

Effect of Leader Peptides on the Permeability of Mitochondria*

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Peptides with sequences based on the leader sequence of yeast cytochrome *c* oxidase subunit IV (pCOX IV-(1–25)) activate the electrophoretic uptake of K⁺ and other cations such as tetraethylammonium and lysine by rat liver mitochondria with EC₅₀ = 11–15 μM. Uptake of these cations is dependent on respiration and is prevented by uncoupling agents, and the V_{max} for K⁺ is 1.2–1.5 μmol/min/mg. Albeit more slowly, the non-electrolytes mannitol and sucrose are also transported by this pathway. Treatment of the peptides with proteinase K eliminates the stimulatory effect. Since the stimulated rate is not inhibited by ATP or by cyclosporin, we conclude that this pathway is not related to the mitochondrial K_{ATP} channel or the Ca²⁺-dependent permeability transition pore. Transport is stimulated by pCOX IV-(1–23), pCOX IV-(1–22), and pCOX IV-(1–12)Y, but not by a 13-amino acid peptide representing the nuclear location sequence of the SV40 large T antigen, which is responsible for directing that protein to the nucleus. Spermine, which has four positive charges, also has no stimulatory effect, and an amphiphilic 22-residue peptide derived from antithrombin III with seven net charges is only one-twentieth as effective as pCOX IV-(1–22). Thus, these data indicate that the sequence/structure is important for activation of transport. We also demonstrate that mitochondrial uncoupling, previously reported to be induced by these peptides, actually reflects coupled accumulation of salt. In view of our findings, it is also likely that the lytic effects attributed to these peptides are secondary to swelling and are not due to membrane damage *per se*. Finally, we show that, in non-ionic media, the peptide is an inhibitor of cytochrome *c* oxidase.

Proteins synthesized in the cytosol are imported into mitochondria if they possess an appropriate signal sequence. This is usually a sequence of 20–30 amino acids with 3–5 lysine or arginine residues, each separated by 2–5 hydrophobic residues. They can be presequences, cleaved after import, or can be part of the mature protein (see Ref. 1 for review). Synthetic peptides based on these signal sequences, frequently referred to as leader peptides, have been shown to competitively inhibit import of proteins (2, 3) and have also been shown to inhibit certain mitochondrial K⁺ channels (4–10). These findings have led to the suggestion that these channels may be part of the mitochondrial protein import machinery (6–10). In a previous

paper (11), we examined the properties of a K⁺ transport pathway in intact mitochondria that is stimulated by *N*-ethylmaleimide and inhibited by adenine nucleotides. Although we have suggested that this pathway may be involved in volume homeostasis, its exact function has not been established. Thus, in view of the reports mentioned above, the question arose as to whether this transport pathway may be one of those involved in protein import. Thus, we began this study by examining the effect of a synthetic leader peptide on K⁺ uptake by mitochondria.

Despite the apparent importance of leader sequences in the translocation of proteins, the effects of leader peptides on isolated mitochondria have not been fully characterized. Several groups have reported that these peptides, which can form amphiphilic helices (12), uncouple mitochondria (3, 13–16) and can cause membrane lysis (14, 16). Glaser and Cumsy (3), however, have reported that the peptide pCOX IV-(1–22), which represents the first 22 amino acids of the leader sequence of yeast cytochrome *c* oxidase subunit IV, does not uncouple, whereas pCOX IV-(1–23), which has a C-terminal tyrosine, does uncouple.

In this paper, we demonstrate that pCOX IV-(1–22) and related peptides have no effect on the ATP-dependent K⁺ channel. We do show, however, that they activate a transport pathway that not only conducts K⁺, tetraethylammonium, and lysine cations, but also the non-electrolytes sucrose and mannitol. Activation of this pathway does not uncouple mitochondria *per se*; however, the respiration-coupled uptake of salts probably explains the reported respiratory stimulation (3, 13, 14, 16). Moreover, the swelling that ensues probably explains the reported peptide-induced disruption of mitochondria (16). We suggest that this pathway could be involved in protein import.

EXPERIMENTAL PROCEDURES

Assay of Transport—Transport was assayed by following swelling that accompanies net solute transport using the light scattering technique as described in detail elsewhere (17, 18). Using this technique, we generated a light scattering variable, β , which normalizes reciprocal absorbance for mitochondrial protein concentration. The rate of solute transport is calculated from the rate of change of β .

Pretreatment of Mitochondria for Assay of Transport—The normal mitochondrial stock suspension (50 mg of protein/ml) in 0.25 M sucrose was treated with rotenone (0.5 μg/mg) and oligomycin (2 nmol/mg).

Assay Media—The media for assay of salt transport contained the K⁺, tetraethylammonium⁺, or lysine⁺ salts of acetate (30 mM), Cl[−] (45 mM), EGTA (0.1 mM), and MOPS¹ (5 mM) plus MgCl₂ (0.3 mM) and cytochrome *c* (10 μM). Ascorbate (2.5 mM) and TMPD (0.25 mM) or succinate (3 mM) were added as indicated in the figure legends. For the sucrose and mannitol assay media, acetate and Cl[−] salts were replaced by sucrose or mannitol (125 mM). The KCl assay medium contained 75 mM KCl and no acetate. All media had an osmolality close to 146 milliosmolal.

