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Effects of Amphipathic Peptides, Including Presequences, on the Functional Integrity of Rat Liver Mitochondrial Membranes

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A number of amphipathic peptides were tested for their effects on structural and functional properties of isolated rat liver mitochondria. The peptides included the matrix targeting sequence of subunit IV of (yeast) cytochrome *c* oxidase. Titration experiments in which the mitochondria were incubated with increasing concentrations of the peptides revealed two major stages in the interaction. First, at low peptide/mitochondria ratios, peptide binding to the outer membrane occurred which was accompanied by gradual lysis of the outer membrane at higher ratios. The latter was deduced from the release of adenylate kinase, the classical marker enzyme of the intermembrane space. Secondly, at still higher peptide/mitochondria ratios, the permeability of the inner membrane progressively increased, as evidenced by measurements of respiratory control and of the membrane potential. Complete uncoupling of respiration seemed to precede dissipation of the membrane potential.

KEY WORDS: Rat liver mitochondria; amphipathic peptides; presequences; membrane structure and function; respiration; membrane potential; protein import.

INTRODUCTION

The majority of the mitochondrial proteins is encoded on the nuclear DNA and translated in the cytosol with a temporary amino terminal extension, the so-called presequence (for reviews see Hartl *et al.*, 1989, Baker and Schatz, 1991, Pfanner *et al.*, 1991, Neupert *et al.*, 1990, and Horwich, 1990). The presequence is both necessary and sufficient to direct mitochondrial localization. Matrix-targeting sequences of mitochondrial precursor proteins have no primary structure homology, but are all rich in basic amino acids and essentially lack acidic residues. It has been predicted that they adopt an α-helical conformation which, by virtue of the distribution of positively charged and neutral amino acids along the peptide chain, would result in an amphipathic structure with the charged and hydrophobic residues facing opposite sides of the helix. The amphipathic character of the presequence has been suggested to aid in the initial binding of the precursor protein to the outer membrane (Von Heijne, 1989; Roise *et al.*, 1986; Roise and Schatz, 1988; Roise *et al.*, 1988; Lennart-Nilsson and Von Heijne, 1988): the nonpolar face of the presequence might partially penetrate into the hydrophobic region of the outer leaflet of the outer membrane, while the basic residues on the polar face would interact with the headgroups of the negatively charged phospholipids. Experimental support for these suggestions was obtained from studies which demonstrated that the initial interactions of both isolated presequences and full-length precursor proteins were strongly stimulated by a negatively charged membrane interface (Roise *et al.*, 1986; Endo *et al.*, 1989; Eilers *et al.*, 1989; Swanson and Roise, 1992). Experiments with model membranes

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and purified biological membranes (see, for example, Ou *et al.*, 1988 for data on the adrenodoxin precursor) provided evidence that, among the negatively charged phospholipids, cardiolipin especially supports this initial interaction. It is of interest that we have recently shown that in rat liver mitochondria significant amounts of cardiolipin, although predominantly localized in the inner membrane, are present in the outer leaflet of the outer membrane (Hovius *et al.*, 1990, 1993). Furthermore, based on model membrane data, Leenhouts *et al.*, (1993) have recently proposed a novel property of presequences, i.e., the cardiolipin-dependent formation and dissociation of mitochondrial intermembrane contact sites.

Maduke and Roise (1993) have recently shown that the 25-residue presequence of cytochrome *c* oxidase subunit IV can be imported in a membrane potential-dependent manner into protein-free phospholipid vesicles. This implies that presequence translocation is not strictly dependent upon a hydrophilic proteinaceous transmembrane channel (Hartl *et al.*, 1989). Furthermore, a number of groups have reported that synthetic presequences are efficiently translocated into mitochondria and that this process is stimulated by an electrical potential across the inner membrane (Roise, 1992; Furuya *et al.*, 1991).

In summary, considerable knowledge has been gathered on the structural requirements of mitochondrial presequences and on their interaction with model membranes. Recent results suggest that synthetic presequences may serve as useful probes of the normal protein translocation pathway. Very little is known about the effects of presequences on the structural and functional integrity of the outer and inner membrane. This is particularly relevant in view of the pronounced interfacial properties of the isolated peptides. In order for these peptides to be meaningful tools for dissecting the protein import pathway, basic knowledge of their effects on mitochondrial properties is crucial. The present study was aimed at providing this basic information by studying the interaction of isolated presequences with rat liver mitochondria under *in vitro* conditions. Apart from the presequences, the analysis included the well-studied amphipathic peptide mastoparan. We especially focused on the effects of the peptides on the structural integrity of the mitochondrial membranes and on the functional integrity of the inner membrane.

