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Critical segment of apocytochrome c for its insertion into membrane

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Abstract

Apocytochrome c has a potent ability to insert spontaneously into membrane. To identify which sequences were critical for this insertion activity, a series of peptides N19, C8, C15 and C21, corresponding to sequences 1–19, 81–88, 74–88 and 68–88 of apocytochrome c, respectively, were synthesized and purified. Insertion ability into phospholipid monolayer, intrinsic fluorescence emission spectra, and the accessibility of peptide C21 to fluorescence quenchers: KI, acrylamide and HB showed that only segment 68–88 could insert into membrane, while other segments did not. CD spectra demonstrated that its interaction with liposomes containing negatively charged phospholipid could induce a partial α-helical conformation in peptide C21. It is interesting to note that a cooperation exists between segment 68–88 and 1–19 in the insertion of apocytochrome c and consequently translocation across membrane. (Mol Cell Biochem **262**: 61–69, 2004)

Key words: apocytochrome c, critical segment, membrane insertion, conformational changes, binding to mitochondria

Introduction

Apocytochrome c is the heme-free precursor of cytochrome c. It is synthesized in the cytosol and post-translationally imported into mitochondria by following a quite unique pathway compared to other mitochondrial precursor proteins [1]. It does not possess a cleavable N-terminal presequence [2], and neither a membrane potential nor ATP is required for its import [3, 4]. Further, in the outer mitochondrial membrane no proteinaceous component responsible for its import has ever been identified [5, 6]. In the intermembrane space the imported apocytochrome c is converted into holocytochrome c by cytochrome c heme lyase (CCHL).

Model membrane studies have demonstrated that apocytochrome c binds with high affinity, preferentially to the negatively charged lipid component, followed by penetration and translocation into liposomes [7]. It was proposed by Neupert *et al*. that instead of utilizing receptor system apocytochrome c might directly insert into the mitochondrial membrane system by its interaction with phospholipid [8]. Thus, it is interesting to identify which sequences are crucial for its penetration and translocation across membrane.

Although much effort has been made to answer this question, a conclusive mechanism remains ambiguous. For example, Margoliash *et al*. found that only carboxyl-terminal segment 66–104 was able to compete with apocytochrome c import into mitochondria [9]. However, de Kruijff *et al*. found that only amino-terminal fragment 1–38 but not carboxyl-terminal fragment 66–104 was able to translocate across lipid bilayer [10].

The purpose of our research is to figure out which sequences are crucial for apocytochrome c insertion into membrane and consequently translocation across membrane. By comparing the effects of hydrophobic segments deletion on the interaction of apocytochrome c with lipids, we have found that C-terminal segment 72–86 plays an important role [11]. Further, a series of peptides, N19, C8, C15 and C21, corresponding to the sequences 1–19, 81–88, 74–88 and 68–88 (Fig. 1), respectively, were synthesized by solid-phase method and purified by reverse phase HPLC. It was

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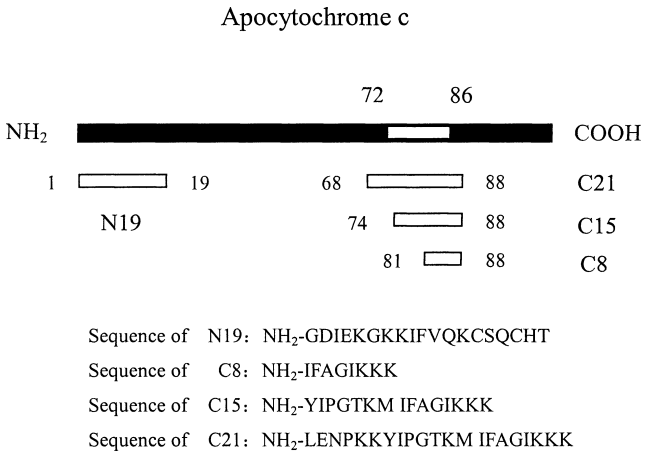

Fig. 1. Structure and abbreviation of synthesized peptides. The blank denotes the deleted region. Peptides N19, C8, C15 and C21 correspond to the sequences 1–19, 81–88, 74–88 and 68–88 of apocytochrome c, respectively.

demonstrated preliminarily that segment 68–88 plays a critical role in the binding of apocytochrome c to lipids [12]. In the present paper a detailed study on this aspect will be reported.