Drugs and Reagents—Most drugs were obtained from Sigma. The

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¹ The abbreviations used are: MOPS, 3-(*N*-morpholino)propanesulfonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

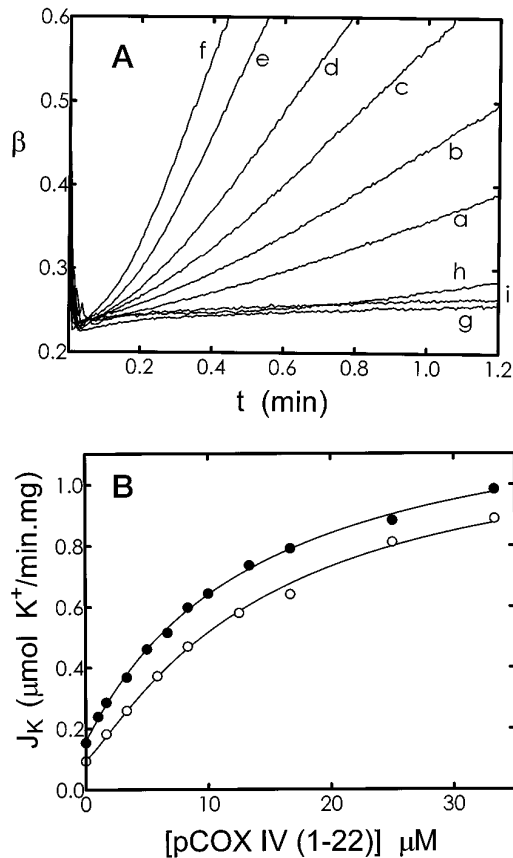


FIG. 1. pCOX IV-(1-22) stimulates K^+ uniport in mitochondria. A, light scattering traces are shown of mitochondria (0.1 mg/ml) respiring on ascorbate/TMPD suspended in KOAc/Cl assay medium. The following doses of pCOX IV-(1-22) were added at zero time: trace a, 0 μM ; trace b, 1 μM ; trace c, 3.3 μM ; trace d, 6.6 μM ; trace e, 16.6 μM ; trace f, 33 μM . For traces g-i, 20 μM pCOX IV-(1-22) was added, but for trace g, ascorbate and TMPD were omitted; for trace h, nigericin (7 nmol/mg) was added; and for trace i, CCCP (0.4 μM) was added. B, the rates of K^+ uptake were determined from traces similar to those shown in A. The mitochondria were energized using ascorbate/TMPD (\bullet) or succinate (\circ) as the substrate. The curves fitted to the data have EC_{50} values and Hill coefficients of 13.8 μM and 1.0 for ascorbate/TMPD and 14.5 μM and 1.2 for succinate, respectively.

ionophores and rotenone were dissolved in ethanol. Rat liver mitochondria were prepared as described previously (17), and rat liver mitoplasts were prepared as described by Schnaitman and Greenawalt (19).

Synthesis of Peptides—The peptides pCOX IV-(1-12)Y, pCOX IV-(1-22), and pCOX IV-(1-23) used in this study were synthesized by Dr. Katherine Wall (University of Toledo) using essentially the same procedure described in Ref. 3. The purified peptides were analyzed for amino acid composition, and molecular weights were confirmed by electrospray ionization mass spectrometry by the University of Texas Health Science Center at the San Antonio Biopolymer Facility. pAT III was a generous gift from Dr. K. W. Kinnally and was synthesized by the Wadsworth Center Biochemistry Core.

RESULTS

pCOX IV-(1-22) Stimulates K^+ Uptake in Respiring Mitochondria—In a previous study (11), we demonstrated that respiring mitochondria swell in K^+ salts of permeant acids and that this transport can be partially inhibited by ATP. Using a similar assay, we have now examined the effect of the leader sequence of cytochrome oxidase subunit IV on K^+ transport in intact mitochondria.

The data in Fig. 1A show the effect of pCOX IV-(1-22), a peptide that represents the first 22 amino acids of the leader peptide and that has the sequence MLSLRQSRFFK-PATRTLCSRS. Contrary to expectations, the peptide stimulated swelling in a dose-dependent manner. The effect of the

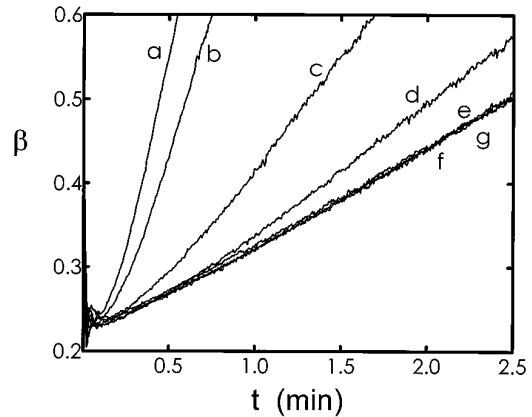


FIG. 2. Proteinase K eliminates stimulation by pCOX IV-(1-22). Light scattering traces are shown of mitochondria (0.11 mg/ml) respiring on succinate suspended in KOAc/Cl assay medium. pCOX IV-(1-22) (5 μM) was added to the medium for all traces except traces f and g. Traces a-e show the effect of treating the stock solution of pCOX IV-(1-22) (3 mM) with proteinase K (0.1 mg/ml) on ice for 0, 0.5, 3, 7, and 60 min, respectively, prior to the assay. Trace f is a control with no pCOX IV-(1-22) and no proteinase K, and trace g shows the effect of proteinase K in the absence of pCOX IV-(1-22). The assay medium and other conditions are described under "Experimental Procedures."

peptide was not observed in the absence of ascorbate and TMPD (trace g) and was abolished by nigericin, an electroneutral K^+ / H^+ antiporter (trace h), and also by the uncoupler CCCP (trace i). These findings indicate that the swelling is dependent on electrophoretic K^+ uptake driven by the membrane potential and does not represent lysis of the mitochondria. Similar results were obtained using succinate as respiratory substrate (data not shown).

