High-affinity binding sites involved in the import of porin into mitochondria

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The specific recognition by mitochondria of the precursor of porin and the insertion into the outer membrane were studied with a radiolabeled water-soluble form of porin derived from the mature protein. High-affinity binding sites had a number of 5—10 pmol/mg mitochondrial protein and a k_a of 1—5 × 10^8 M^{-1}. Binding was abolished after trypsin pretreatment of mitochondria indicating that binding sites were of protein-aceous nature. Specifically bound porin could be extracted at alkaline pH but not by high salt and was protected against low concentrations of proteinase K. It could be chased to a highly protease resistant form corresponding to mature porin. High-affinity binding sites could be extracted from mitochondria with detergent and reconstituted in asolectin-ergosterol liposomes. Water-soluble porin competed for the specific binding and import of the precursor of the ADP/ATP carrier, an inner membrane protein. We suggest that (i) binding of precursors to proteinaceous receptors serves as an initial step for recognition, (ii) the receptor for porin may also be in-volved in the import of precursors of inner membrane pro-teins, and (iii) interaction with the receptor triggers partial insertion of the precursor into the outer membrane.

Key words: mitochondria/protein import/mitochondrial outer membrane/porin/import receptors

Introduction

Mitochondrial porin is an integral protein of the outer membrane that forms aqueous channels of about 2 nm diameter (Zalman *et al.*, 1980; Colombini, 1979; Freitag *et al.*, 1982a,b). This nuclear encoded protein is made as a precursor which has the same apparent mol. wt as the mature protein. Porin precursor can be synthesized *in vitro* in reticulocyte lysates programmed with poly-A^+ from *Neurospora* or yeast (Freitag *et al.*, 1982b; Mihara *et al.*, 1982; Gasser and Schatz, 1983). It can be imported into isolated mitochondria in a posttranslational manner. In con-trast to most nuclear encoded mitochondrial proteins, transfer of porin into mitochondria does not require a potential across the inner membrane. Assembly into the outer membrane leads to a mature form of porin that is resistant to digestion by high amounts of added protease, even when mitochondria are solubil-ized with Triton X-100. Import *in vitro* is abolished after pretreat-ment of mitochondria with low amounts of precursors (Zwizinski *et al.*, 1984). This indicates the involvement of proteinaceous components ('import receptors') in the import process. The re-quirement for protease-sensitive structures has also been found for other mitochondrial precursor proteins (Zwizinski *et al.*, 1984). Import receptors, however, have not yet been character-ized or purified.

Porin isolated from *N. crassa* mitochondria can be converted into a water-soluble form free of detergents and lipids (Pfaller *et al.*, 1985). Using appropriate conditions it was possible to insert water-soluble porin into planar lipid bilayers where it showed the same pore characteristics as does the detergent purified porin (e.g. specific conductance and voltage dependence). Furthermore, water-soluble porin bound to mitochondria in a specific manner and inhibited import of the *in vitro* synthesized porin precursor. These import sites were degraded by pretreatment of mitochon-dria with low amounts of trypsin. Taken together, water-soluble porin behaved essentially the same as the precursor form of porin synthesized in a cell-free system.

Since water-soluble porin is available in chemical amounts and can be radiolabeled by reductive methylation with [^{14}C]formal-dehyde, the experimental prerequisites were at hand to character-ize import sites for the precursor form of porin in more detail. Affinity and number of binding sites were determined by Scatch-ard analysis. High-affinity binding was found to be abolished by protease pretreatment of mitochondria. High-affinity binding sites were reconstituted into liposomes. Water-soluble porin interfered with the import of the ADP/ATP carrier to the inner mitochon-drial membrane which suggests that porin and other mitochondrial proteins use common components, probably the same receptor, of the mitochondrial import machinery. We suggest a role for the putative receptors which includes recognition by specific binding and the triggering of partial insertion of the precursor into the outer membrane.

Results

Characteristics of binding of [^{14}C]water-soluble porin to mito-chondria

Water-soluble porin was radiolabeled with [^{14}C]formaldehyde by reductive methylation using NaBH$_3$CN. This yielded porin with a specific radioactivity of 0.5 Ci/mmol which was then employed as a ligand for binding to mitochondria.

In order to obtain reproducible binding experiments several artifacts had to be eliminated. From *in vitro* studies it was known that the import of porin precursor cannot be blocked by un-couplers of mitochondrial oxidative phosphorylation (Freitag *et al.*, 1982b), in contrast to the import of most mitochondrial precursor proteins. Therefore, in order to prevent further trans-port into the outer membrane, binding of [^{14}C]water-soluble porin to mitochondria had to be carried out for short periods of time at low temperature. Titration of binding sites at 25°C did not lead to saturation; a much higher amount of porin was associ-ated with mitochondria at 25°C as compared with 0°C. Water-soluble porin has a strong tendency to adhere to surfaces thus causing a high degree of unspecific binding. Therefore, binding was analyzed in the presence of 180 mM KCl and bovine serum albumin (BSA).