MATERIALS AND METHODS

Materials

Acetonitrile (Far UV) was purchased from FSA Laboratory Supplies. Mastoparan was obtained from Bissendorf Biochemicals Gmbh (Hannover, Germany) and used without further purification. Phenylmethylsulfonylfluoride (PMSF),[4] leupeptin, benzamidin, carbonyl cyanide *m*-chlorophenylhydrazone, oligomycin, and valinomycin were purchased from Sigma Chemical Co. (St. Louis, Missouri). Soybean trypsin inhibitor and digitonin were obtained from Merck (Darmstad, Germany). Myxothiazol was from Boehringer Mannheim. The fluorescent membrane potential dye diS-C_3-(5) was from Molecular Probes. All other (bio)chemicals were of the highest purity commerically available.

Synthesis and Purification of the Presequences

Three different synthetic peptides derived from the presequence of cytochrome *c* oxidase subunit IV were used: (i) p25 corresponding to the entire presequence (Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Tyr-Leu-Leu); (ii) p25(W), as p25 except that the leucine residue at position 18 was replaced by a tryptophan; (iii) p17, the first 17 residues of p25. P25 and p25(W) were made with ^{14}C-labeled Ala at position 14 (518 dpm/mg peptide). P17 was prepared by solid-phase synthesis at the Hubrecht Laboratory (Utrecht University), while p25 and p25(W) were obtained from Dr. D. Olshevski (University of California, San Diego). The peptides were purified by reverse-phase HPLC on a Nucleosil C_4 column (Macherey-Nagel, Düren, Germany) and eluted with linear water-acetonitrile gradients containing 0.1% (v/v) TFA.

[4] Abbreviations: DHFR, dihydrofolate reductase; pCoxIV-DHFR, fusion protein of the presequence of cytochrome *c* oxidase subunit IV and DHFR; p25, wild-type presequence of cytochrome *c* oxidase subunit IV; p25(W), presequence of cytochrome *c* oxidase subunit IV in which the leucine at position 18 is replaced by a tryptophan residue; p17, synthetic peptide comprising the first 17 N-terminal amino acids of the presequence of cytochrome *c* oxidase subunit IV; diS-C_3-(5), (3,3′)-dipropylthiocarbocyanine iodide; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; HEPES, 2-[4-(2-hydroxyethyl-1-piperazinyl)] ethanesulfonic acid; Tris, tris(hydromethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

Characterization of the Presequences

The purity of the presequences was determined with HPLC using an analytical polygosyl C_{18} column (Macherey-Nagel, Düren, Germany) and eluted as above with acetonitrile–water gradients. The purity was further confirmed by polyacrylamide gel electrophoresis. SDS-PAGE was done in a MiniProtean system from BioRad with a gel system consisting of three parts, i.e., a stacking gel, a spacer gel, and a separating gel containing 3.8, 9.8, and 15.5% (w/v) acrylamide, respectively. The anode buffer contained 0.2 M Tris-HCl (pH 8.9) while the cathode buffer contained 0.1 M Tricine, 0.1 M Tris-HCl, and 0.1% SDS (pH 8.25). The molecular weight markers used ranged from 12.5 (cytochrome *c*) to 1.45 kDa (bacitracin). Peptide bands were visualized with Coomassie Brilliant Blue staining. All peptides appeared at least 95% pure as judged both from HPLC and SDS-PAGE. The identity of the peptides was confirmed by amino acid analysis according to standard procedures.

Peptide Solutions

Stock solutions (1 mM) were prepared in distilled water and stored at −30°C in aliquots which were thawed only once. The concentrations of mastoparan, p25, and p17 were determined by the micro-BCA assay (Pierce, Rockford, Illinois) using known amounts of peptide as a standard. The concentration of p25(W) was measured with spectrophotometry using $\epsilon^{280} = 5,600\,M^{-1}\,cm^{-1}$.