The dose-response curves for pCOX IV-(1-22) with mitochondria respiring on ascorbate/TMPD and succinate are compared in Fig. 1B. Although the control rate with succinate was only about half that found with ascorbate/TMPD, the maximum rates were very similar. In both cases, influx rates approached a J_{max} of 1.2 $\mu\text{mol}/\text{min}/\text{mg}$ with an EC_{50} of 14 μM and a Hill coefficient of 1. This represents a 7.5- and 14.6-fold stimulation of the flux with ascorbate/TMPD and succinate, respectively. Since it has been reported that pCOX IV-(1-23), which has a C-terminal tyrosine, is an uncoupler (3), we also tested this peptide. The data obtained with pCOX IV-(1-23) were very similar to those obtained with pCOX IV-(1-22) and yielded EC_{50} values of 11.0 and 12.5 μM using ascorbate/TMPD and succinate, respectively. Again, J_{max} values were similar and close to 1.4 $\mu\text{mol}/\text{min}/\text{mg}$ (data not shown).

Proteolysis Abolishes the Effect of pCOX IV-(1-22)—The data in Fig. 2 show that pretreatment of the peptide solution with proteinase K completely abolishes the effect of the peptide with a half-time of ~ 2 min at 4 $^{\circ}\text{C}$. Over the time course of the traces, the protease alone had no effect (trace g). Thus, an intact peptide is necessary to induce stimulation. Similar results were obtained with both succinate and ascorbate/TMPD as substrates.

ATP Does Not Inhibit Peptide-induced K^+ Transport—In view of our previous studies under similar conditions showing potent inhibition of K^+ transport by ATP ($K_I = 2.3 \mu\text{M}$) (11), we asked the question whether ATP would also inhibit transport induced by the peptide. The data in Fig. 3 show that this is not the case. Although ATP (0.2 mM) inhibited the control rate in the absence of pCOX IV-(1-22) (traces a and b), no inhibition was observed in the presence of pCOX IV-(1-22) (8.3 μM) (traces c and d). In fact, we consistently observed a small stimulation. Two possible explanations for the lack of inhibition by ATP are 1) that the pathway induced by pCOX IV-(1-22) is independent

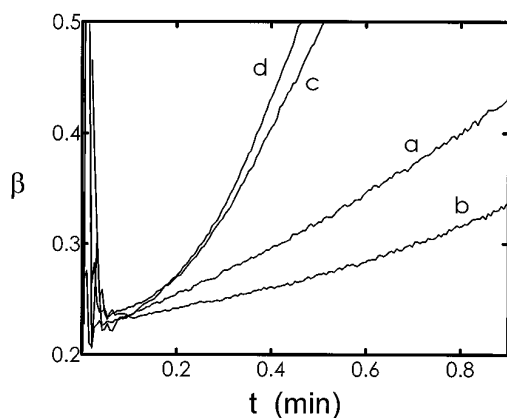


FIG. 3. **ATP does not inhibit pCOX IV-(1-22)-induced K^+ transport.** Light scattering kinetics are shown for mitochondria (0.1 mg/ml) respiring on ascorbate/TMPD suspended in KOAc/Cl assay medium. Trace a, control; trace b, plus ATP (0.2 mM); trace c, plus pCOX IV-(1-22) (8.3 μ M); trace d, plus pCOX IV-(1-22) (8.3 μ M) and ATP (0.2 mM). The assay medium and other conditions are described under "Experimental Procedures."

of the ATP-regulated pathway and 2) that the peptide prevents ATP from inhibiting the channel. Although formation of a complex between the positively charged peptide and negatively charged ATP could lead to a mutual neutralization of effects, this cannot explain these results because ATP was added in excess of peptide, and consequently, formation of a complex could only enhance the inhibitory effect of ATP.

Is the Effect of pCOX IV-(1-22) Specific for the Leader Sequence?—In view of the role of leader peptides in directing the import of proteins into mitochondria, the possibility arises that pCOX IV-(1-22) is activating a channel involved in protein import. If this were the case, the effect should only be observed with authentic leader sequences. To begin a study of the specificity of the effect, we first examined the effect of spermine on K^+ uptake since spermine, like the peptide, is polycationic. Spermine, however, did not stimulate, but rather inhibited uptake (data not shown).

We have also examined the effect of a 13-residue peptide (which we will refer to as Q13) that contains the nuclear location sequence responsible for directing the import of the SV40 large T antigen into the nucleus (20). This peptide has the sequence CGGGPKKKRKVED, which, like pCOX IV-(1-22), contains 5 basic amino acids, but also 2 acidic amino acids. For comparison, we also examined the effect of pCOX IV-(1-12)Y, which has a net charge of 3+ and includes the first 12 amino acids of pCOX IV-(1-22), which are sufficient to direct import of proteins into mitochondria (21). As shown by the traces in Fig. 4, pCOX IV-(1-12)Y stimulated K^+ uptake, but was less potent than pCOX IV-(1-22). In contrast, Q13 had no effect at least up to a concentration of 33 μ M.