These three requirements, (i) low temperature, (ii) high-salt buffer, (iii) presence of BSA, had to be fulfilled to be able to titrate saturable binding sites for water-soluble porin on mitochon-
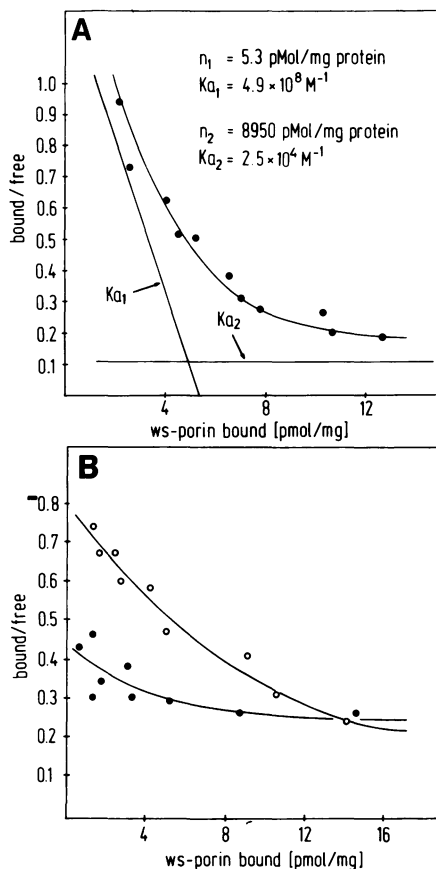
Fig. 1. Water-soluble porin occupies two different types of binding sites on mitochondria, one of which is sensitive to trypsin digestion.
A: Mitochondria (0.5 mg/ml) were incubated with increasing amounts of ^{14}C-labeled water-soluble porin for 20 min at 0°C to equilibrate free and bound ligand. Incubation was carried out in 0.5 ml of a buffer containing 2.4% (w/v) BSA, 0.2 M KCl, 20 mM NaPi, 0.2 mM PMSF, 8 mM MOPS/KOH, pH 7.2. ^{14}C-Labeled water-soluble porin had a specific radioactivity of 1000 c.p.m./pmol water-soluble porin. After incubation, mitochondria were reisolated by centrifugation and aliquots of the supernatants were removed to determine free radioactivity. Mitochondrial pellets containing bound ligand were resuspended in KMBP-buffer [3% (w/v) BSA, 180 mM KCl, 0.2 mM PMSF, 10 mM MOPS/KOH, pH 7.2], transferred to new tubes and reisolated again by centrifugation. Radioactivity associated with mitochondria was determined by liquid scintillation counting. Scatchard analysis of binding data (Scatchard, 1949) was carried out by plotting bound/free versus bound ligand. Binding parameters were determined from the Scatchard plot by computerized least-square parameter fitting. B: Trypsin pretreatment of mitochondria was carried out essentially as described by Zwizinski et al. (1984). A mitochondrial suspension (2.5 mg/ml) in a buffer containing 180 mM KCl, 10 mM MOPS/KOH, pH 7.2 (KM-buffer) was treated with trypsin at a ratio of 5 μg trypsin per mg mitochondrial protein. After incubation for 15 min at 25°C, 100 μg of soybean trypsin inhibitor (STI) and PMSF (0.2 mM final concentration) were added. The reaction mixture was incubated for three more minutes at 25°C followed by a 10 min incubation at 0°C. Control mitochondria were incubated in parallel with trypsin that had been inactivated by pretreatment with STI (mock treatment). PMSF was also added at the beginning of the incubation. Both mitochondrial preparations were reisolated by centrifugation. The mitochondrial pellet was resuspended in ice cold KMBP-buffer containing 100 μg STI/ml. The mitochondria were sedimented again and then resuspended in ice cold KMBP-buffer for the binding assay which was performed as described in A. —O—O—O—: titration of mitochondria mock treated with trypsin. —●—●—●—: titration of mitochondria treated with 5 μg tryspin/mg mitochondria.

dria. The binding curve was biphasic indicating at least two kinds of binding sites which are different in affinity and number (Figure 1A). The constant (k_a) for high-affinity binding sites was deter-
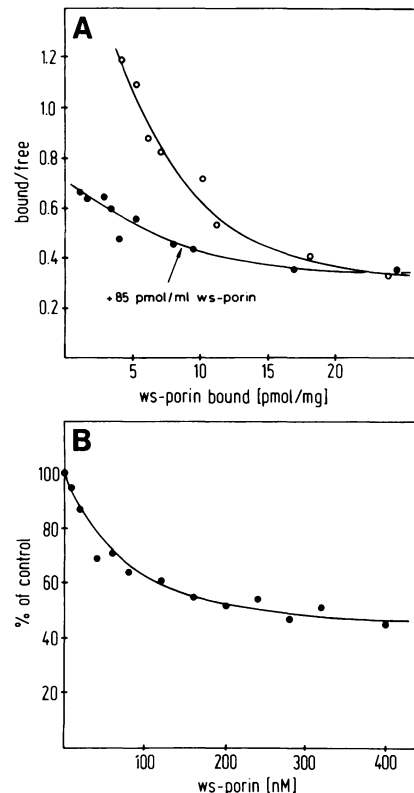
Fig. 2. Competition of high-affinity binding of [^{14}C]water-soluble porin by unlabeled water-soluble porin. A: Scatchard plot analysis for binding of [^{14}C]water-soluble porin to mitochondria was performed as described in Figure 1A. There was either no unlabeled water-soluble porin (—O—O—O—) or 85 nM water-soluble porin (—●—●—●—) present during incubation. B: Mitochondria (0.5 mg/ml concentration), resuspended in the same buffer system as described in Figure 1A, were incubated with [^{14}C]water-soluble porin (4 nM final concentration) and increasing amounts of unlabeled water-soluble porin (up to 400 nM final concentration). After incubation for 20 min at 0°C, the mitochondria were reisolated by centrifugation. An aliquot of the supernatant was removed to determine free radioactivity. The pellet was resuspended in KMBP-buffer, transferred to new tubes and mitochondria were spun down again. The resulting mitochondrial pellet contained bound radioactivity. For each sample, total radioactivity was determined from the sum of free and bound radioactivity and percentage bound was calculated for each sample. Bound radioactivity in the sample without added unlabeled water-soluble porin was set at 100%.

mined to be $1-5 \times 10^8$ M^{-1} with a number of around $5-10$ pmol/mg mitochondrial protein, whereas the k_a of low-affinity sites was $2-3 \times 10^4$ M^{-1} and the respective number was approximately 9×10^3 pmol/mg (average of 20 experiments). Binding was complete within about 10 min at 0°C. The binding data rely on the assumption that the ligand is in a monomeric state. This, however, is not actually known. In fact water-soluble porin forms a dimer (Pfaller et al., 1985). If binding of dimer would occur, the figures for binding would have to be changed accordingly.

Trypsin sensitivity of high-affinity binding

In a previous report it has been shown that import of the in vitro synthesized porin precursor was drastically reduced when mitochondria were pretreated with low amounts of trypsin (Zwizinski et al., 1984). In order to show that high-affinity binding is abolished under the same conditions, mitochondria were pretreated with trypsin (5 μg/mg mitochondrial protein) for 15 min at 25°C (Figure 1B). Trypsin treatment was then stopped by adding soybean trypsin inhibitor (STI; 20-fold weight excess) and

phenylmethylsulfonyl fluoride (PMSF). Control mitochondria were treated under the same condition with trypsin that had been preincubated with STI. The binding sites were then titrated with both mitochondrial preparations. Mock trypsin treatment did not affect high-affinity binding while trypsin pretreated mitochondria showed only low-affinity binding (Figure 1B). High-affinity binding was destroyed by trypsin concentrations as low as 1 μg/mg mitochondrial protein at 0°C. Endoprotease V-8 from *Staphylococcus aureus* abolished high-affinity binding completely at 10 μg/mg mitochondrial protein (15 min at 25°C).