Isolation of Mitochondria

Rat liver mitochondria were isolated from the livers of adult male Wistar rats as described elsewhere (Hovius *et al.*, 1990) except that the isolation media contained a cocktail of protease inhibitors (1 mM PMSF, 2 μM leupeptin, 0.2 mM benzamidin, and 50 μg soybean trypsin inhibitor/ml). The inhibitors were omitted during the final washing and resuspension.

Mitochondrial Respiration

Respiration was measured at 30°C with an oxygraph as described earlier (Hovius *et al.*, 1990) in a medium containing sucrose (70 mM), mannitol (200 mM), HEPES (25 mM), potassium phosphate (2.5 mM), EDTA (1 mM), $MgCl_2$ (2 mM), rotenone (2 μM), and succinate (10 mM) at pH 7.4 (medium A). Mitochondria were added to 0.8 mg protein/ml in a final volume of 2 ml. State III respiration was induced by the addition of 900 nmol ADP. After respiratory rates had returned to basal state IV levels, peptides were added to variable concentrations and the steady-state rates of peptide-induced respiration were measured. Finally, the influence of the peptides on state III respiration was determined by a further addition of 900 nmol ADP.

Membrane Potential Measurments

The membrane potential ($\Delta\Psi$) maintained by the mitochondria was estimated with the fluorescent dye diS-C_3-(5) (Sims *et al.*, 1974) on an SLM Aminco SPF 500 fluorimeter interfaced to a Laser computer. Mitochondria (100 μg/ml) and 2 μM dye were added to 2 ml medium A in a stirred cuvette at 30°C. Excitation and emission wavelengths were 620 and 680 nm, respectively. Different amounts of peptide were probed for their effects on $\Delta\Psi$. The peptides were also tested for their influence on $\Delta\Psi$ generated by ATP hydrolysis in the absence of respiration by adding 1 mM KCN and 4 mM ATP to the medium.

Effects of Peptides on Release of Adenylate Kinase

Mitochondria (0.8 mg protein/ml) were incubated with varying concentrations of peptide at 30°C in medium A. At different time points, the mitochondria were sedimented by centrifugation (5 min at 14,000 rpm in an Eppendorf microcentrifuge). The supernatant was removed and the pellet resuspended in medium A. Adenylate kinase activity was measured in both fractions.

Binding of p25 and p25(W) to Rat Liver Mitochondria

Mitochondria (0.8 mg/ml) were incubated with increasing concentrations of p25 or p25(W) ranging from 0 to 5 nmol/mg of mitochondrial protein. After 5 min incubation at 30°C in medium A the mitochondria were reisolated by centrifugation and the amount of peptide in pellet and supernatant determined by scintillation counting. Control experiments on the time dependence of binding showed that binding remained unaltered when increasing the incubation time from 1 to 5 min.

Intactness of p25 and p25(W) after Incubation with Mitochondria

The possible proteolytic digestion of the presequences p25 and p25(W) upon incubation with rat

liver mitochondria was assessed by SDS-PAGE. Peptides were incubated with mitochondria as above, after which the mitochondria were separated from the medium by centrifugation. Next, both pellet and supernatant samples were analysed by SDS-PAGE using the gel system detailed above. Because of the high resolving power of the gel system used, it was possible to selectively detect the p25 and p25(W) bands by Coomassie Blue staining also in the presence of mitochondrial protein. The relative mobility and the staining intensities of the peptide bands demonstrated that the presequences were essentially recovered in their original form, i.e., that proteolytic digestion was not significant under the present experimental conditions.

Mitochondrial Localization of p25 and p25(W)

The localization of the presequences in relation to the mitochondrial compartments was assessed by controlled digitonin fractionation (Hovius *et al.*, 1990) followed by marker enzyme analysis and liquid scintillation counting to quantify the radiolabeled peptides in the fractions.

Enzyme Assays

The activities of adenylate kinase, monoamine oxidase, cytochrome *c* oxidase, and fumarase were measured according to published procedures (Hovius *et al.*, 1990).

Miscellaneous

All experiments were performed in vials coated with dimethyldichlorosilane (Pierce).

RESULTS

Structural and Functional Effects of Amphipathic Peptides on Rat Liver Mitochondria

Four different peptides were examined for their effects on rat liver mitochondrial functions, i.e., p25, p25(W), p17, and mastoparan. The first three are representative of the matrix-targeting sequence of the cytosolically synthesized precursor of subunit IV of cytochrome *c* oxidase. The fluorescence properties of the non-natural p25(W) peptide have previously been used by De Kroon *et al.* (1991) to investigate the $\Delta\Psi$ dependence of its interaction with model membranes. Mastoparan, a tetradecapeptide from wasp venom, has pronounced membrane-seeking properties and a strong tendency to adopt an amphipathic α-helix conformation when membrane associated (Higashijima *et al.*, 1983).