As a more appropriate control, we examined the effect of pAT III, which is a 22-amino acid peptide derived from antithrombin III with the sequence RNASVLKSSKNKRYLRNLKA. This peptide contains 7 basic amino acids and can also form an amphiphilic helix, but, unlike pCOX IV-(1-22), is reported not to inhibit protein import in yeast (3). The data in Fig. 4B show that although pAT III does stimulate K^+ transport, it is only about one-twentieth as potent as pCOX IV-(1-22). From these data, we conclude that the presence of positive charges alone is insufficient to activate transport and that some additional property of pCOX-(1-22) is responsible for its potency.

Selectivity of the Permeability Induced by pCOX IV-(1-22)—If the " K^+ channel" activated by pCOX IV-(1-22) is normally involved in the transport of proteins, e.g. when the peptide is attached to a protein, one would expect a rather broad

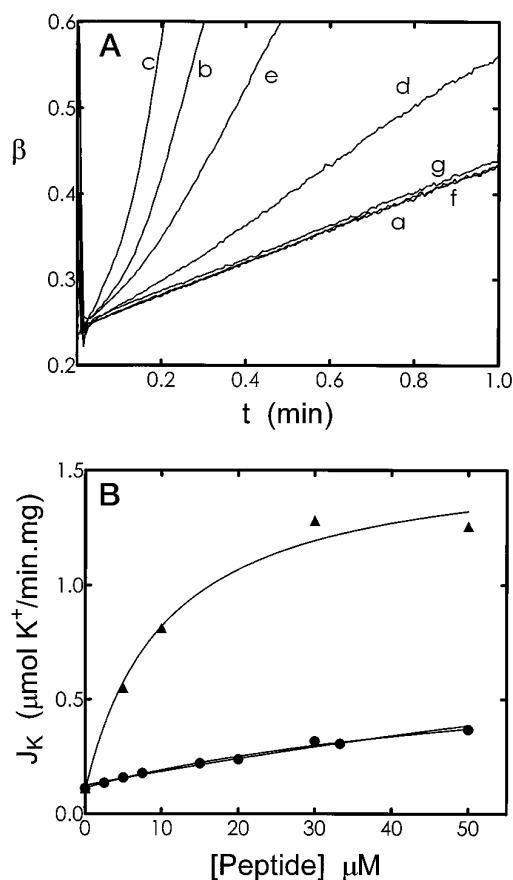


FIG. 4. **Selectivity of peptide effect on K^+ permeability.** A, light scattering kinetics are shown for mitochondria (0.12 mg/ml) respiring on ascorbate/TMPD suspended in KOAc/Cl assay medium. Trace a, control; trace b, plus 8 μ M pCOX IV-(1-22); trace c, plus 33 μ M pCOX IV-(1-22); trace d, plus 8 μ M pCOX IV-(1-12)Y; trace e, plus 33 μ M pCOX IV-(1-12)Y; trace f, plus 8 μ M Q13; trace g, plus 33 μ M Q13. In each case, the peptide was added at zero time. B, dose-response curves for pAT III (\bullet) and pCOX IV-(1-22) (\blacktriangle) are compared. Data were obtained as described for A using the same preparation of mitochondria. The curves fitted to the data yield $J_{\max} = 1.58 \mu\text{mol}/\text{min}/\text{mg}$ and $EC_{50} = 10.6 \mu\text{M}$ for pCOX IV-(1-12) and $J_{\max} = 0.77 \mu\text{mol}/\text{min}/\text{mg}$ and $EC_{50} = 79 \mu\text{M}$ for pAT III. Also shown is the curve obtained if it is assumed that the value of J_{\max} for pAT III is 1.58, and this gives an EC_{50} of 228 μM . Hill coefficient = 1.0 for all curves. The assay medium and other experimental details are described under "Experimental Procedures."

selectivity to permit passage of the variety of amino acid side chains found in proteins. Thus, we have investigated the effect of pCOX IV-(1-22) on mitochondria respiring in a variety of media. The dose-response curves shown in Fig. 5 show that transport can be observed in respiring mitochondria when the K^+ in the assay medium is replaced by tetraethylammonium ions or by the amino acid lysine. We also observed swelling, although at a much lower rate, in the non-electrolytes sucrose and mannitol. One would not expect the uptake of these non-electrolytes to be driven by the membrane potential; however, the rate was significantly higher in respiring mitochondria (data not shown). One explanation for this effect may be that, in the absence of a membrane potential, K^+ is lost from the mitochondria. It is also possible that the membrane potential may play a role in the binding of the peptide to the putative channel or may affect the open probability of the channel. The lowest rate of swelling was observed in KCl medium. In this case, addition of an uncoupler or omission of substrates actually stimulated swelling. This suggests that Cl^- may be able to enter the mitochondria under these conditions, but that in energized mitochondria, this flux is prevented by the membrane potential.

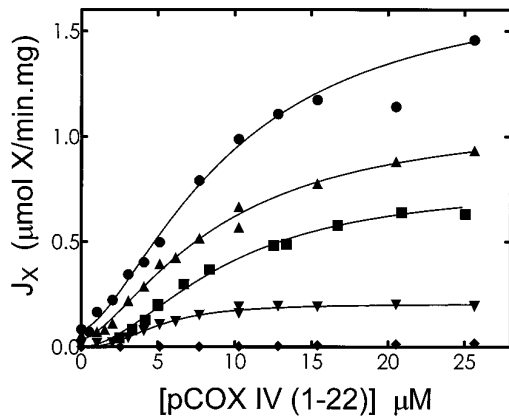


FIG. 5. **Selectivity of permeability induced by pCOX IV-(1-22).** Rates of K^+ (●), tetraethylammonium $^+$ (▲), lysine (■), and sucrose (▼) uptake are plotted versus the concentration of pCOX IV-(1-22) for mitochondria respiring on ascorbate/TMPD suspended in the appropriate assay medium. Swelling rates in KCl medium (◆) in the absence of a permeant acid are also shown. The measurements were made from light scattering traces similar to those shown in Fig. 1. The assay media and other experimental details are described under "Experimental Procedures."