Materials and methods

Materials

Soybean phospholipid, trypsin and PIPES (piperazine-N, N-bis(2-ethanesulfonic acid)) were obtained from Sigma. L-α-phosphatidylcholine (PC) and L-α-phosphatidylethanolamine (PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Phosphatidylserine (PS) and phosphatidylglycerol (PG) were obtained from Molecular Probes, Inc. Hypocrellin B (HB) was the generous gift of Dr. J. C. Yue, Institute of Biophysics, Chinese Academy of Sciences. Three-to-four-week old chickens were purchased from Beijing Merial Viral Laboratory Animal Technology Co., Ltd. TNT^{TM} coupled reticulocyte lysate systems were obtained from Promega. ^{35}S-cysteine was obtained from Dupont NEN Inc. Plasmid pSTCN containing the entire coding sequence of chicken apocytochrome c has been constructed by our laboratory. Other chemical reagents were of the best quality commercially available. PIPES buffer consisted of 10 mmol/L PIPES, pH 7.0, 50 mmol/L NaCl, 0.2 mmol/L EDTA. HMSE buffer used in binding measurements consisted of 5 mmol/L Hepes-KOH, pH 7.4, 220 mmol/L mannitol, 70 mmol/L sucrose, 2 mmol/L EGTA. All buffers were prepared from fresh double distilled water.

Synthesis and purification of peptides

A series of peptides N19, C8, C15, C21 and C21w were synthesized by solid-phase method and purified by reverse-phase HPLC as described previously [12]. As shown in Fig. 1, N19, C8, C15 and C21 corresponded to the sequences 1–19, 81–88, 74–88 and 68–88 of apocytochrome c, respectively. Peptide C21w has an additional tryptophan residue at N-terminal of C21.

Preparation of vesicles

The purified soybean phospholipid (in chloroform/methanol (3:1)) was taken to dryness under a stream of nitrogen, and lyophilized overnight. Soybean phospholipid SUVs (small unilamellar vesicles) used in fluorescence experiments were prepared by sonication in PIPES buffer using an MSE sonicator at 0°C under nitrogen gas. After sonication, the solution was centrifuged at 100,000 g for 30 min in a Beckman TL-100 ultracentrifuge to eliminate the multilayer liposomes and titanium residue. The phospholipid concentration was determined by perchloric acid digestion [13].

Insertion ability into phospholipid monolayer

Insertion of peptides into monolayer composed of soybean phospholipid was studied on a homemade film balance, HAN-2000. The experiments were performed as described previously [11]. An increase of the surface pressure $\Delta\pi$ is measured as a function of the initial surface pressure π. A plot of $\Delta\pi$ versus π yields a straight line with negative slope that intersects the abscissa at the value named as the limiting surface pressure (π_c). All measurements were performed at room temperature at peptide concentrations of 2.5 μg/ml.

Tryptophan fluorescence spectra

Fluorescence spectra of the single tryptophan residue contained within peptide C21w were measured before and after addition of soybean phospholipid SUVs. All fluorescence measurements were performed at 30 $\pm$ 0.1°C with a Hitachi F-4010 spectrofluorometer equipped with a thermostatically controlled cuvette holder. The peptide concentration was 2 μmol/L, and that of SUVs was 200 μmol Pi/L. Excitation wavelength was set at 295 nm, and emission from 310 nm to 400 nm was recorded with the bandwidths of 5 nm for both excitation and emission. Each measurement was corrected for the light scattering contribution of vesicles.

Fluorescence quenching

After incubation of peptide C21w with liposomes for 30 min at 30°C, KI, acrylamide or HB quenching experiments were carried out at an excitation wavelength of 295 nm. Fluorescence was monitored at 340 nm. The peptide concentration was 2 μmol/L, and that of SUVs was 200 μmol Pi/L. The

values obtained were corrected for dilution, the scattering contribution and the absorptive screening by quenchers. Data were analyzed according to the Stern-Volmer equation: $F_0/F = 1 + Ksv\,[Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of quencher. [Q] is the molar concentration of quencher and Ksv is the apparent Stern-Volmer quenching constant.

Circular dichroism measurements

CD spectra were measured on a Jasco J-720 spectropolarimeter at room temperature with path length 0.1 mm. The concentrations of apocytochrome c and peptides were 100 μmol/L, and that of SDS was 0.5% (w/v). The final spectra were the average of eight scans with time constant 1 sec and scan speed 50 nm/min and corrected for buffer background.

Competitive inhibition

Chicken apocytochrome c was transcribed and translated *in vitro* with a nuclease-treated rabbit reticulocyte lysate, and radiolabeled by ^{35}S-cysteine. The free ^{35}S-cysteine was removed by dialysis. The translation product was identified by SDS-PAGE and fluorography. Chicken heart mitochondria were isolated according to the method established by our laboratory [14]. Activities of monoamine oxidase, cytochrome c oxidase, adenylate kinase, acid phosphatase and glucose-6-phosphatase were assayed to check the purity of mitochondria and the intactness of mitochondrial outer membrane [14]. To detect the effect of peptides on the binding of apocytochrome c to mitochondria, unlabeled synthesized peptides were preincubated with mitochondria at 25°C for 15 min. Then the binding reaction was started by the addition of ^{35}S-apocytochrome c. These mixtures were incubated for 15 min at 25°C, followed by centrifugation at 10,000 $\times$ g for 4 min to separate bound and unbound protein fractions. The pellet was resuspended in HMSE buffer, washed twice, lysed with 10 μL 10% SDS and filtered through a cellulose acetate membrane. The dried filter membrane was put into 5 mL dimethylbenzene solution. The amount of ^{35}S-apocytochrome c binding to mitochondria was determined using a scintillation counter.