When the trypsin treatment of mitochondria was harsh, the remaining unspecific binding was higher compared with untreated mitochondria. An explanation for this phenomenon could be that excessive protease treatment produced a large number of new unspecific binding sites.

We conclude that specific binding sites are present at the surface of the outer membrane. Loss of binding competence of mitochondria after protease treatment demonstrates the essential role of the specific sites very early in the import pathway.

High-affinity binding of radiolabeled water-soluble porin can be competed for by unlabeled water-soluble porin

Only high-affinity binding was competed for by unlabeled water-soluble porin added to the binding reaction while unspecific binding was unaffected (Figure 2A). The presence of increasing amounts of unlabeled water-soluble porin during incubation with relatively low concentrations of [14C]water-soluble porin (5 pmol/ml) decreased the amount of labeled porin associated with mitochondria (Figure 2B). Thus, under these conditions ~60% of the label bound can be considered as specific. The concentration of unlabeled water-soluble porin at which 50% of specific binding was inhibited was 60 nM (about 10 times the k_d of the high-affinity binding).

[14C]Water-soluble porin that had bound to mitochondria could not be displaced by excess unlabeled water-soluble porin (not shown). This indicates either that the specific association with the binding site is very tight, or that the precursor is already beyond the protease sensitive binding site (see below).

Water-soluble porin bound to high-affinity sites is resistant to low concentrations of proteinase K

From initial binding studies it was obvious that specific binding of [14C]water-soluble porin to mitochondria was accompanied by a considerably higher amount of unspecific interaction with the membrane. Therefore it was desirable to find a way to distinguish between specific and unspecific binding.

Bound water-soluble porin, as a fraction of total water-soluble porin in the assay, was determined either directly or following treatment of the samples with proteinase K after the binding reaction had taken place. After treatment with proteinase K, a saturation curve for the binding was observed, whereas without proteinase K treatment mitochondria showed additional unspecific, i.e. non-saturable binding (Figure 3A and B). SDS–PAGE of proteinase K treated mitochondria carrying [14C]water-soluble porin revealed no degradation products but only intact radiolabeled porin. Unspecifically bound porin was degraded readily by proteinase K (as was unbound water-soluble porin) while degradation of specifically bound water-soluble porin occurred to a much lesser degree. Only when higher concentrations of proteinase K were applied, or when incubation with lower concentrations was performed for longer time periods, was specifically bound porin completely degraded.

Specific interaction of water-soluble porin with mitochondria apparently leads to an intermediate form which is resistant to
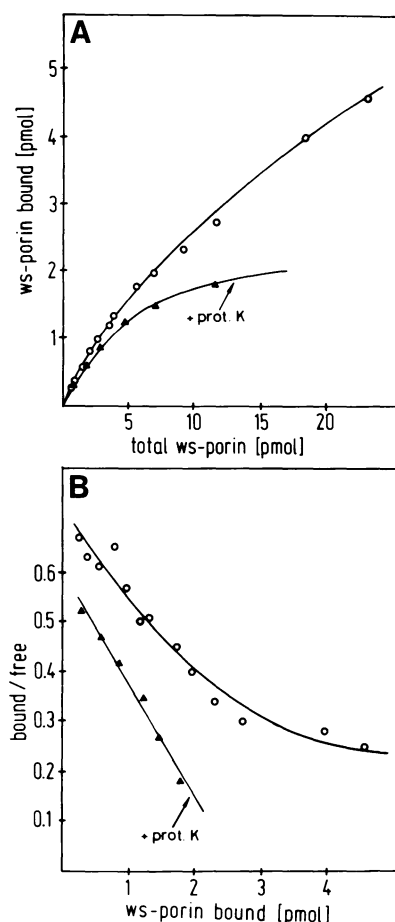

Fig. 3. Water-soluble porin bound to high-affinity binding sites is resistant to treatment with low amounts of proteinase K. Titration of binding sites for [14C]water-soluble porin to mitochondria was carried out as described in Figure 1A except PMSF was omitted during incubation. After 20 min incubation at 0°C, proteinase K was added to each sample to a final concentration of 2 μg/ml. A control binding curve was obtained in a different experiment where no protease was added. Protease treatment was stopped after 1 h at 0°C with PMSF (0.4 mM final concentration). After 5 min at 0°C, mitochondria were reisolated by centrifugation. Then free and bound radioactivity was determined as described in Figure 1A. **A:** Plot of bound versus total. **B:** Scatchard plot. —O—O—O—: without proteinase K treatment. —▲—▲—▲—: treatment with 2 μg/ml proteinase K.

moderate proteolytic attack. This protection may be due to a tight contact with the outer membrane, perhaps involving the lipid phase. In contrast, mature porin was not degraded even by treatment with excessive amounts of proteinase K.

Water-soluble porin associated with mitochondria at low temperature can be chased into mitochondria at higher temperature