Does pCOX IV-(1-22) Induce Proton Permeability?—The data presented in Figs. 1 and 5 suggest that the uptake of K^+ induced by pCOX IV-(1-22) is coupled to respiration and is not the result of mitochondrial damage or uncoupling. To investigate this further, we examined the effect of pCOX IV-(1-22) on the rate of oxygen consumption in KOAc/Cl, sucrose, and KCl media in experiments carried out in parallel to those shown in Fig. 5. As shown by the data in Fig. 6, pCOX IV-(1-22) stimulated respiration substantially in KOAc/Cl medium, but negligibly in sucrose or KCl. Thus, at least over this dose range, pCOX IV-(1-22) does not appear to uncouple mitochondria. A number of reports, however, have suggested that leader peptides do uncouple mitochondria (3, 13–16). To try to resolve this issue, we have investigated the effect of pCOX IV-(1-22) on mitochondria respiring in a medium with the same salt composition as that used by Nicolay *et al.* (16). Although the major osmolytes are non-electrolytes, this medium does contain about 39 mM K^+ , 10 mM succinate, and 2.5 mM P_i ; therefore, the stimulation of respiration reported by Nicolay *et al.* (16) may in fact reflect respiration-driven uptake of potassium succinate permitted by an increased K^+ permeability and the activities of the dicarboxylate carrier and phosphate carrier. The data shown in Fig. 7 support this hypothesis. *Traces a* and *b* in Fig. 7A show that pCOX IV-(1-22) stimulates respiration 3.7-fold; however, *traces c* and *d* show that the stimulation is dependent on the presence of P_i , and *trace e* shows that the stimulation can be blocked by *N*-ethylmaleimide, an inhibitor of the phosphate carrier. Comparison of *traces a* and *f* reveals that, in the absence of pCOX IV-(1-22), P_i stimulates respiration 1.4-fold, and comparison of *traces d* and *f* shows that, in the absence of added P_i , the peptide stimulates respiration 1.6-fold. Since this latter stimulation is also blocked by *N*-ethylmaleimide (initial parts of *traces e* and *d*), this probably reflects the existence of endogenous P_i . These data also show that the rate of pCOX IV-(1-22)-stimulated respiration is essentially the same whether P_i is added at the start (*trace b*) or at 1.25 min (*trace c*). Thus, the effect of pCOX IV-(1-22) must be induced very rapidly.

These conclusions are fully supported by light scattering traces obtained in parallel experiments shown in Fig. 7B. Comparison of *traces a*, *c*, and *d* shows that, in the presence of both P_i and pCOX IV-(1-22), there is rapid swelling, which can be blocked by *N*-ethylmaleimide (*trace e*). Moreover, consistent

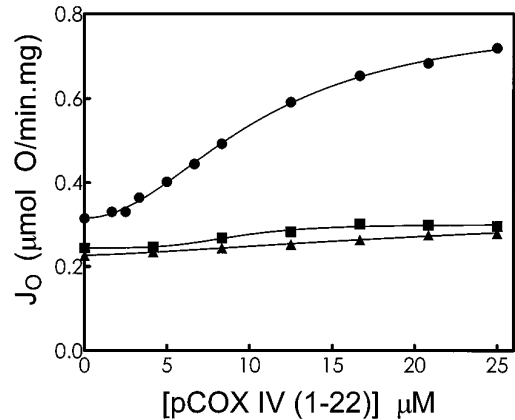


FIG. 6. **Stimulation of respiration by pCOX IV-(1-22) is dependent on influx of cations.** The rate of oxygen consumption (J_O) by mitochondria (1.2 mg/ml) suspended in various assay media containing ascorbate/TMPD is plotted versus the concentration of pCOX IV-(1-22). The assay media were the same composition as those used in Fig. 5: ●, KOAc/Cl; ■, sucrose; ▲, KCl. The composition of the assay media and other experimental details are described under "Experimental Procedures."

with the effects on respiration, in the absence of exogenous P_i , pCOX IV-(1-22) stimulated swelling slightly (*traces d* and *f*). In this experiment, we also show that CCCP, a true uncoupler, abolished the swelling induced by pCOX IV-(1-22) and phosphate (*traces c* and *g*). Thus, the swelling induced by the peptide under these experimental conditions cannot be attributed to an uncoupling effect. This does not rule out the possibility that pCOX IV-(1-22) increases proton permeability; however, it does mean that the H^+ flux must be insignificant compared with the K^+ flux.

Peptide-induced Permeability Is Not the Permeability Transition Pore—In view of our finding that the pCOX IV-(1-22)-induced pathway can carry a variety of solutes, the question arose as to whether it could represent the so-called permeability transition pore (22), which is frequently assayed through its ability to transport sucrose. Since the opening of the permeability transition pore is potently inhibited by cyclosporin A, we examined the effect of cyclosporin on sucrose permeability induced by pCOX IV-(1-22). For this experiment, cyclosporin was added at zero time, and 2 min were allowed to elapse before addition of pCOX IV-(1-22). As shown by the data in Fig. 8, addition of the peptide induced swelling; moreover, the traces obtained with 0.17 and 1.7 μ M cyclosporin are indistinguishable from the control trace. From this result, we conclude the pCOX IV-(1-22) does not activate the permeability transition pore.