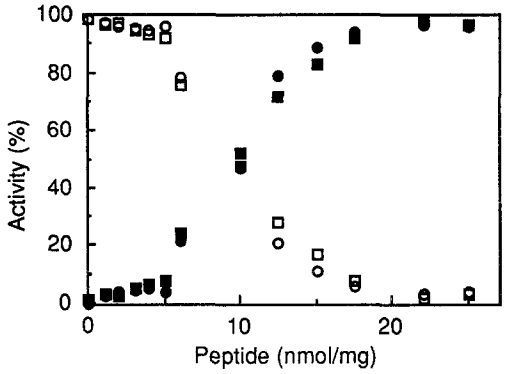

Fig. 1. Release of adenylate kinase from rat liver mitochondria induced by p25 and p25(W). Rat liver mitochondria were incubated for 5 min with increasing concentrations of prepeptide. The release of adenylate kinase was determined by measuring the distribution of enzyme activity between supernatant and pellet after centrifugation. At all peptide concentrations, the sum of the activity in the two fractions added up to 100% of that detected in the absence of peptide. Symbols: (○, ●) p25; (□, ■) p25(W). Open symbols: pellet; closed symbols: supernatant. Further details in Materials and Methods.

Outer Membrane Lysis

First, the effects of the peptides on the intactness of the outer membrane were measured. For that purpose, the peptide-induced release of the intermembrane space enzyme adenylate kinase was determined as a function of the ratio of peptide/mitochondrial protein. Figure 1 shows a representative example of such an experiment for p25 and p25(W). Starting from 5 nmol peptide/mg mitochondrial protein the integrity of the outer membrane was increasingly compromised such that at 12–13 nmol peptide/mg protein adenylate kinase was fully released. The efficiency of p25 and p25(W) appeared almost identical. Mastoparan had a similar potency in releasing adenylate kinase (data not shown). By contrast, p17 appeared considerably less effective, with 50% of total adenylate kinase being released at approximately 20 nmol peptide/mg protein (data not shown).

Uncoupling of Respiration

Next the peptides were tested for their effects on mitochondrial respiration. Figure 2 shows a comparison of p25, p25(W), p17, and mastoparan. Above

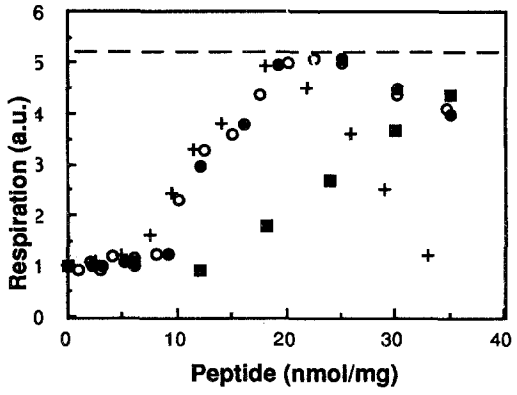

Fig. 2. Peptide-induced uncoupling of respiration. Rat liver mitochondria respiring on succinate in state IV were presented with peptide at the levels indicated. Respiratory rates observed in the presence of peptides are plotted relative to the rate of state IV respiration measured prior to peptide addition. Symbols: ●, p25; ○, p25(W); ■, p17; +, mastoparan. The broken line represents the rate of state III respiration, in the absence of peptide. For further details see Materials and Methods.

5 nmol/mg mitochondrial protein, p25, p25(W), and mastoparan caused an increase in state IV respiration. Stimulation to state III rates was reached around 20 nmol/mg protein. Further increasing the level of peptide led to a gradual inhibition of respiration. The latter effect was most pronounced for mastoparan. It is important to note that below 20 nmol peptide/mg mitochondrial protein state III respiration as induced by excess ADP remained unaffected. Above the latter ratio, ADP-stimulated respiration was identical to the peptide-induced state IV rates for p25, p25(W), and mastoparan (not shown). P17 induced uncoupling at considerably higher concentrations than the other peptides.