Loss of high-affinity binding by pretreatment of mitochondria with trypsin indicated the involvement of proteinaceous components. To further demonstrate high-affinity binding sites to be specific sites for import of porin precursor into mitochondria, water-soluble porin was bound to mitochondria (5 min at 0°C) which were either pretreated (Figure 4, lanes 4−6) or not pretreated (Figure 4, lanes 1−3) with 1 μg trypsin/mg mitochondrial protein. To produce selectively high-affinity bound water-soluble porin, mitochondria after binding were treated with 5 μg proteinase K/ml (Figure 4a). Parallel samples were treated with 100 μg proteinase K/ml to show the protease sensitivity of water-soluble porin associated with mitochondria at low temperature (Figure 4b). From a third set of samples, mitochondria were reisolated
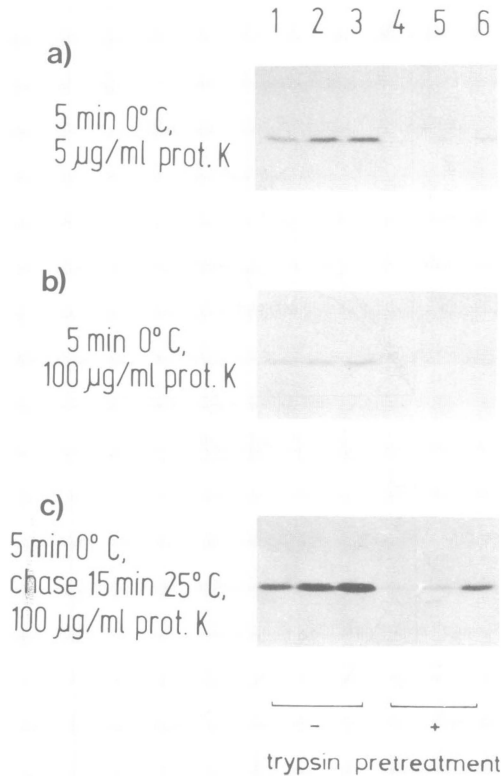
Fig. 4. Water-soluble porin, specifically bound to trypsin-sensitive binding sites, can be chased into a location where it is resistant to treatment with high proteinase K concentrations. Three mg of isolated mitochondria (at a concentration of 1 mg/ml) were treated in KM-buffer with 1 μg tryspin/mg for 15 min at 25°C. Protease treatment was stopped with 0.2 mM PMSF and 60 μg soybean trypsin inhibitor (STI, 20-fold weight excess over trypsin). Another 3 mg of mitochondria were treated in the same way but received no trypsin (control), and PMSF was added at the beginning of the incubation. After 3 min at 25°C followed by 5 min at 0°C, mitochondria were reisolated from the two samples and washed once in KMP-buffer containing 0.1 mg/ml STI. The mitochondria were resuspended in ice cold KMB-buffer at a protein concentration of 1 mg/ml. Then, 0.2 mg of mitochondria, either pretreated (lanes 4−6) or not pretreated with trypsin (lanes 1−3) were incubated with three increasing concentrations of [^{14}C]water-soluble porin (4 nM, 8 nM and 16 nM, respectively) under the same buffer conditions as described in Figure 1A. (a) After 5 min at 0°C, proteinase K was added to 5 μg/ml final concentration; (b) after 5 min at 0°C, proteinase K was added to 100 μg/ml final concentration; (c) after 5 min at 0°C, mitochondria were reisolated, pellets were resuspended in 0.5 ml of KMB and the samples were incubated for 15 min at 25°C (chase). Then mitochondria were cooled to 0°C and proteinase K was added to 100 μg/ml final concentration. Protease treatment was stopped after 1 h at 0°C by 0.4 mM PMSF. Mitochondria were reisolated and washed once in KMP-buffer. The resulting mitochondrial pellets were lysed and subjected to SDS gel electrophoresis. Water-soluble porin associated with mitochondria was detected by fluorography of the dried gels. Note: the amounts of radioactive porin associated with mitochondria should be the same in panels (a) and (c), lanes 1−3. However, this is not the case, apparently because mitochondria in panel (c) were reisolated before the 'chase' and proteinase K treatment; effectively, this results in a longer binding period as compared with panel (a) where proteinase K was added immediately after 5 min incubation.

after 5 min incubation at 0°C, resuspended in the same volume of KMB-buffer and incubated at 25°C for 15 min. After cooling the samples to 0°C, proteinase K was added to 100 μg/ml final concentration to determine water-soluble porin associated with mitochondria that had become resistant to high concentrations of proteinase K and therefore should correspond to mature porin (Figure 4c).

Pretreatment of mitochondria with trypsin reduced the amount
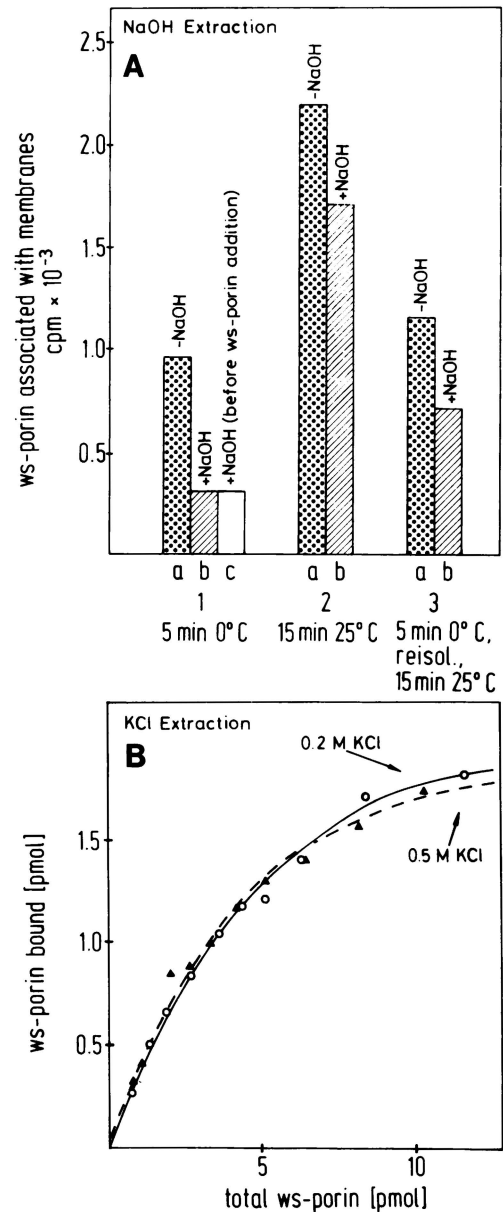
Fig. 5. Water-soluble porin bound to high-affinity binding sites can be extracted with 0.1 M NaOH but not with 0.5 M KCl. **A:** Mitochondria (0.5 mg/1.0 ml) were reacted with 5 pmol [^{14}C]water-soluble porin (see Figure 1A) under three different conditions. Reaction 1: incubation for 5 min at 0°C; reaction 2: incubation for 15 min at 25°C; reaction 3: incubation for 5 min at 0°C, reisolation of mitochondria resuspension in 0.5 ml KMBP-buffer and further incubation for 15 min at 25°C. From one half of each reaction mitochondria were reisolated and free and bound radioactivity was determined (1a, 2a, 3a). The second halves (1b, 2b, 3b) were cooled to 0°C, NaOH was added to a final concentration of 0.1 M and incubation was continued for 30 min at 0°C followed by centrifugation at 100 000 × g for 1 h to separate bound from free radioactivity. In order to determine the fraction of water-soluble porin which was pelleted after the NaOH treatment in the absence of specific binding, NaOH was added to one sample before addition of water-soluble porin. After 35 min at 0°C radioactivity in the pellet was determined (1c). **B:** Extraction with 0.5 M KCl: binding sites for [^{14}C]water-soluble porin on mitochondria were titrated in two parallel assays as described in Figure 1A. After 20 min incubation at 0°C, proteinase K-resistant bound water-soluble porin was produced as described in Figure 3. Protease was then inhibited with PMSF. In one assay, KCl was added to 0.5 M final concentration (−▲—▲—▲—), and in another assay to 0.2 M KCL (−○—○—○—). After 15 min incubation at 0°C bound and free radioactivities were determined as described in Figure 1A. Total water-soluble porin in each sample was determined from the sum of bound and free and plotted versus bound water-soluble porin.