Leader Peptides Inhibit Cytochrome c Oxidase—In comparing data obtained in the various media, we observed that, unlike in salt media, in sucrose medium, the V_{max} and EC_{50} values for pCOX IV-(1-22) and pCOX IV-(1-23) appeared to be lower when ascorbate/TMPD was used as a substrate than when succinate was used (data not shown). This difference appears to be related to the lower ionic strength since, as shown in Fig. 9, a similar result was obtained in mannitol medium. Since, at the highest peptide doses, the rates actually appear to decrease, we suspected that the effect may be related to inhibition of cytochrome *c* oxidase.

To investigate this possibility, we examined the effect of pCOX IV-(1-22) and pCOX IV-(1-23) on the rate of respiration of mitoplasts suspended in sucrose medium and KCl assay medium with CCCP added to ensure respiration was limited by the activity of the respiratory chain. As shown by the data in Fig. 10, pCOX IV-(1-22) significantly inhibited oxidation of ascorbate/TMPD, but had a relatively small effect on oxidation

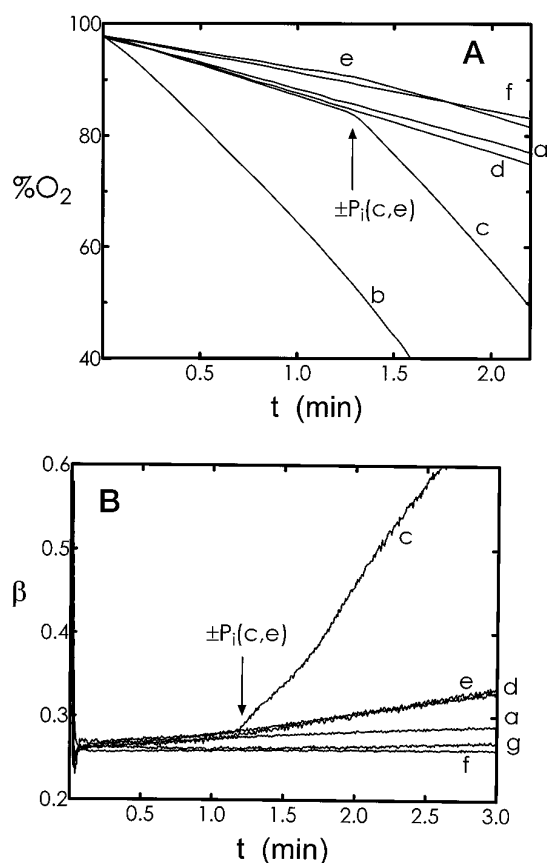


FIG. 7. Low concentrations of phosphate are sufficient to support pCOX IV-(1-22)-dependent respiratory stimulation and uptake of K⁺. Oxygen electrode recordings (A) and light scattering kinetics (B) are shown for mitochondria (1.3 mg/ml (A) and 0.12 mg/ml (B)) respiring on succinate. For both panels: trace a, control with P_i (2.5 mM); trace b, plus pCOX IV-(1-22) (25 μM) and P_i at zero time; trace c, plus pCOX IV-(1-22) (25 μM) at t = 0 and P_i at t = 1.3 min; trace d, plus pCOX IV-(1-22) (25 μM) at t = 0; trace e, N-ethylmaleimide-treated mitochondria, plus pCOX IV-(1-22) at t = 0 and P_i at t = 1.3 min; trace f, no pCOX IV-(1-22) and no P_i; trace g, plus pCOX IV-(1-22) (25 μM), P_i, and CCCP (0.26 μM) at t = 0 (B only). The assay medium contained sucrose (250 mM), MgCl₂ (2.5 mM), cytochrome c (10 μM), and rotenone (1 μg/ml) plus the K⁺ salts of HEPES (25 mM), succinate (10 mM), and EDTA (1 mM) and, where indicated, P_i (2.5 mM). This medium is essentially the same as that used by Nicolay *et al.* (16), with the mannitol replaced by sucrose.

of succinate. These results are consistent with cytochrome *c* oxidase being the site of inhibition since oxidation of succinate is limited by succinate dehydrogenase and not by cytochrome *c* oxidase. The higher potency in sucrose *versus* KCl suggests that ionic interactions between the positively charged peptides and their site of action may be involved in binding. The IC₅₀ obtained in this experiment was 31 μM with a mitoplast concentration of 0.4 mg/ml. Further experiments revealed that the IC₅₀ decreased as the mitoplast concentration was decreased, yielding an extrapolated value of 7 μM at zero concentration (data not shown). This result is consistent with the existence of a large number of low affinity binding sites on the mitoplasts, which will affect the free concentration of peptide. This finding is consistent with the report of Pak and Weiner (23).