Dissipation of the Membrane Potential Across the Inner Membrane

Measurements of the membrane potential ($\Delta\Psi$) were used as a sensitive marker for the functional integrity of the inner membrane. $\Delta\Psi$ was estimated with the fluorescent dye diS-C_3-(5), which is accumulated in the matrix under the influence of the $\Delta\Psi$, leading to quenching of its fluorescence (Bunting *et al.*, 1989). The possible peptide-induced dissipation of $\Delta\Psi$ might be expected to lead to (partial) relief of this quenching. Figure 3 shows two typical experimental tracings in the absence (A) and in the presence of (an intermediary concentration of) p25 (B). The peptide led to fluorescence dequenching, which was almost identical during respiration on succinate and in the

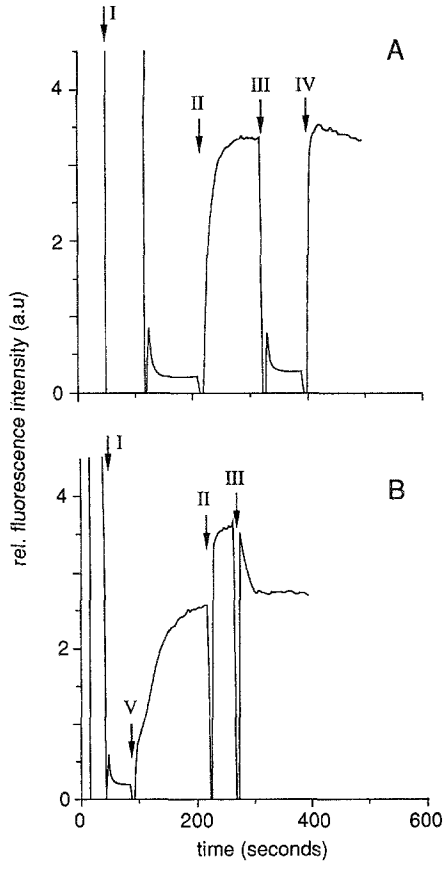

Fig. 3. Peptide-induced dissipation of the membrane potential across the inner membrane as estimated from dequenching of diS-C_3-(5) fluorescence. (A) Control; (B) in the presence of 30.2 nmol p25/mg mitochondrial protein. At the arrows the following additions were made: I, diS-C_3-(5) was added to the cuvette containing rat liver mitochondria respiring on succinate; II, 1 mM KCN to inhibit respiration; III, 4 mM ATP to allow $\Delta\Psi$ generation through H^+-ATPase activity; IV, 50 μg oligomycin to inhibit ATP hydrolysis; at the arrow labeled V in Fig. 3B, p25 was injected. Note that the $\Delta\Psi$ generated by succinate oxidation was affected by p25 to a similar extent as that generated by ATP hydrolysis. Further details in Materials and Methods.

presence of ATP with respiration inhibited by KCN. Figure 4 depicts the results of titrations with p25 and p25(W) for mitochondria respiring on succinate. At high peptide concentrations the fluorescence increased to levels seen upon KCN addition. Fluorescence intensities attained with ATP showed an essentially identical behavior (Fig. 3B; data not shown). These experiments suggest that the peptide–mitochondria interaction caused a gradual dissipation of the $\Delta\Psi$ due to an increase of the H^+-permeability of the inner membrane and not due to interference with $\Delta\Psi$ generation by respiratory or ATPase activity as such. These data imply that $\Delta\Psi$ became negligible

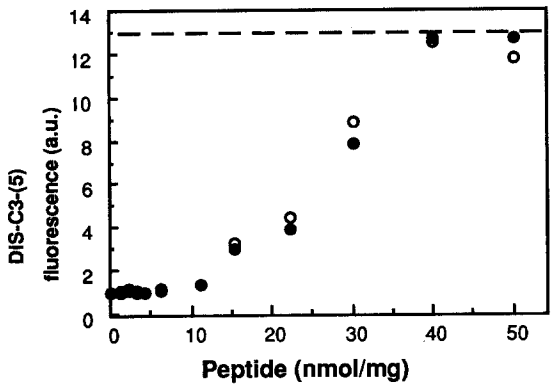

Fig. 4. Peptide-induced dissipation of $\Delta\Psi$ in rat liver mitochondria respiring on succinate. Data were obtained from a series of experiments shown partly in Fig. 3. The fluorescence intensity measured at each peptide concentration is plotted as a function of the peptide concentration. The broken line represents the fluorescence intensity measured upon addition of KCN. Symbols: (●), p25; (○), p25(W). For clarity, only the data for p25 and p25(W) are shown. Other experimental details in the legend to Fig. 3 and in Materials and Methods.