Results

Insertion of different synthesized peptides into phospholipid monolayer

The insertion abilities of synthesized peptides were studied by comparison of their penetration into soybean phospholipid monolayer. The important parameter in a monolayer experiment is the limiting pressure (π_c), which is defined as the pressure at which a protein can no longer penetrate. In other words this is the point at which the change in surface pressure is zero. In monolayer the 'equivalence pressure' of bilayer may be defined as that pressure at which the lipid density in the monolayer is identical with that in the bilayer. The bilayer equivalence pressure of a phospholipid monolayer is thought to be 32–35 mN/m [15], hence the π_c is a more influential factor in predicting the insertion ability of a protein into membrane.

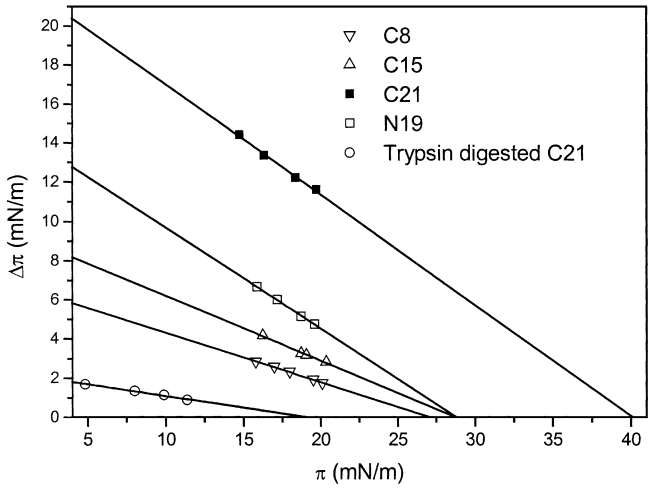

Fig. 2. Interaction of synthesized peptides with soybean phospholipid monolayer. Displayed is the $\Delta\pi$–π plot of surface pressure changes after injection of synthesized peptides (2.5 μg/ml) underneath soybean phospholipid monolayer at different initial surface pressures. The limiting surface pressures of peptides N19, C8, C15 and C21 were 28.78, 27.09, 28.74 and 40.13 mN/m, respectively, and that of trypsin-digested peptide C21 was 19.13 mN/m.

Figure 2 shows the numerical value of π_c for peptide C21 was 40.13 mN/m, and those for C8, C15 and N19 were 27.09, 28.74 and 28.78 mN/m, respectively. It can be seen the π_c of peptide C21 was obviously higher than the 'equivalence pressure' of bilayer (32–35 mN/m), while values for peptides C8, C15 and N19 were no higher than that. These results indicated that peptide C21, corresponding to sequence 68–88 of apocytochrome c, may insert into the membrane.

Treatment of peptide C21 with trypsin at a ratio of 40:1 (weight:weight) resulted in complete digestion of peptide after incubation at room temperature for 40 min as shown by SDS-PAGE. As a result, the π_c of digested peptide C21 was decreased to 19.13 mN/m. This indicated that the insertion ability of peptide C21 depended on the intact sequence from 68 to 88.

Additionally, the penetration of full-length apocytochrome c or peptide C21 into monolayer composed of different phospholipid was compared. As shown in Fig. 3A, the numerical values of π_c for apocytochrome c on PG, PS, PE and PC