To further investigate the nature of this inhibition, we examined the effect of varying cytochrome *c* concentration. In these experiments, inhibition by 8.6 μM pCOX IV-(1-22) decreased from 75% to 65, 55, and 35% as the concentration of exogenous cytochrome *c* was raised from 0 μM to 10, 26, and 129 μM, respectively (data not shown). Most of this effect can be explained by an increase in the apparent *K_m* for cytochrome *c* from 2.6 to 16 μM, which would require a *K_i* of 1.7 μM for the

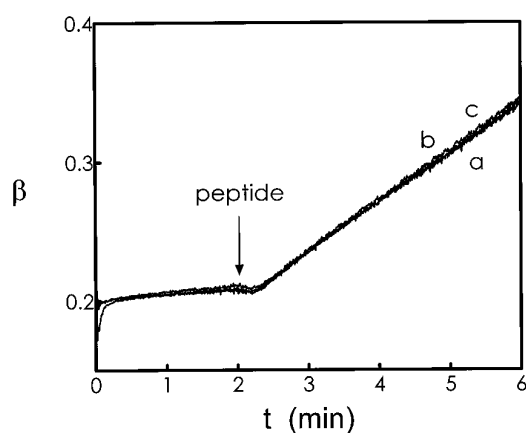


FIG. 8. Effect of cyclosporin on permeability induced by pCOX IV-(1-22). Light scattering traces are shown of mitochondria (0.1 mg/ml) respiring on succinate suspended in sucrose assay medium. pCOX IV-(1-22) (10 μM) was added at 2 min. Trace a, control; trace b, plus pCOX IV-(1-22) (10 μM) added at t = 0; trace c, plus cyclosporin (1.6 nmol/mg) added at t = 0. The composition of the assay medium and other experimental details are described under "Experimental Procedures."

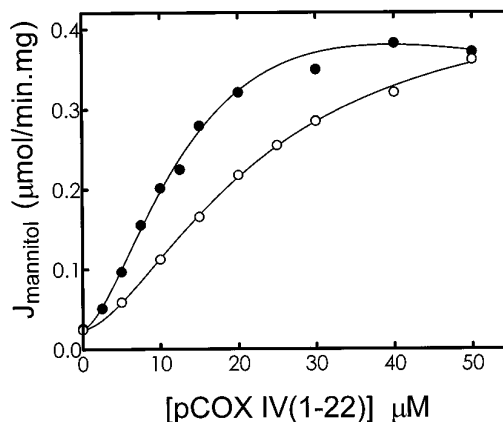


FIG. 9. Peptide dose-response curves differ using succinate *versus* ascorbate/TMPD as substrates. Rates of mannitol influx are plotted *versus* the dose of pCOX IV-(1-22) using ascorbate/TMPD (●) and succinate (○) as substrates. The rates were determined from light scattering kinetics similar to those shown in Fig. 1, but using mannitol assay medium. The composition of the assay medium and other experimental details are described under "Experimental Procedures."

peptide. From these results, it can be predicted that, in intact mitochondria, in which the concentration of cytochrome *c* between the membranes must be very high, the activity of cytochrome *c* oxidase should be relatively insensitive to inhibition by pCOX IV-(1-22). This proved to be the case. Addition of 50 μM pCOX IV-(1-22), which inhibited cytochrome *c* oxidase by 65% in the presence of 10 μM cytochrome *c*, inhibited respiration by only 20% in intact mitochondria oxidizing ascorbate/TMPD (data not shown).

DISCUSSION

In this paper, we have examined the effect of the leader sequence of yeast cytochrome oxidase subunit IV on the permeability and respiratory chain of rat liver mitochondria. This leader sequence is responsible for directing the import of cytochrome oxidase subunit IV into the mitochondrial matrix (1), but can also direct the import of totally unrelated proteins (2, 21, 24). Several groups have begun investigations of the effects of synthetic peptides based on mitochondrial leader sequences on membranes and isolated channels. These peptides, which form amphiphilic helices (12), interact with lipid membranes (13, 25-27) and have been reported to uncouple mitochondria

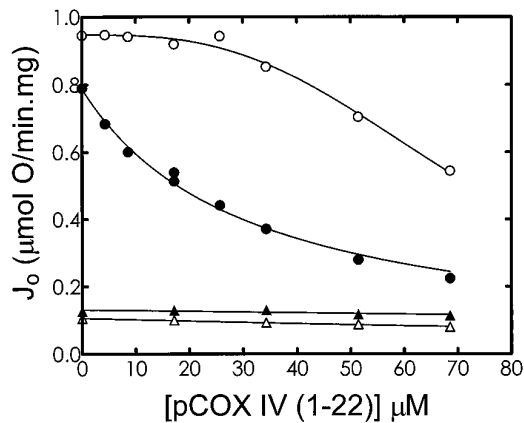


FIG. 10. Inhibition of cytochrome oxidase by pCOX IV-(1-22). The rate of respiration of mitoplasts (0.43 mg/ml) is plotted versus the dose of pCOX IV-(1-22) using ascorbate/TMPD (●, ○) and succinate (▲, △) as substrates in sucrose medium (●, ▲) and KCl medium (○, △). The composition of the assay media and other experimental details are described under "Experimental Procedures."

(3, 13-16). Singer *et al.* (28) have suggested that this latter effect may result from the peptides themselves forming pores. Others have shown that these synthetic peptides competitively inhibit protein import (3, 15, 23) and can be transported (29). Thieffry and co-workers (4-6, 9) have identified a K^+ channel, believed to reside in the outer mitochondrial membrane, that can be blocked by pCOX IV-(1-12)Y.

In view of the reported properties of leader peptides, we were surprised when we first found that pCOX IV-(1-22) is able to stimulate the uptake of K^+ in respiring mitochondria. Moreover, since this uptake is prevented by uncouplers, nigericin, and omission of respiratory substrates, it is clearly not a consequence of membrane lysis. The EC_{50} for this process, which lies between 10 and 15 μM , is in the range reported for inhibition of protein import (3, 15, 23). Unlike the K^+ uptake pathway previously characterized (11), the peptide-induced pathway is not blocked by ATP; therefore, we tentatively conclude that the pathways are not related.

