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Change of apocytochrome c translocation across membrane in consequence of hydrophobic segment deletion

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Abstract

Wild-type apocytochrome c and its hydrophobic segment deleted mutants, named Δ28–39, Δ72–86 and Δ28–29/72–86 were constructed, expressed and highly purified respectively. Insertion ability into phospholipid monolayer, inducing leakage of entrapped fluorescent dye fluorescein sulfonate (FS) from liposomes, and translocation across model membrane system showed that the wild-type apoprotein and Δ28–39 almost exhibited the same characteristics, while mutants with segment 72–86 deletion did not. Furthermore, CD spectra, intrinsic fluorescence emission spectra, and the accessibility of the protein to the fluorescence quenchers: KI, acrylamide and HB demonstrated that the segment 72–86 deletion has a significant effect on the conformational changes of apocytochrome c following its interaction with phospholipid. On the basis of these results it is postulated that the C-terminal hydrophobic segment 72–86 plays an important role in the translocation of apocytochrome c across membrane. (Mol Cell Biochem **233**: 39–47, 2002)

Key words: apocytochrome c, deleted mutation, membrane insertion, membrane- leakage, conformational changes, translocation across membrane

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, no proteinaceous component responsible for its import has ever been identified [5, 6]. Although much effort has been made to uncover the mystery of its import [7–10], a conclusive mechanism remains ambiguous.

It has been proposed that the direct interaction between apocytochrome c and phospholipid, especially the hydrophobic interaction should play an important role at least in some steps of the translocation process. Apocytochrome c has the strong ability to insert spontaneously into membrane [11, 12]. 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 preprotein inserts into the lipid bilayer, followed by perturbation the acyl chain packing [13, 14], and leads to translocation across membrane. We have therefore sought to study the role of hydrophobic segment of apocytochrome c in its insertion into phospholipid monolayer and translocation across membrane.

In the present work, deleted mutants of apocytochrome c, namely, Δ28–39, Δ72–86 and Δ28–29/72–86 were obtained by site-directed mutagenesis, and the effects of deletions on the interaction of apoprotein with phospholipid and hence its translocation across membrane were determined and compared.

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Materials and methods

Materials

Restriction endonucleases including BamHI, EcoRI and NcoI were purchased from Promega. T_4 DNA ligase, T_4 DNA polymerase and CM-Sepharose 6B were purchased from Pharmacia. α-^{35}S-dATP was from Dupont NEN Inc. Phagemid pALTER-1 and helper phage M13KO7 were obtained from Promega. The other plasmids and strains used in this paper were conserved by our laboratory. Soybean phospholipid, trypsin, soybean trypsin inhibitor and PIPES (piperazine-N,N-bis(2-ethanesulfonic acid)) were obtained from Sigma. FS (fluorescein sulfonate) was purchased from Molecular Probes (Junction City, OR, USA). HB (Hypocrellin B) was the generous gift of Dr. J.C. Yue, Institute of Biophysics, the Chinese Academy of Sciences. 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. All buffers were prepared from fresh double distilled water.

Preparation and purification of chicken apocytochrome c and its deleted mutants

The entire coding sequence of chicken apocytochrome c has been cloned into pUC19 (pUCC1) [15]. For site-directed mutagenesis, the EcoRI-BamHI fragment from pUCC1 containing the coding sequence was first cloned into phagemid pALTER-1, and site-directed mutagenesis was carried out according to the instruction manual of the Promega Altered Sites System. Helper phage M13KO7 was used. Clones were screened directly by sequencing on alkali-denatured, double-strand DNA using dideoxy chain termination method. In order to facilitate cloning the gene into the expression plasmid pET-3d, a NcoI site at the start codon was first introduced by altering one base from A to C, and subsequent mutations were constructed on the NcoI-containing plasmid pSTCN. Primers for Δ28–39 and Δ72–86 were 5′-GGAGGCAAGCACAAGACAGGACAAGCTGAGG-3′ and 5′-GAGTATTTGGAAAATCCAAAGAAGTCTGAGAGAGTA-3′, respectively. The dual-deleted mutation of Δ28–39/72–86 was generated on the basis of Δ72–86 with the primer of Δ28–39. The structure and abbreviation of the deleted mutants are listed in Fig. 1.

Wild-type chicken apocytochrome c and mutants Δ28–39, Δ72–86 and Δ28–39/72–86 were expressed in *E. coli* strain B121-(DE3) containing the plasmid pET-3d-C. These plasmids are derivatives of pET-3d and contain the entire coding sequence of chicken apocytochrome c or the mutated versions cloned into the NcoI-BamHI sites of this vector. Preparation of inclusion bodies and purification of the proteins were carried out as reported [15], and purity of the proteins was identified by SDS-PAGE. The accurate molecular weight of the proteins was measured by electrospray mass spectrum (VG Platform II). The purified proteins were lyophilized and were subjected to a renaturation procedure as described previously [16]. Renatured proteins were dissolved in PIPES buffer and stored in aliquots at –70°C, and used immediately after thawing. Protein concentration was determined using a molar absorption coefficient of 10,580 $M^{-1}\,cm^{-1}$ at 277 nm.