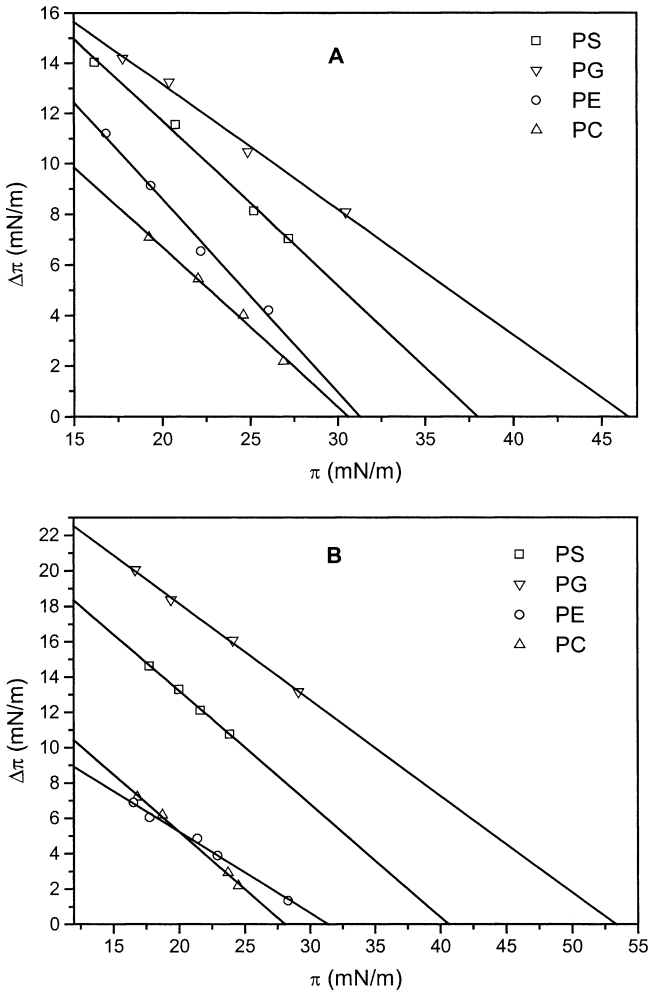

Fig. 3. Interaction of apocytochrome c or synthesized peptide C21 with different phospholipid monolayer. Displayed is the $\Delta\pi$–π plot of surface pressure changes after injection of apocytochrome c (A) or peptide C21 (B) (2.5 μg/ml) underneath different phospholipid monolayer at different initial surface pressures. The limiting surface pressures of apocytochrome c on PG, PS, PE and PC monolayer are 46.51, 37.96, 31.26 and 30.60 mN/m, respectively. π_c of peptide C21 on PG, PS, PE and PC monolayer are 53.32, 40.63, 31.40 and 28.06 mN/m, respectively.

monolayer were 46.51, 37.96, 31.26 and 30.60 mN/m, respectively. In the case of peptide C21 (Fig. 3B), the order of π_c on PG, PS, PE and PC is PG > PS > PE $\approx$ PC. Hence, the insertion ability of peptide C21 depends on negatively charged phospholipid (PG, PS) as that of full-length apocytochrome c.

Intrinsic fluorescence measurement of synthesized peptide C21w following its interaction with liposomes

The insertion ability of peptide C21 into membrane was further studied by monitoring the intrinsic fluorescence spectra of peptide C21w, which has an additional tryptophan residue

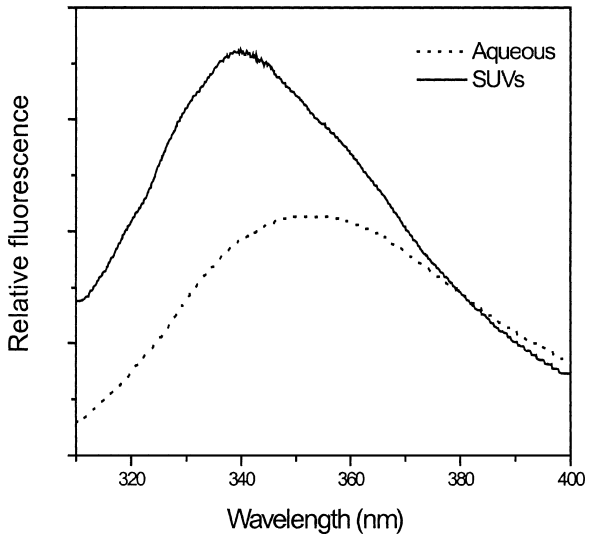

Fig. 4. Fluorescence emission spectra of peptide C21w in aqueous solution (dotted line) and following its interaction with soybean phospholipid SUVs (solid line). The peptide concentration was 2 μmol/L and that of SUVs was 200 μmol Pi/L. Excitation wavelength was set at 295 nm, and the emission spectra were recorded from 310 nm to 400 nm with bandwidths of 5 nm for both excitation and emission.

at N-terminal of peptide C21. The tryptophan residue has no effect on the insertion of peptide C21 into phospholipid monolayer (data not shown).

The fluorescence emission spectra of peptide C21w following its interaction with soybean phospholipid SUVs are shown in Fig. 4. In aqueous solution, the highest peak of tryptophan fluorescence emission spectrum was observed at 349 nm. However, upon interaction with liposomes, the fluorescence maximum was blue shifted to 338 nm. It has been shown that association of apocytochrome c with membrane results in a blue shift of peak (λ_{max}) of fluorescence intensity [11], indicating that tryptophan residue is shifted to a more hydrophobic environment [16]. Hence, from the tryptophan fluorescence emission spectra it could be deduced that peptide C21 inserts into a more hydrophobic environment following its interaction with membrane in a similar way as full-length apocytochrome c.