of specifically bound water-soluble porin drastically (Figure 4a). Furthermore, in contrast to mature porin, specifically bound water-soluble porin was still sensitive to proteolytic attack with high concentrations of proteinase K (Figure 4b). However, even after short times of incubation a small but distinct amount of porin was resistant to high protease; most probably this is an experimental artifact, since manipulation after the 5 min incubation at 0°C was such that partial movement of receptor bound porin into the membrane could occur.

After binding of porin at 0°C, mitochondria were incubated at elevated temperature (25°C) ('chase'). This led to a substantial increase of porin which was resistant to high amounts of proteinase K (Figure 4c). This suggests the transport from an intermediate location into a form where it shows protease resistance properties comparable with mature porin. Furthermore, pretrypsinized mitochondria failed not only to bind but also to import water-soluble porin efficiently. This confirms the existence of a protease-sensitive component on the mitochondrial surface which is involved in the import pathway of porin.

Specifically bound water-soluble porin is released from the membrane by treatment with 0.1 M NaOH but not with 0.5 M KCl

Extractability with alkali and with high salt was studied to investigate the nature of the interaction between water-soluble porin and its specific binding sites.

Alkaline extraction was carried out in the following way: mitochondria were incubated with [^{14}C]water-soluble porin at a concentration which yields mainly specific binding for 5 min at 0°C followed by addition of NaOH to 0.1 M final concentration. After 30 min at 0°C the samples were centrifuged to obtain a soluble and a pellet fraction. Most of the bound water-soluble porin could be extracted into the soluble fraction when binding was carried out at 0°C (Figure 5A, columns 1a and b). Radioactivity recovered in the pellet fraction appeared to be associated with mitochondria in an unspecific manner as shown by NaOH treatment of a mixture of water-soluble porin and mitochondria without incubation (Figure 5A, column 1c). In contrast, when mitochondria were incubated with water-soluble porin under the same conditions except at 25°C, most of the porin was not extracted (Figure 5A, columns 2a and b). Furthermore, water-soluble porin bound at 0°C could be chased into a NaOH resistant form by raising the temperature prior to alkaline treatment (Figure 5A, columns 3a and b). Pre-existent assembled porin was not released from the outer membrane upon alkaline treatment (not shown).

In order to determine whether specifically-bound water-soluble porin can be extracted with high salt, mitochondria were treated with either 0.2 M KCl or 0.5 M KCl for 15 min at 0°C following binding and treatment wth low proteinase K. Only minor amounts of the bound water-soluble porin were released (Figure 5B). This supports the view that hydrophobic interactions, rather than ionic interactions, play an important role in binding. Interestingly, pretreatment of mitochondria with 0.5 M KCl under the same conditions as described led to a complete loss of high-affinity binding although there were only minute alterations in the mitochondrial protein pattern (not shown). It is not known whether this loss of binding is due to denaturation of binding sites or extraction of a component essential for specific binding.

These data, taken together, suggest that binding of water-soluble porin at low temperature leads to a tight interaction with the outer membrane. Contact with the protease sensitive binding site may lead to a partial insertion into the outer membrane. This partially inserted intermediate would be in close association with
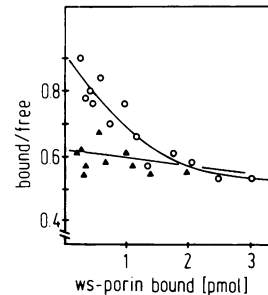
Fig. 6. Reconstitution of high-affinity binding sites into asolectin/ergosterol liposomes. Liposomes from asolectin containing 9% ergosterol were prepared as described in Materials and methods. Liposomes were diluted in KMB-buffer and aliquots were used instead of mitochondria in the binding assay described in Figure 1A. —O—O—O—: titration of liposomes prepared with mitochondrial extracts. —▲—▲—▲—: titration of liposomes prepared without mitochondrial extracts.

the saturable high-affinity site, since it can be extracted at alkaline pH and is not embedded in the lipid bilayer in the same manner as the mature porin.

Reconstitution of high-affinity binding sites into liposomes

One aim of this study was to establish a functional test for the purification of the porin receptor. A possible way to provide such a test is to reconstitute high-affinity binding sites into liposomes and employ these in a binding assay.