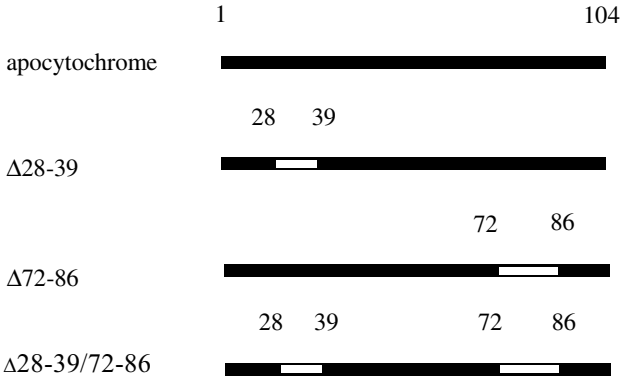

Fig. 1. Deletion of apocytochrome c. The blank denotes the deleted region. Δ28–39, Δ72–86 and Δ28–39/72–86 represent the deletions of the sequence 28–39, 72–86 and the dual deletions of 28–39 and 72–86, respectively.

Preparation of vesicles

The purified soybean phospholipid (in chloroform/methanol (3:1)) were taken to dryness under a stream of nitrogen, and lyophilized overnight.

Trypsin-enclosed soybean phospholipid LUVs (Large Unilamellar Vesicles) used in the following translocation assay, and FS-enclosed soybean phospholipid LUVs used in FS leakage measurements were prepared by the reverse-phase evaporation method of Szoka and Papahajopolous as previously reported [17].

Soybean phospholipid SUVs (Small Unilamellar Vesicles) used in fluorescence experiments were prepared by sonication in PIPES buffer on MSE sonicator at 0°C under nitrogen purge. After sonication, the solution was centrifuged at 100,000 g for 30 min on Beckman TL-100 ultracentrifuge to eliminate the multilayer liposomes and titanium residue.

The phospholipid concentration was determined by perchloric acid digestion [18].

Insertion ability into monolayer

A film balance, type Han-2000, designed and made in our laboratory was used to study the membrane penetrating ability of proteins. Briefly, 3 ml of PIPES buffer as a subphase

was added into the mini-trough, which has been described previously [19]. The monomolecular lipid layer was spread to give the desired initial surface pressure by dropping aliquots of lipids dissolved in chloroform on the aqueous surface. The surface pressure of the monolayer was measured by the Wilhelmy-plate method using plates cut from filter paper and rinsed with methanol prior to use. After the initial surface pressure had stabilized to a plateau value, the desired amount of protein was injected into the mixing chamber (which contains a magnetic stir bar) through a 0.7-cm^2 hole in the edge. The samples were then rapidly mixed with bulk solution, and radically diffused to the upper monolayer-spreading disk. The change in surface pressure was recorded as a function of time. All measurements were performed at room temperature. After each experiment the trough was thoroughly cleaned with distilled water. Usually, an increase of the surface pressure $\Delta\pi$ is measured as a function of the initial surface pressure π. A plot of $\Delta\pi$ vs. π yields a straight line with negative slope that intersects the abscissa at the value named as the limiting surface pressure (π_c).

Inducing leakage of entrapped FS from liposomes

LUVs containing FS (10 mmol/L) were prepared by reverse-phase evaporation, and separated from extravesicular FS by washing. Following incubation of increasing amounts of apocytochrome c or its deleted mutants with 50 nmol LUVs in 100 µl of PIPES buffer at 30°C for 30 min, the vesicles were sedimented by centrifugation (35,000 × g, 25 min, 4°C). Half of the supernatant (S) was sampled out, to which, and to the remainder (R) in the centrifugation tube as well, 350 µl of PIPES buffer containing 0.1% Triton X-100 was added to dilute and dissolve the samples. The control followed the same procedure except for the addition of buffer instead of apocytochrome c or its deleted mutants. FS contained in the two parts of the solution was quantified using a spectrofluorimeter, and the fluorescence intensity was corrected for self-quenching according to the standard curve of fluorescence vs. [FS]. FS was excited at 490 nm and emission at 520 nm was recorded with the bandwidths of 1.5 and 3 nm for excitation and emission, respectively. The percentage of FS leakage, therefore, was determined by:

$$FS\ Leakage\ (\%) = \left(\frac{2 \times S}{S + R} - B\right) \times 100$$

Where B is the leakage extent of the control.

Circular dichroism measurements

CD spectra were measured on a Jasco J-720 spectropolarimeter at room temperature with path length 0.1 mm and protein concentration 100 µmol/L. SDS was added from a 10% (w/v) stock solution to a final concentration of 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 with appropriate background.

Tryptophan fluorescence spectra

Intrinsic fluorescence spectra of the single tryptophan residue (at position 59) of apocytochrome c or its deleted mutants were measured before and after addition of soybean phospholipid SUVs. All fluorescence measurements were performed at 30 ± 0.1°C with a Hitachi F-4010 spectrofluorometer equipped with a thermostatically controlled cuvette holder. The protein concentration was 2 µmol/L, and that of SUVs was 200 µmol/L. Excitation wavelength was set at 295 nm, and emission from 310–400 nm was recorded with the bandwidths of 5 nm for both excitation and emission. Each measurement was corrected for the light scattering contribution of the signal due to the vesicles.