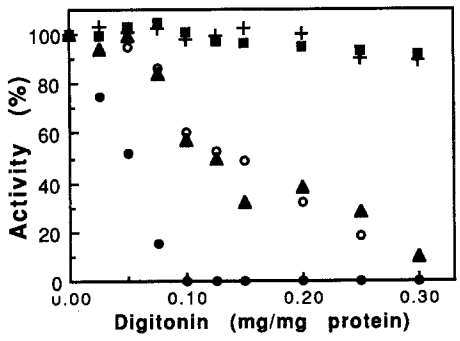

Fig. 5. Digitonin treatment of mitochondria to assess the location of bound p25 in relation to marker enzymes. Rat liver mitochondria (0.8 mg/ml) were incubated with 4 nmol p25/mg in medium A for 5 min at 30°C. Subsequently, the mitochondria were subjected to digitonin treatment. Marker enzymes and peptide were quantified in the resulting subfractions as detailed in Materials and Methods. Symbols: ●, adenylate kinase; ○, monoamine oxidase; ■, fumarase; +, cytochrome *c* oxidase; ▲, ^{14}C counts from p25. Data for the pellet fraction are given relative to the control without digitonin.

above approximately 40 nmol/mg protein for p25 and p25(W). Mastoparan behaved essentially identical to p25 and p25(W). By contrast, p17 proved considerably less potent, with complete dissipation of $\Delta\Psi$ being reached at ca. 60 nmol peptide/mg protein (data not shown).

Intramitochondrial Localization of p25 and p25(W)

We next determined the location of mitochondrion-associated p25 and p25(W). This experiment was restricted to p25 and p25(W) since these were synthesized with a radiolabeled amino acid and thus could be conveniently monitored by liquid-scintillation counting. P17 and mastoparan were not radiolabeled. The localization experiment was done under conditions where both membranes remained fully intact. In control experiments we first measured the extent of binding of p25 and p25(W) to the rat liver mitochondria. It appeared that up to 5 nmol prepeptide/mg protein, binding was linear with the amount of peptide added, i.e., the percentage peptide bound remained constant. The binding amounted to $28 \pm 4\%$ ($n = 6$) and did not increase when extending the incubation time from 1 to 5 min. In extensive studies on p25 binding to phospholipid vesicles and yeast mitochondria, Swanson and Roise (1992) also observed that binding was linear throughout a large range of peptide concentrations. This shows that binding sites do not saturate under these conditions and also suggests that binding can be treated as a simple partitioning of the prepeptide between the aqueous phase and the membrane phase.

The location of the mitochondrion-associated peptides was determined by controlled lysis of the mitochondria with digitonin (Hovius *et al.*, 1990). A typical experiment with p25 is shown in Fig. 5. Interestingly, the digitonin-induced release of bound p25 coincided with that of the outer membrane marker monoamine oxidase, suggesting that the peptide was exclusively associated with that membrane. Incubations under other conditions (e.g., upon energization with succinate) did not alter the location of the peptide (not shown). Similar observations were made with p25(W) (not shown).

We did not attempt to determine the intramitochondrial localization of p25 and p25(W) at high peptide concentrations because the selectivity of the digitonin fractionation critically depends on the integrity of the mitochondrial boundary membranes (Hovius *et al.*, 1990; Nicolay *et al.*, 1990).

DISCUSSION

This study addressed the effects of amphipathic peptides on the structural and functional integrity of rat liver mitochondrial membranes. Going from low to high ratios of peptide-to-mitochondrial protein, the following sequence of events was observed: (i) peptide binding to the outer membrane (Fig. 5); (ii) lysis of the outer membrane as evidenced by the release of