CHAPS ((3-(3-cholamidopropyl)-dimethylammonio)-1-propane-sulfonate) was chosen as detergent for extraction of membrane proteins together with soybean lecithin (asolectin). For reconstitution, detergent extracts were mixed with a suspension of asolectin containing 9% ergosterol. Ergosterol was added to the asolectin lipids because the mitochondrial outer membrane of *N. crassa* contains a high amount of this sterol (Hallermayer and Neupert, 1974). Another reason to include a sterol was to increase the size of the liposomes in order to allow reisolation by low speed centrifugation. This turned out to be necessary because part of the water-soluble porin was pelleted at high *g*-values (Pfaller *et al.*, 1985).
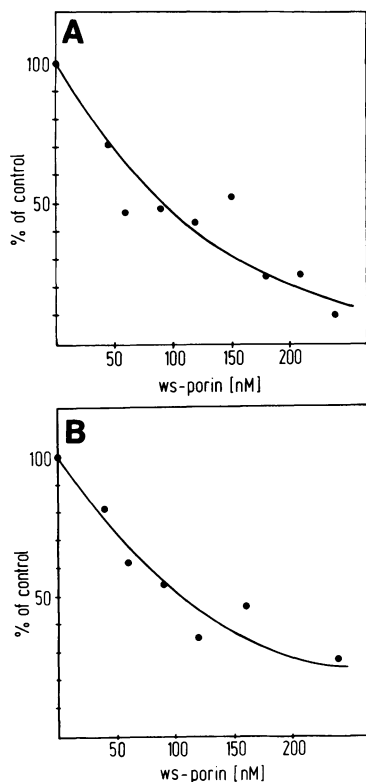
Asolectin-ergosterol liposomes without protein showed binding with low affinity but with high capacity (Figure 6). Obviously there is a considerable degree of unspecific binding. Liposomes containing protein from mitochondrial extracts, however, showed high-affinity binding (in addition to the unspecific binding) which was comparable with that observed with mitochondria (Figure 6). The affinity constant, determined by Scatchard analysis, was around 10^8 M^{-1}. With liposomes derived from extracts of pretrypsinized mitochondria no high-affinity binding was observed. Unspecific binding to these liposomes, however, was higher than that to control liposomes. The presence of tryptic fragments may enhance the unspecific interaction of water-soluble porin with membranes.

In conclusion, it appears possible to reconstitute specific binding sites into artificial membranes.

Competition of water-soluble porin with the precursor of ADP/ATP carrier for import

Are the binding sites described above involved in the import of only porin precursor, or are they also used by other mitochondrial precursor proteins? Competition experiments were carried out by incubating reticulocyte lysates containing radiolabeled precursors with mitochondria in the presence of water-soluble porin.
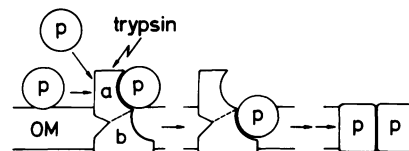
Recent studies in our laboratory led to the identification of distinct steps of the import pathway of the ADP/ATP carrier

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Fig. 8. Working hypothesis on the role of a mitochondrial import receptor for porin. Porin precursor binds to a trypsin-sensitive component a exposed on the surface of the outer membrane. Following this binding, porin precursor is partly inserted into the outer membrane. It is then present at high-affinity saturable sites b. Sites a and b may be present in one component, or in different components of the outer membrane. From site b, complete insertion into the membrane and oligomerization to the functional porin takes place. The initial binding may occur either directly from the cytosolic form or after the precursor has made loose contact with the lipids of the outer membrane. p: porin.

Fig. 7. Influence of water-soluble porin on binding and $\Delta\psi$ dependent import of the ADP/ATP carrier. **A:** Competition of specific carrier binding: antimycin A (Sigma) and oligomycin (Sigma) (4 μM and 10 μM final concentration, respectively) were added from a 100-fold concentrated stock solution (in ethanol) to a mitochondrial suspension [0.5 mg/ml concentration, suspended in a buffer containing 250 mM sucrose, 80 mM KCl, 5 mM MgCl$_2$, 10 mM MOPS and 3% BSA (w/v), adjusted to pH 7.2 with KOH]. Aliquots of 20 μg (corresponding to 70 μl final volume) mitochondrial suspension were incubated with 60 μl of increasing concentrations of unlabeled water-soluble porin (resolubilized in 100 mM NaP$_i$, pH 6.8) and 20 μl of reticulocyte lysate containing radiolabeled precursor to the ADP/ATP carrier. After 40 min incubation at 0°C, mitochondria were reisolated, resuspended in 0.2 ml of ice cold BSA-containing buffer (described above) and then proteinase K was added to a final concentration of 10 μg/ml. After 30 min at 0°C the protease treatment was stopped by 1 mM PMSF. Mitochondria were reisolated and bound carrier was analyzed by immunoprecipitation, SDS gel electrophoresis and fluorography of the dried gel. Bands were quantitated by densitometry. **B:** Competition of carrier import: competition of carrier import into mitochondria was carried out essentially as described in A. Antimycin A and oligomycin, however, were added after the 40 min incubation at 0°C (4 μM and 10 μM final concentrations, respectively). Then mitochondria were reisolated, resuspended in 0.2 ml of BSA-containing buffer (containing 1 mM PMSF and 1.2 μM CAT) and incubated for 15 min at 25°C for completion of assembly of imported carrier. The mitochondria were reisolated and assembled carrier was assessed by HTP chromatography (Schleyer and Neupert, 1984), immunoprecipitation of the pass-through fractions, gel electrophoresis, fluorography and densitometry.

(Pfanner and Neupert, 1987). Binding of the precursor in the absence of a membrane potential produced a species, located in the outer membrane, that was protected from attack by low concentrations of protease and could be chased into the assembled form in the presence of a membrane potential.

In order to test whether water-soluble porin competes for the formation of this early intermediate, de-energized mitochondria were incubated together with reticulocyte lysate containing radiolabeled precursors and with water-soluble porin. After reisolation, mitochondria were treated with proteinase K to remove unspecifically bound ADP/ATP carrier and the amount of ADP/ATP carrier associated with the mitochondria was determined. When

the incubation was performed at 0°C, reduction of specific binding was observed (Figure 7A). At 100 nM of water-soluble porin, specific binding of labeled ADP/ATP carrier was reduced by 50%. This must be compared with the concentration necessary to reduce high-affinity binding of [^{14}C]water-soluble porin by unlabeled water-soluble porin where a 50% reduction was achieved at about 60 nM. In contrast to measuring specific binding at 0°C, competition efficiency at 25°C was lower (not shown). This would indicate the turnover of bound water-soluble porin at higher temperature.

In a second experiment, complete import of ADP/ATP carrier was followed in an assay in which carboxyatractyloside was added to the incubation mixtures after stopping import (Schleyer and Neupert, 1984). This inhibitor of the carrier interacts only with the mature form which then no longer binds to hydroxylapatite. Therefore, import and correct assembly of the carrier can be monitored.

The presence of increasing amounts of water-soluble porin decreased the import of ADP/ATP carrier at 0°C (Figure 7B). Concentrations of water-soluble porin necessary to reduce import by 50% were around 90 nM. This suggests that binding and import of carrier were competed with similar efficiencies. At 25°C, however, incubation of mitochondria with water-soluble porin (at a concentration of 100 nM) and precursor to the ADP/ATP carrier led to only 25% reduction of carrier import.

