |
| ΔN3–20 porin | (1–2.5) | 4 | 1.5 | 0.66 | 2 ± 0.2 |
| ΔC269–283 porin | (1.5) | 3 | 1.3 | 0.42 | 1.3 ± 0.3 |
| ΔN2–12/ΔC269–283 porin | (12.5) | 2 | 1 | 0.96 | 0.8 ± 0.3 |
The conductance increase produced by the native and the His6porins occurred in distinct steps, the N-terminal deletion mutants showed "noisy" single channel behavior (Fig. 4, data not shown for ΔD2–12porin). Each single channel showed fast fluctuations on top of a stable state. After insertion of several channels into a lipid bilayer membrane, the fluctuations added up to noisy current recordings, in which single steps could no longer be discriminated. This current noise made it difficult to distinguish more than a few steps in a given membrane. De-spitethecurrentnoiseweobservedconductancestepsofabout 4 nS for both the ΔD2–12porin and the ΔD3–20porin (Fig. 5). This indicatesthattheN-terminalmutantscangeneratepores of the same dimensions as the wild-type protein. The large number of low conductance steps in the histograms (Fig. 5, A and B) probably results from the instability of the pores rather than the insertion of smaller channels.

The mutant ΔC269–283porin did not show a similar current
noise as did all the N-terminal mutants (Fig. 4C). As with the wild-type porin, the conductance increase occurred in distinct steps, although they were significantly smaller than that of the wild-type and the His6 porin. The histogram of the single channel conductances obtained with the ΔC269–283 porin mutant had two maxima at 1.5 and 3 nS (Fig. 5C), instead of 2 and 4 nS for the wild type (Fig. 2B). The double mutant ΔN2–12/ΔC269–283 porin produced very noisy current recordings. The histogram of the single channel distribution of this mutant contained two maxima at 1 and 2.5 nS, which were even smaller than those measured for the C-terminal mutant (data not shown). Thus, the characteristics of both single mutants are present in the double mutant.

Voltage Dependence and Ion Selectivity of the Mutant Channels—It has been proposed that the N-terminal α-helix is directly involved in the voltage-dependent gating of mitochondrial porins (Peng et al., 1992; Thomas et al., 1993). To test this hypothesis, the voltage dependence of the channels formed by the N-terminal deletion mutant porins was examined. ΔN2–12 porin had a slightly reduced voltage dependence (Fig. 6) as compared with wild-type and His6 porins, but it was still voltage-dependent. G_U/G_0 was about 0.60 at 50 mV in comparison to 0.40–0.50 for the wild type (Table I). This effect was even stronger for porin channels lacking the whole N-terminal part (ΔN3–20 porin); for these channels the apparent conductance at high potential was only about 30% smaller than at 10 mV (G_50 mV/G_0 = 0.66).

Whereas the pore size was altered by the C-terminal deletion, the gating of this mutant was not changed. A comparison of Figs. 3 and 6 demonstrates that the voltage-dependent closure was not influenced by the deletion at the C terminus. The double mutant ΔN2–12/ΔC269–283 porin showed no voltage dependence in 1 m KCl (Fig. 6). Only slight closure (about 20%) was observed in low salt (50–150 mM KCl). The closure of the native, the His6 porin, and the other mutant channels occurred already at lower voltages in less concentrated salt solutions (data not shown). This has been observed previously for other mitochondrial porins (Troll et al., 1992).

The N and C termini of porin contain charged residues and thus could influence the ion selectivity of the channel. Therefore we investigated the effect of the deletion of these parts of the porin by performing selectivity measurements with the different deletion mutants. The ion selectivity of the ΔN2–12 porin mutant was not significantly different from that of the wild-type protein, whereas the ΔN3–20 porin mutant had a slightly higher cation selectivity, probably because more channels were in a closed, cation-selective configuration. The double mutant ΔN2–12/ΔC269–283 porin displayed slight anion selectivity. It is possible that it was not capable of switching into a closed configuration and remained in an anion-selective, open state most of the time. In ΔC269–283 porin the ion selectivity was not influenced as measured by the application of a salt gradient (Table I). The determination of the voltage dependence in 1 m LiCl or 1 m potassium acetate supported this observation (see Fig. 7). The difference between the G_U/G_0...
curves in various salts is caused by the different ion selectivity of the open versus the closed states of the porins (Ludwig et al., 1989). The curves in Fig. 7, A and B, are very similar, and thus the relative ion selectivity of the different states of the ΔC269–283porin and the native porin seem to be essentially the same.

**DISCUSSION**

Channel Formation by Expressed His-tagged Mitochondrial Porins—Previously, mutational analysis was only performed on yeast mitochondrial porin (Hamasjima et al., 1988; Blachly-Dyson et al., 1990) due to the availability of molecular biology techniques for this organism. In this article, we show that N. crassa porin can be expressed as a His-tagged protein very efficiently in E. coli. This expression system is not only valuable for obtaining mutant porins but also for producing the large amounts of protein that are necessary for most spectroscopic techniques. Overexpression of eukaryotic porins from other sources, e.g. porin from pea amyloplasts and different porins from potato, is also possible in E. coli.