Quenching of the intrinsic fluorescence by KI, acrylamide or HB

To further confirm the insertion ability of peptide C21 into membrane, the change in quenching of the intrinsic fluorescence by KI, acrylamide or HB following the interaction of peptide C21w with soybean phospholipid SUVs was compared. KI and acrylamide were used as aqueous quenchers. Hydrophobic quenching was accomplished by HB [17].

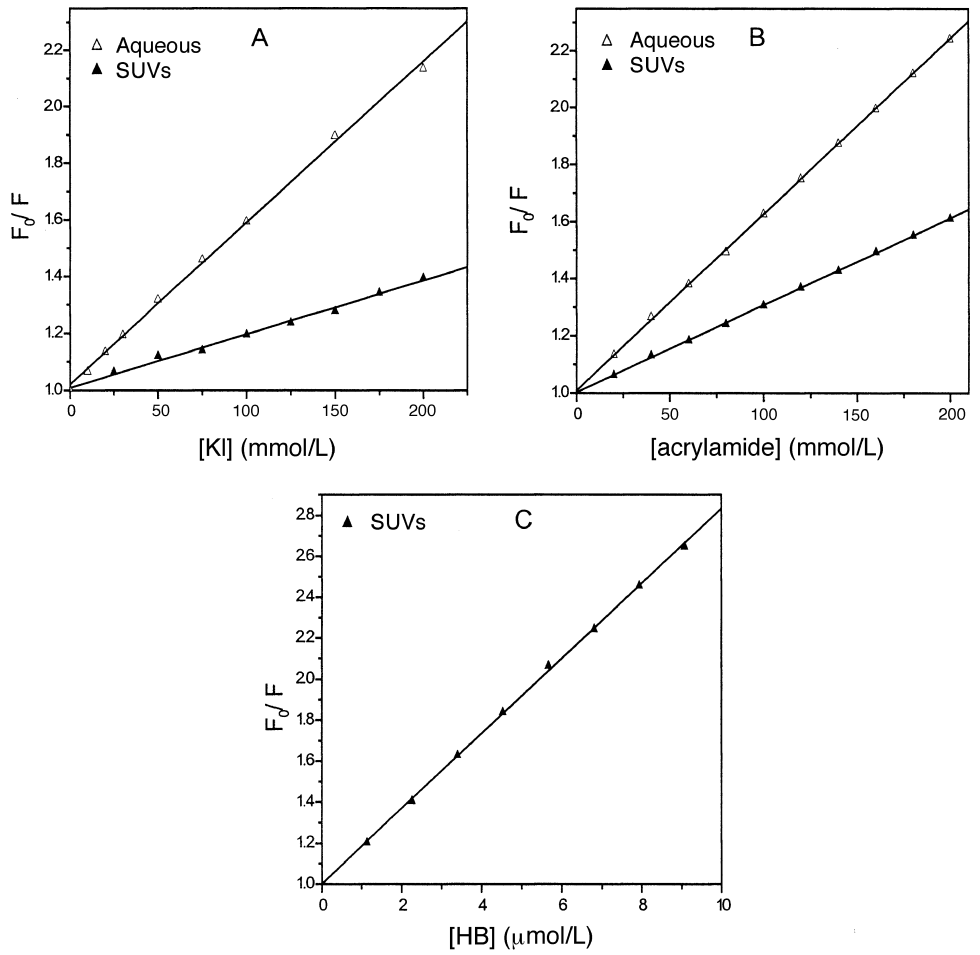

Fig. 5. Stern-Volmer plots of quenching of intrinsic fluorescence of peptide C21w by I^- (A), acrylamide (B) and HB (C) in the presence of soybean phospholipid SUVs. Peptide (2 μmol/L) was incubated with soybean phospholipid SUVs (200 μmol Pi/L) in PIPES buffer at 30°C. Spectra were recorded at 30°C. Spectra bandwidths were 5 nm for both excitation and emission.

Figure 5 shows the tryptophan fluorescence quenching of peptide C21w by KI (Fig. 5A), acrylamide (Fig. 5B) or HB (Fig. 5C). The efficiency of quenching calculated according to the Stern-Volmer equation was shown in Table 1. These results indicated clearly that, following the interaction with soybean phospholipid SUVs, the intrinsic fluorescence of peptide C21w can be quenched by hydrophobic quencher HB. However, the quenching efficiency by aqueous quencher KI or acrylamide was decreased. This may further indicate that peptide C21 has ability to penetrate into the hydrophobic core of the lipid bilayer following its interaction with phospholipid.

Conformational change following the interaction of peptide C21 with phospholipid

It has been generally suggested that the conformation of apocytochrome c has been changed remarkably as a consequence of its interaction with the phospholipid in model membranes. Such changes are thought to be the driving force for the transfer of apocytochrome c from an aqueous compartment into membranes [18]. Thus it can be questioned whether there is similar conformational change following the interaction of peptide C21 with lipids. Circular dichroism (CD) spectra were applied to such study.