It was not possible to displace ADP/ATP carrierd bound to de-energized mitochondria by excess unlabeled water-soluble porin. When water-soluble porin, however, was first bound to mitochondria, specific binding of ADP/ATP carrier to these mitochondria was decreased. The effect of water-soluble porin on the import of ADP/ATP carrier from the bound state was also analyzed. Precursor to the carrier was first bound to de-energized mitochondria, then these mitochondria were re-energized in the presence of water-soluble porin. Import of the carrier from the bound state (Pfanner and Neupert, 1985) was not affected (not shown). This is evidence for the water-soluble porin having no effect on the membrane-potential-dependent step of carrier import, but rather on the initial binding reaction.

The following observations speak strongly against the possibility that inhibition of binding and import of the precursor to the ADP/ATP carrier by water-soluble porin is simply due to depletion of free precursor caused by association with water-soluble porin: (i) binding and import of the ADP/ATP carrier was also reduced, when mitochondria were preincubated with water-soluble porin and reisolated before binding and import of ADP/ATP carrier was performed. (ii) When competition was carried out at 25°C instead of 0°C, inhibition of binding and import of ADP/ATP carrier was strongly reduced (see above).

Discussion

In this study we addressed the problem of how the precursor of porin is specifically directed to mitochondria. Rather than using porin produced by cell-free translation, a water-soluble form was employed which was prepared from the isolated mature membrane protein. The reason for this was that precursors synthesized *in vitro* are not available in sufficient amounts to saturate binding sites and, moreover, are not in a pure state. These drawbacks are thus circumvented by employing water-soluble porin which can easily be prepared from the purified membrane protein in nmol amounts.

Water-soluble porin has the properties of a precursor according to several lines of evidence, including competition of water-soluble porin with *in vitro* synthesized porin precursor for import into mitochondria (Pfaller *et al.*, 1985). Here we show that interaction of water-soluble porin with mitochondria at 0°C leads to a protease sensitive form which is comparable with *in vitro* synthesized porin precursor associated with mitochondria at low temperature (Freitag *et al.*, 1982b). At higher temperature, however, water-soluble porin became resistant to protease treatment reflecting further transport into the mitochondrial outer membrane.

Scatchard analysis reveals the existence of at least two kinds of binding sites for water-soluble porin on mitochondria which differ in affinity and number. High-affinity binding sites are easily destroyed by mild pretreatment of mitochondria with protease. In an earlier investigation, import of porin was found to be inhibited after trypsin pretreatment of mitochondria (Zwizinski *et al.*, 1984).

Several criteria are to be fulfilled so as to suggest a role of these high-affinity binding sites as receptors: they are of limited number and can be competed by excess unlabeled ligand; the affinity of these receptor sites is comparable with that of hormone receptors and other surface receptors of cytoplasmic membranes (Kahn, 1975); binding occurs rapidly, even at 0°C, and leads to saturation; susceptibility to mild proteolysis shows that binding involves proteinaceous components exposed on the surface of the outer mitochondrial membrane; binding to high-affinity sites and subsequent incubation at elevated temperature leads to a protease resistant form with characteristics of mature porin.

What is the nature and function of the high-affinity binding sites described here? The information available at present is rather scarce. A working hypothesis to describe the role of the high-affinity sites is proposed in Figure 8. According to this hypothesis, porin precursor is specifically recognized and bound to a trypsin-sensitive site exposed to the surface of the outer membrane. This binding may occur either from the free cytosolic state of the precursor, or porin precursor may first interact with the lipid phase of the outer membrane and then bind to the trypsin-sensitive site. (However, if the latter is the case, the binding to the lipids would occur with rather low affinity.) The binding to the trypsin-sensitive site may trigger a partial insertion of the precursor into the membrane. Apparently this is the stage, which is assessed in our titration experiments. It is not known whether at this point porin precursor is still bound to the same component that is responsible for initial recognition, or whether the precursor has moved to another component with a specific binding site. In Figure 8 this is indicated by leaving open the possibility that components a and b are segments of a single protein component, or that a and b are two separate entities which may either be physically linked or may even be two unlinked components of the outer membrane. Assembly of functional porin would finally occur by complete insertion of the precursor and by folding into the oligo-

meric channel-forming protein. Progress in verifying this working hypothesis requires essentially two achievements, namely the ability to further dissect the pathway by blocking at distinct steps and, furthermore, the isolation and reconstitution of the receptor-like component(s).

The requirement for nucleoside triphosphates in the import of proteins into the mitochondrial inner membrane and matrix has been demonstrated (Pfanner and Neupert, 1986; Eilers *et al.*, 1987; Pfanner *et al.*, 1987). Recently, we have found that import of porin precursor synthesized from cloned cDNA in a coupled transcription and translation system does also require nucleoside triphosphates (Kleene *et al.*, accompanying paper). The role of nucleoside triphosphates in porin import (and in import of proteins in general) is thought to be in conferring an import-competent conformation to precursors in the cytosol. Preliminary data indicate that import of water-soluble porin does not depend on nucleoside triphosphates. An explanation for this discrepancy might be that the denaturation-renaturation protocol used in the preparation of water-soluble porin results in the generation of an 'unfolded' form of porin; this form may not require the continued presence of nucleoside triphosphate-dependent protein factors which may help in keeping precursors in an 'unfolded' (i.e. import-competent) form.

Competition of water-soluble porin for the import of other precursor proteins raises some questions as to the specificity of the porin receptor. Not only import but also specific and import-competent binding of the precursor to the ADP/ATP carrier was efficiently competed for by water-soluble porin. This observation rules out the possibility that inhibition of import is simply due to uncoupling of mitochondria. A synthetic peptide representing the complete presequence of mitochondrial ornithine transcarbamylase was reported to inhibit the import of a number of mitochondrial precursor proteins (Gillespie *et al.*, 1985). This was proposed to reflect competition for components on the import pathway and thus may be related to our observations. On the other hand, synthetic peptides of presequences of different precursor proteins were shown to have a deteriorating effect on the membrane potential (Gillespie *et al.*, 1985; Ito *et al.*, 1985; Roise *et al.*, 1986). The finding that water-soluble porin does not inhibit $\Delta\psi$ dependent uptake of ADP/ATP carrier from the bound state also shows that water-soluble porin has no effect on the membrane potential. In the case of the ADP/ATP carrier, competition for import appears to take place at the stage of specific binding. The binding reaction does not require a membrane potential (Pfanner and Neupert, 1987).