One important question regarding the action of pCOX IV-(1-22) is, what structural features are necessary to induce the effect? Although it is difficult to answer this question without synthesizing a large number of peptides, we have demonstrated the following. Both pCOX IV-(1-22) and pCOX IV-(1-12)Y, which have five and three net positive charges, respectively, stimulate transport, while other positively charged molecules such as spermine, which has four positive charges and a 13-residue peptide (Q13) that contains the sequence Lys-Lys-Lys-Arg-Lys, did not. More interesting is the finding that pAT III, which has many properties similar to pCOX IV-(1-22), is much less potent than pCOX IV-(1-22). For example, if it is assumed that the J_{max} values for the two peptides are equal, the EC_{50} for pAT III is 20-fold higher than for pCOX IV-(1-22). Thus, it is quite possible that the structural requirements for activating protein import and the K^+ channel are the same.

The selectivity of the peptide-activated pathway appears to be fairly broad. It not only carries K^+ and other cations including tetraethylammonium and lysine, but also non-electrolytes such as sucrose and mannitol. In the absence of salts that can be accumulated, the increased permeability does not appear to be associated with an increase in the rate of respiration. Thus, although the peptide-activated pathway is able to conduct molecules as large as sucrose, it seems to be relatively impermeable to protons. Contrary to this conclusion, many others have reported that leader peptides uncouple intact mitochondria (3,

13-16). Thus, in an attempt to reconcile these disparate conclusions, we investigated the effect of pCOX IV-(1-22) on the respiration rate and volume of mitochondria in a medium with the same salt composition as that used by Nicolay *et al.* (16). We confirmed their finding that the peptide stimulates respiration, but we disagree with their conclusion. Our data clearly demonstrate that, in this medium, the immediate effect of the peptide is the activation of a K^+ influx pathway and that it is the respiration-dependent uptake of K^+ phosphate and succinate that is responsible for the stimulation of respiration. In view of the rapid swelling associated with the influx of the K^+ salts, the outer membrane will be broken and intermembrane enzymes lost. Eventually, the inner membrane may itself become damaged; and volume homeostatic mechanisms involving the K^+/H^+ antiporter (31) and the inner membrane anion channel (32) will become active, and oxidative phosphorylation will be uncoupled. Thus, we believe that the conclusions that these peptides solubilize the outer membrane at low doses and make the inner membrane leaky at high doses should be reassessed. It is most likely that these effects are secondary to mitochondrial swelling. Since we have shown that pCOX IV-(1-22) also increases the permeability to sucrose and mannitol, which are frequently used as the major osmolytes in studies with isolated mitochondria, it is likely that, even in the absence of permeant acids, swelling due to influx of these non-electrolytes will also cause breakage of the outer membrane and eventual loss of respiratory control.

In the course of these studies, the question arose as to whether pCOX IV-(1-22) was activating a pathway specific to the mitochondrial membrane or whether, as suggested by Singer *et al.* (28), it itself was forming channels. In view of the five positive charges carried by the peptide, it is hard to imagine that it would form a cation-conducting channel; however, we investigated whether we could induce the formation of channels in a planar lipid bilayer by adding 100 μM peptide to the chamber. No channels, however, were observed (data not shown). This finding is consistent with the report of Henry *et al.* (5), who found that pCOX IV-(1-12)Y does not form channels in lipid bilayers, and the report of Simon and Blobel (33), who found that the LamB signal peptide, which opens protein-conducting channels in *Escherichia coli*, does not change the conductance of planar lipid bilayers in the absence of proteins.

Another property of pCOX IV-(1-22) is its ability to inhibit respiration at the level of cytochrome *c* oxidase. Our data suggest that the peptide competes with cytochrome *c* for binding to the oxidase since its effectiveness is decreased by raising the concentration of cytochrome *c* and is negligible in intact mitochondria, which maintain a high concentration of cytochrome *c* between their membranes. An ionic interaction is suggested by the fact that, in the 0-30 μM range, inhibition is only significant at low ionic strength. Although this effect can complicate the interpretation of data obtained utilizing these peptides in intact mitochondria, it probably has no physiological significance because cytochrome *c* oxidase does not play a significant limiting role when succinate or NAD-linked substrates are being oxidized.

It is tempting to speculate that the peptide-activated transport pathway is in fact part of the protein import machinery. It makes sense that the leader peptide should open the channel through which the protein has to pass since, in the absence of a substrate, one would expect the channel to be closed. Although it has been reported that channels isolated from mitochondria can be blocked by pCOX IV-(1-22) (4-6, 9, 10), it has also been reported that leader peptides open channels responsible for the secretion of proteins in bacteria (33). The increase in K^+ conductance induced by the peptide may be apparent

only in the absence of a protein attached to the leader sequence. On the other hand, it is possible that the increase in K^+ conductance is not a coincidence, but is important for the growth of mitochondria. When mitochondria divide, it is not only necessary for new proteins to be synthesized, but they must also increase their K^+ content. Another possibility is that the cation conductance may somehow be necessary for the conduction of negatively charged residues in translocated peptides. One may also question whether the concentration of peptide necessary to open the pore is not too high. It is possible, however, that the binding affinity may be much higher when it is attached to a protein. A decrease in affinity after cleavage, e.g. due to exposure of a C-terminal carboxyl group, could then prevent the peptide from keeping the channel open after protein translocation is complete. One could also argue that a relatively low affinity is necessary to enable the leader peptide to pass through the channel. If it were to bind too tightly, it may actually block transport of the protein. Although these considerations are very interesting, it remains to be proved whether or not the effects of these peptides on mitochondrial permeability are related to their interaction with the protein import machinery or are an unrelated phenomenon.

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