Functional reconstitution of the recombinant porins was only possible in the presence of ergosterol and detergent. Native mitochondrial porins co-purify with sterols, such as ergosterol in the case of N. crassa (Freitag et al., 1982) and cholesterol in the case of porin from bovine heart mitochondria (De Pinto et al., 1989). Furthermore, it has been demonstrated that sterols play a crucial role in the reconstitution of sterol-free, water-soluble mitochondrial porins (Pfaller et al., 1985; Popp et al., 1995; Carbonara et al., 1996). Possibly the sterols shield exposed charges and thus facilitate insertion into the bilayer or formation of a structure competent for membrane insertion. This hypothesis is supported by our observation that sterols are particularly essential in low salt concentrations (50 or 150 mM KCl), whereas in high salt concentrations channel formation can occur in the absence of sterol, albeit less efficiently.

All the biophysical properties of reconstituted recombinant mitochondrial porin from N. crassa expressed in E. coli were indistinguishable from porin isolated from mitochondria. We have shown in previous studies that reconstitution of other sterol-free, water-soluble porins does not always result in channels with the same properties as the native channels (Popp et al., 1995; Carbonara et al., 1996). Interestingly, the additional six histidinyl residues attached to the N terminus had no detectable influence on the biophysical properties, viz. single channel conductance, channel gating, and channel selectivity of mitochondrial porin from N. crassa. This result indicates that the His-tag is not localized in a crucial position of the channel structure, and thus the His-tagged fusion products can be directly compared with the native porin. Furthermore, it is unlikely that the N terminus undergoes transmembrane movement during potential-induced gating, as suggested by others (Peng et al., 1992; Thomas et al., 1993). Such a model would predict that the gating characteristics would be altered in porin molecules containing an altered N terminus.

What Can Be Learned from the Deletion Mutants about the Channel Structure?—According to secondary structure predictions and comparison to mitochondrial presequences, the N-terminal part of the protein may form an α-helix (Fig. 8) (Mihara and Sato, 1985; Kleene et al., 1987; Kayser et al., 1989). This was confirmed by CD spectroscopy of peptides with a sequence corresponding to residues 1–20 of the N. crassa mitochondrial porin (Guo et al., 1995). It has been suggested that the N-terminal α-helix is part of the channel wall (Blachly-Dyson et al., 1990; Peng et al., 1992; Thomas et al., 1993; Zizi et al., 1995). According to our experiments neither the addition of a His-tag nor the deletion of residues 3–20 prevented the formation of normal-sized channels. If either of these alterations were within sequences that form the wall, they would be expected to disturb the channel structure or result in smaller channels. Therefore, we suggest that the N terminus of porin is exposed to either the intermembrane space or the cytosol.

Surprisingly, the mitochondrial porin seems to be quite insensitive to the deletion of its C terminus. The resulting channels had decreased single channel conductance but otherwise unchanged biophysical properties. Secondary structure predictions suggest that the deleted region consists of one or two of the β-strands that build the channel wall (Fig. 8). Assuming that the mitochondrial porin is a water-filled channel with a wall built by 16 β-strands, one would expect that the removal of 2 strands would result in a decrease of the cross-section by 23% and thus a reduction of the single channel conductance by a similar value. This is in good agreement with what we observed. The ΔC269–283porin channels had a conductance of about 3 nS, about 25% smaller than that obtained for the wild-type channels. However, we cannot exclude the possibility that the removal of only one β-strand by the C-terminal deletion would result in a similar decrease of the conductance, even though the cross-section would be decreased by only 12%. The reduction in single channel conductance could be greater than predicted because the model of an undisturbed passage of the ions through a cylindrical channel is only a rough approximation. When the cross-section of the channel is smaller, more interactions of the permeating ions with the channel wall have

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2 K. Fischer, personal communication.
3 U. K. Schmitz, personal communication.
to be taken into account. Furthermore, we have to consider that the topology of the channel may not be a perfect cylinder.

The crystal structures of several bacterial porins indicate that the β-barrel of these proteins is closed by a salt bridge between the N- and C-terminal amino acids (Cowan et al., 1992). Mitochondrial porins are predicted to have a similar β-barrel structure (for a review see Benz, 1994). Thus, it is expected that this structure would be destabilized in the ΔC269–283 porin, if a similar interaction is required to close the pore. However, the deletion of the C-terminal 15 amino acids and the introduction of a glutamine residue at the C terminus does not prevent channel formation. Possibly, other interactions are responsible for closing the barrel structure.

Voltage-dependent Gating and Ion Selectivity of the Mutant Channels—The ΔC269–283 porin forms stable open channels and displays normal voltage-dependent gating. Thus, this region of the protein is not directly responsible for gating. Furthermore, it does not seem to play any role in the ion selectivity. The double mutant ΔN2–12/ΔC269–283porin is still able to form channels in the bilayer but has lost the gating behavior. This could be caused by a drastic disturbance of the overall channel structure. The channels are slightly anion-selective as expected from their lack of closing behavior.

The N terminus itself seems to be important for overall channel stability, rather than being directly involved in gating. The gating characteristics of the ΔN-channels were only slightly different from those of the wild-type channels, suggesting that the structures responsible for the gating process are slightly different from those of the wild-type channels, suggesting that the structures responsible for the gating process are slightly different from those of the wild-type channels, suggesting that the structures responsible for the gating process are slightly different from those of the wild-type channels, suggesting that the structures responsible for the gating process are slightly different from those of the wild-type channels, suggesting that the structures responsible for the gating process are slightly different from those of the wild-type channels, suggesting that the structures responsible for the gating process are slightly different from those of the wild-type channels, suggesting that the structures responsible for the gating process are slightly different from those of the wild-type channels.