adenylate kinase (Fig. 1); (iii) an increase in the permeability of the inner membrane as deduced from uncoupling of respiration (Fig. 2); and (iv) dissipation of $\Delta\Psi$ (Figs. 3 and 4). A comparison of the response of respiration (Fig. 2) and of diS-C_3-(5) fluorescence (Fig. 4) to p25 and p25(W) addition suggested a lack of correspondence between the stimulation of oxygen consumption and the decrease in membrane potential. It should be noted, however, that the diS-C_3-(5) fluorescence signal has been reported to deviate from a linear dependence on $\Delta\Psi$ in some cases, especially at high potentials (Smith, 1990). This would account for the stimulation of respiration at peptide levels which appear not to affect potential. By contrast, Rottenberg (1979) has reported a linear relationship between $\Delta\Psi$ (as measured by ^{86}Rb distribution) and quenching of diS-C_3-(5) fluorescence in rat liver mitochondrial suspensions. In view of these apparent contradictions on diS-C_3-(5) signal calibration, no definitive conclusions can be drawn as to the precise relation between the peptide-induced effects on respiration and membrane potential.

According to the aforementioned criteria, p25, p25(W), and mastoparan compromised mitochondrial integrity similarly, while the shorter p17 prepeptide was less potent. The present data highlight the strong membrane-seeking nature of amphipathic peptides. Previous model membrane studies showed that the membrane interaction of CoxIV presequences and mastoparan is greatly stimulated by negatively charged phospholipids and by the presence of a membrane potential (inside negative) (Roise *et al.*, 1986; De Kroon *et al.*, 1991). Using NMR, Jordi *et al.* (1990) showed that binding of p17 to model membranes containing negatively charged phospholipids had a strong disordering effect on the acyl chains and the polar headgroup. These data most probably are relevant for understanding the mechanism by which amphipathic peptides compromise membrane integrity. In mitochondria this may lead to a loss of the permeability barrier of both boundary membranes as evidenced by leakage of adenylate kinase through the outer membrane (this study), uncoupling of respiration and dissipation of $\Delta\Psi$ across the inner membrane (this study; Roise *et al.*, 1986; Glaser and Cumsky, 1990a; Cyr and Douglas, 1991). Similarly, presequence-induced release of contents and $\Delta\Psi$ dissipation have been reported for protein-free phospholipid vesicles (Roise *et al.*, 1986; Roise *et al.*, 1988; Skerjanc *et al.*, 1987; Endo and Schatz, 1988).

Westerhoff *et al.* (1989) have recently reported on the effects of a class of eukaryotic antimicrobial peptides, the magainins, on mitochondrial free-energy transduction. While these peptides compromise mitochondrial oxidative phosphorylation in a similar manner as the peptides used here, these authors found an interesting positive cooperativity from the concentration dependence of these effects. This was interpreted as an indication for the formation of an oligomeric structure constituting a hydrophilic pore through the inner membrane. Such effects have not been described so far for mitochondrial presequences or mastoparan.

It has previously been reported that presequences cause a dose-dependent inhibition of the *in vitro* mitochondrial import of a variety of precursor proteins (Glaser and Cumsky, 1990a; Cyr and Douglas, 1991; Hoyt *et al.*, 1991; Ito *et al.*, 1985; Glaser and Cumsky, 1990b). We have similarly assessed the effects of p25 and p25(W) on the import of the purified pCoxIV-DHFR precursor protein (Eilers and Schatz, 1986; Vestweber and Schatz, 1988) into rat liver mitochondria (F.D. Laterveer and K. Nicolay, unpublished observations). As expected, the presequences seriously compromised the import reaction: the import activity had been reduced by 50% at approximately 8 nmol p25 or p25(W)/mg mitochondrial protein. This implies that protein import was greatly diminished by the prepeptides at concentrations where the structural and functional integrity of the outer and inner membrane was still largely unaffected. This strongly suggested that the early phase of import inhibition was not through dissipation of $\Delta\Psi$ or some other overall effect. As such, this finding is fully in line with earlier reports (Glaser and Cumsky, 1990a; Cyr and Douglas, 1991; Hoyt *et al.*, 1991; Ito *et al.*, 1985; Glaser and Cumsky, 1990b).

It should be noted, however, that previous studies on the specificity of the prepeptide-induced inhibition of the mitochondrial import of full-length precursor proteins have largely based the above conclusion on the finding that inhibition of import occurs at peptide levels lower than those causing dissipation of $\Delta\Psi$. Our study clearly shows that this may lead to erroneous conclusions: dissipation of the membrane potential is a late event that is preceded by lysis of the outer membrane and most probably also by (partial) uncoupling of respiration.

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