In conclusion, common binding sites appear to exist for outer membrane proteins such as porin and inner membrane proteins such as the ADP/ATP carrier. Analysis of binding of apocytochrome *c* to mitochondria, however, clearly showed the existence of a different import receptor (Hennig *et al.*, 1983). With cytochrome *c*, the affinity of specific binding sites is lower, their number is higher and their protease sensitivity is much lower as compared with porin. Since cytochrome *c* follows an import pathway which differs considerably from that of all other proteins studied so far, the involvement of different binding sites is not unexpected.

Materials and methods

Growth of N. crassa and preparation of mitochondria

Neurospora crassa (wild-type 74A) was grown as described (Schleyer *et al.*, 1982). Mitochondria were isolated from *N. crassa* hyphae by differential centrifugation after breaking up the cells either by using a grind mill for large scale isolation in the case of porin purification (Sebald *et al.*, 1979), or by grinding with sand

(Korb and Neupert, 1978). For binding studies employing [^{14}C]water-soluble porin and for competition experiments, KM-buffer (180 mM KCl, 10 mM MOPS, adjusted to pH 7.2 with KOH) was used as isolation medium. Mitochondrial protein was determined according to Bradford (1976).

Preparation of [^{14}C]water-soluble porin

Porin was purified from isolated mitochondria, precipitated with TCA/methanol and resolubilized with 0.1 M NaOH followed by neutralization with 0.2 M NaH$_2$PO$_4$ as described (Pfaller *et al.*, 1985). Reductive methylation with [^{14}C]-formaldehyde (specific radioactivity 52.5 mCi/mmol, New England Nuclear) and NaBH$_3$CN (Serva) was carried out as reported previously (Pfaller *et al.*, 1985) to a specific radioactivity of 0.5 Ci/mmol water-soluble porin. Aliquots were stored at −80°C as pellets (obtained by TCA precipitation and washing with acetone); they were then resolubilized in 0.1 M NaOH, and after 60 s neutralized with 0.2 M NaH$_2$PO$_4$ before each experiment. Protein determination of labeled and unlabeled porin was carried out according to the Lowry method (1951).

Preparation of asolectin-ergosterol liposomes

Preparation of lipid/CHAPS suspensions: 91 mg asolectin (soybean lecithin, Sigma) and 9 mg ergosterol (Fluka) were dissolved together with 80 µg butylated hydroxy-toluene (Sigma) in 3 ml chloroform/methanol (2:1, v/v) and aliquots corresponding to 10 mg total lipid were placed in Eppendorf tubes. The solvent was removed under vacuum and the lipid pellets were stored at −30°C. To 10 mg of lipid, 0.5 ml of KM-buffer together with glass beads (1 mm diameter) were added and the tubes were flushed with nitrogen. The mixtures were then vortexed until all the lipid was suspended. CHAPS (Sigma) was dissolved in the resulting suspensions to a final concentration of 7% (w/v) and the mixtures were pulse-sonified three times at 1 min intervals (Branson sonifier) followed by a 15 s spin in an Eppendorf centrifuge to remove large particles.

Preparation of mitochondrial extracts: mitochondrial pellets (6−10 mg of protein) were resuspended in 1.5 ml of a 0.8% (w/v) CHAPS solution in KM-buffer containing 5 mg/ml asolectin, protease inhibitor fraction from *N. crassa* (purified according to Schmidt *et al.*, 1984) and PMSF (0.2 mM final concentration). Extraction was carried out for 15 min at 0°C followed by 30 min centrifugation at 50 000 × *g* to pellet non-solubilized material. The supernatants were then used for preparing liposomes.

Preparation of liposomes: to 1.5 ml of extract, 1 ml of asolectin-ergosterol-CHAPS suspension was added. The resulting mixture was kept at 0°C for 20 min followed by gel filtration (at 8°C) over Sephadex G-25 (PD-10 columns, Pharmacia) equilibrated with KM-buffer. Turbid fractions were collected and CaCl$_2$ was added from a 0.5 M stock solution to a final concentration of 20 mM. After 15 min at room temperature, EDTA was added from a 0.25 M stock solution to 40 mM final concentration. After 15 min incubation at room temperature, liposomes were collected by 10 min of centrifugation at 30 000 × *g*. The pellets were suspended in ice cold KM-buffer containing 0.2 mM PMSF and were centrifuged again. The resulting pellets were then resuspended in KMB-buffer containing 0.2 mM PMSF and used instead of mitochondria in binding studies. Control liposomes without protein were prepared the same way but the mitochondrial extract was not added.

In vitro synthesis of precursor proteins

Precursor proteins were synthesized in rabbit reticulocyte lysates in the presence of L-[^{35}S]methionine (500 Ci/mmol, Amersham, Buchler) as described (Pfanner and Neupert, 1987).

Analytical procedures

Liquid scintillation counting: 0.2 ml aliquots of supernatants from binding assays were mixed with 0.1 ml of 10% Triton X-100 and then diluted with 12 ml of a scintillation cocktail containing 60% (v/v) toluene, 40% (v/v) methylglycol and 6 g/l Permablend III (Packard, Zurich, Switzerland). Mitochondrial pellets were dissolved in 0.1 ml of 10% Triton X-100, 0.1 M NaOH and diluted with 1.2 ml of a scintillation cocktail containing 70% (v/v) toluene, 30% (v/v) Triton X-100 and 6 g/l Permablend III. Samples were mixed and radioactivity was determined in a Packard counter.

Published procedures were used for immunoprecipitation of ADP/ATP carrier (Schleyer *et al.*, 1982; Pfanner and Neupert, 1985), SDS−PAGE (Laemmli, 1970) and fluorography of dried gels (Chamberlain, 1979). Quantitation of fluorographed protein bands was carried out on a LKB Ultroscan XL laser densitometer.

Assessment of import of ADP/ATP carrier by hydroxylapatite (HTP) chromatography was performed as described (Schleyer and Neupert, 1984).

Acknowledgements

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