Considering a quantitative interpretation of the CD spectra was difficult due to vesicle aggregation induced by

Table 1. Effective Stern-Volmer quenching constants (Ksv) for tryptophan fluorescence of peptide C21w by I^- and acrylamide in aqueous medium or in the presence of SUVs

	KI (M^{-1})	Acrylamide (M^{-1})
Aqueous medium	5.71	6.18
With SUVs	1.89	3.05

The Stern-Volmer quenching constants (Ksv) of three experiments were calculated as described in 'Materials and methods' section.

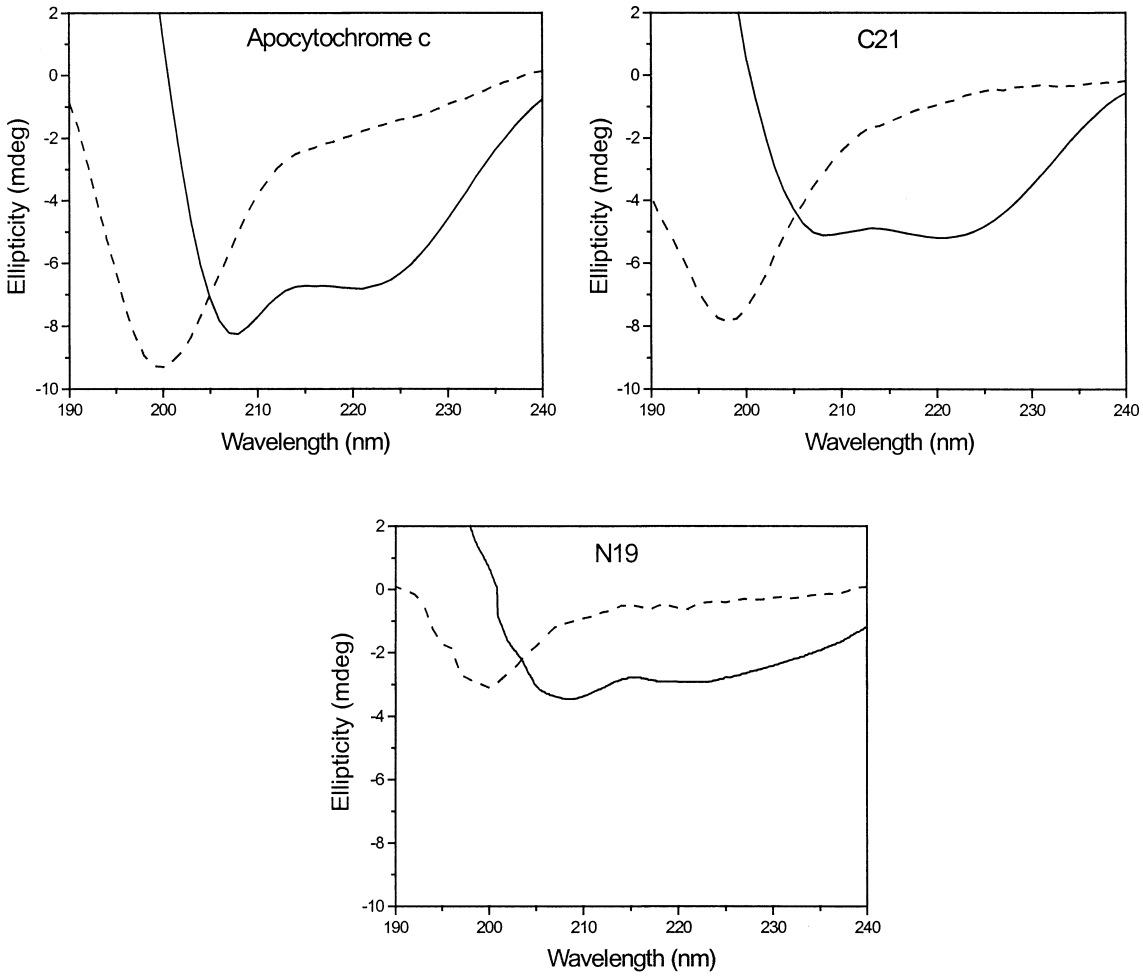

Fig. 6. Circular dichroism spectra of apocytochrome c, peptide C21 and N19 in aqueous solution (dashed line) and in the presence of 0.5% SDS (solid line). The concentration of protein or peptide was 100 μmol/L. The spectra were scanned from 250 to 190 nm and eight scans were averaged. Measurements were carried out at room temperature using cells with pathlength of 0.1 mm. All spectra were acquired with a resolution of 0.5 nm.

apoprotein, so the detergent SDS was used instead of negatively charged phospholipid [19, 20]. From Fig. 6 it can be seen a partial α-helical conformation induced in peptide C21 following its interaction with SDS, which showed characteristics quite similar to that of apocytochrome c.

Interaction between peptide C21 and N19

Results obtained from insertion into phospholipid monolayer, intrinsic fluorescence emission spectra and fluorescence quenching all indicated that peptide C21 could insert into membrane. However, by comparing its π_c (40.13 mM/m) with that of apocytochrome c, which is 51.50 mN/m on soybean phospholipid monolayer [11], we found that the insertion ability of peptide C21, corresponding to the sequence 68–88 of apocytochrome c, into membrane is weaker than that of full-length apocytochrome c. So the next question to be answered was: In addition to sequence 68–88 were there any other segments also required for the insertion of apocytochrome c into membranes and finally translocation across membranes?

To investigate this possibility, deletion mutant Δ72–88 and peptide C21 were mixed at a 1:1 molar ratio. The limiting surface pressure (π_c) of this mixture on soybean phospholipid monolayer was measured as shown in Fig. 7. The numerical value of π_c is 50.74 mN/m, which is not only higher than that of peptide C21, but also is as high as that of apocytochrome c. Since it has been shown that deletion mutant Δ72–88 has no insertion ability into membrane [11], these data suggest that there is cooperation between segment 68–88 and other regions of apocytochrome c during the insertion into membrane.

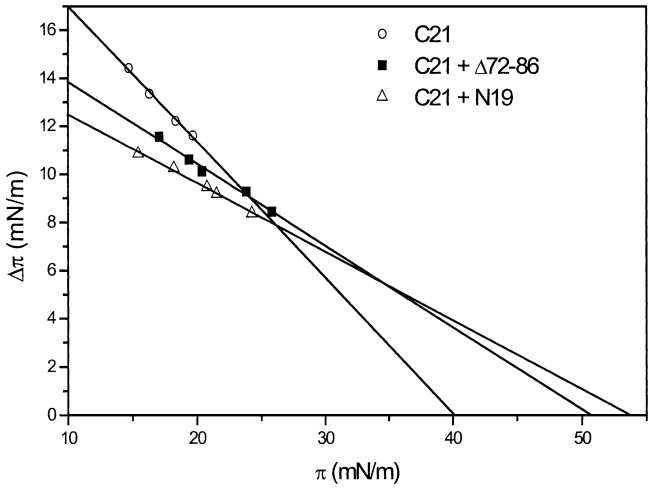

Fig. 7. Cooperative effect of peptide C21 and N19 on its insertion into phospholipid monolayer. Displayed is the $\Delta\pi$–π plot of surface pressure changes after injection of peptide C21 (○), C21 + deletion mutant Δ72–86 (■) or mixture composed of peptide C21 and N19 (△) at a 1:1 molar ratio underneath soybean phospholipid monolayer at different initial surface pressures. The limiting surface pressures were 40.13, 50.74 and 53.78 mN/m, respectively.

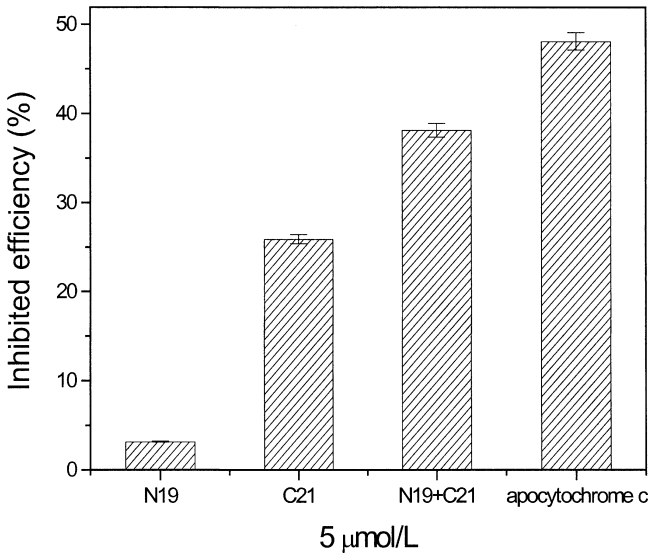

Fig. 8. Competitive inhibition of ^{35}S-apocytochrome c binding to mitochondria by peptide C21, N19 or their mixture at a 1:1 molar ratio. Unlabelled peptides (5 μmol/L) or apocytochrome c was preincubated with mitochondria at 25°C for 15 min. The binding reaction was started by the addition of ^{35}S-apocytochrome c. These mixtures were incubated for 15 min at 25°C, followed by centrifugation (10,000 × *g*, 4 min, 4°C) to separate bound and unbound protein. The amount of ^{35}S-apocytochrome c binding to mitochondria was determined using a scintillation counter.

Then, peptide N19 was tentatively selected and mixed with peptide C21 in a ratio of 1:1 (molar:molar). Obtained results showed that the π_c of this mixture on soybean phospholipid monolayer is 53.78 mN/m (Fig. 7), which is higher than that of peptide C21 alone and as high as that of whole apocytochrome c. This may indicate that the insertion of segment 68–88 into the membrane can be promoted by segment 1–19, even though the latter can't insert into membrane by itself.

Competitive inhibition of ^{35}S-apocytochrome c binding to mitochondria by peptide C21 and N19

As mentioned above, there is a cooperative effect between segments 68–88 and 1–19 on the insertion of apocytochrome c into membrane. We have therefore sought to study whether a similar phenomenon also existed in the translocation of apocytochrome c across mitochondria. The effect of peptides C21, N19 or their mixture on the binding of apocytochrome c to mitochondria was thus studied. It can be seen from Fig. 8 that the binding of ^{35}S-apocytochrome c decreased 25.88% by peptide C21 at 5 μmol/L. Under the same conditions, the binding of ^{35}S-apocytochrome c was very weakly inhibited by peptide N19. However, the inhibition of ^{35}S-apocytochrome c binding to mitochondria by peptide C21 was enhanced by addition of peptide N19. So we may deduce that the interaction between segment 68–88 and 1–19 also exists in the binding of apocytochrome c to mitochondria.

Discussion

Until now, the molecular mechanism of apocytochrome c translocation remains elusive. Although it was proposed by Neupert *et al.* that instead of a receptor system apocytochrome c might directly insert into the mitochondrial membranes by its interaction with phospholipid [8], which sequence of apocytochrome c responsible for this process is still unknown.

The key result of this study is the finding that segment 68–88 of apocytochrome c plays a crucial role in its insertion into membrane. This conclusion has been drawn based on the following results: (i) The limiting surface pressure of peptide C21, which corresponds to sequence 68–88 of apocytochrome c on soybean phospholipid monolayer is higher than the 'equivalence pressure' of bilayer. (ii) Following the interaction of peptide C21 with liposomes, its fluorescence can be quenched by the hydrophobic quencher HB, and the quenching efficiency by aqueous quencher KI or acrylamide was decreased. (iii) Intrinsic fluorescence emission spectra showed that peptide C21 inserts into the hydrophobic core of the lipid bilayer following its interaction with membrane. Furthermore, similar to that of apocytochrome c, the insertion ability of peptide C21 dependents on negatively charged phospholipid.

Model membrane studies have demonstrated that translocation of apocytochrome c across mitochondrial membrane is a multiple steps process. Firstly, it binds with high affinity preferentially to the negatively charged lipid components of the outer mitochondrial membrane. Then in consequence of the hydrophobic interaction between the hydrophobic amino acids of apocytochrome c with the hydrophobic core of the bilayer, the apoprotein inserts into the lipid bilayer, followed by perturbation the acyl chain packing [21, 22], with its concomitant conformational changes, which are thought to be the driving force for apoprotein transfer from an aqueous compartment into membrane [18], and leads to translocation across membrane. By comparison the effects of synthesized peptides association on the barrier properties of vesicles, we have found that peptide C21 has ability to perturb the acyl chain packing of lipid bilayer [12] and in the present paper, the conformational change of peptide C21 following its interaction with negatively charged phospholipid was also proved by CD spectra. Hence, we may conclude that segment 68–88 plays an important role in different steps of translocation: insertion into monolayer, destabilization of lipid bilayer structure and inducing conformational changes of preprotein.

We have noticed that the insertion ability of segment 68–88 into membrane was weaker than that of apocytochrome c, so it was interesting to see if other regions in addition to segment 68–88 also played some role in the insertion of apocytochrome c. Obtained results showed that the insertion of peptide C21 into membrane was enhanced in the presence of peptide N19, which corresponds to sequence 1–19 of apocytochrome c. Similar results were obtained in the binding of apocytochrome c to mitochondria. Here, stronger competitive inhibition could be observed if N-terminal segment 1–19 was presented in addition to segment 68–88.

Then, what is the mechanism of cooperative effect of segment 68–88 and 1–19 on the translocation of apocytochrome c across membrane? Although peptide N19 could not insert into membrane, a little amount α-helix was induced following its interaction with negatively charged phospholipid as shown in Fig. 6. It is tentatively suggested that helix–helix interaction between structure caused by segments 68–88 and 1–19 occurred during translocation of apocytochrome c across the membrane. Such interaction may be essential for the further insertion of segment 68–88 and hence the translocation of whole molecule of apocytochrome c. The detailed molecular mechanism underlying the cooperative effect of segments 68–88 and 1–19 on apocytochrome c translocation across membrane deserves further investigation.

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