Fluorescence quenching

After incubation of apocytochrome c or its deleted mutants with liposomes for 30 min at 30°C, KI, acrylamide or HB quenching experiments were carried out on a Hitachi F-4010 spectrofluorometer. The values obtained were corrected for dilution, the scatter contribution and the absorptive screening by quenchers. Data were analysed according to the Stern-Volmer equation: $F_0/F = 1 + K\text{sv}\ [Q]$, 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.

Translocation assay

Translocation of apocytochrome c or its deleted mutants across the lipid bilayer of the LUVs containing trypsin was assayed essentially as described by Tong *et al*. [16]. Generally, the entrapped volume of trypsin-enclosed LUVs used in translocation assay was more than 30 µl/mmol of Pi [20].

Results

Expression and purification of wild-type apocytochrome c and its deleted mutants

The map of hydrophobicity indicates that apocytochrome c contains four hydrophobic segments: 9–20, 28–39, 72–86 and

94–98. To study the role of the sequence that is rich in hydrophobic amino acids, deleted mutants of apoprotein: Δ28–39, Δ72–86 and Δ28–29/72–86 were obtained by site-directed mutagenesis (Fig. 1). DNA sequencing identified the deleted mutation, and consistent mutation frequency of 30–60% was observed. The purity of the expressed proteins was at least 90% as shown by SDS-PAGE and the accurate molecular weight of the proteins was measured by electrospray mass spectrum. Obtained data showed that the molecular weight of Δ28–39 or Δ72–86 is larger than that calculated theoretically due to an uncleaved N-terminal Met residue after expression in *E. coli*. Thus, the hydrophobicity of apocytochrome c was decreased to –8.28, –9.48 or –9.89, following the deletion of segment 28–39, 72–86 or dual-deletion of 28–29/72–86, respectively.

Insertion of wild-type apocytochrome c and its deleted mutants into phospholipid monolayer

The effect of deletions on the interaction of apocytochrome c with lipids was studied by comparison the penetration of wild-type apocytochrome c into soybean phospholipid monolayer with those of its deleted mutants. The important parameter in monolayer experiment is the limiting pressure (π_c), which is defined as the pressure that the protein can no longer penetrate, in other words the change in surface pressure is zero at that time. 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 phospholipid monolayer is thought to be 32 ~ 35 mN/m [21], hence the π_c is a more influential factor in predicting the insertion ability of a protein into membrane.

Apocytochrome c has high membrane insertion property. The limiting surface pressure (π_c) measured as shown in Fig. 2 is 51.50 mN/m. In consequence of hydrophobic segment deletion an obvious decrease of π_c can be observed. The numerical value of π_c for Δ28–39 is 47.04 mN/m, and those for Δ72–86, Δ28–29/72–86 are 32.84 and 29.99 mN/m, respectively. However, it can be seen the π_c of Δ28–39 is still obviously higher than the 'equivalence pressure' of bilayer (32 ~ 35 mN/m). So, it may indicate that the insertion of apocytochrome c into monolayer was not distinctly affected by the deletion of hydrophobic segment 28–39 in N-terminal. But in the case of Δ72–86 the insertion ability of apoprotein was almost lost at all. So, it implies that its C-terminal hydrophobic segment is crucial to the insertion of apocytochrome c into membrane.

Comparison of FS leakage from LUVs induced by apocytochrome c and its deleted mutants

To further understand the effect of deletions on the interaction of apocytochrome c with lipids, the entrapped-FS release experiments were carried out to investigate the effect of apocytochrome c or its deleted mutants association on the barrier properties of vesicles. As shown in Fig. 3, apocytochrome c and Δ28–39 can induce FS leakage of 90 and 87% at 40 μmol/L, respectively, which indicated that the perturbation of lipid bilayer structure caused by apocytochrome c association was almost not affected by the deletion of segment 28–39. However, in the case of Δ72–86 and Δ28–39/72–86, the leakage of FS is 61 and 71% lower than that of wild-type apocytochrome c, respectively. These results showed that the C-terminal hydrophobic segment 72–86 was also related to the ability of apocytochrome c to perturb the lipid bilayer structure.

Comparison of conformational changes following the interaction of apocytochrome c and its deleted mutants with lipids

It is generally suggested that the protein conformation has been changed remarkably in consequence of the interaction of apocytochrome c with the phospholipid in model membranes, which is initially electrostatic [22, 23], and secondarily hydrophobic [22, 24]. Such changes in the protein conformation are thought to be the driving force for apocytochrome c transfer from an aqueous compartment into membranes [22, 24]. Thus it can be questioned whether there is any difference in the conformational changes resulting from the aforementioned deletions of apocytochrome c. CD spectra, tryptophan fluorescence spectra and fluorescence quenching were applied to such studies.

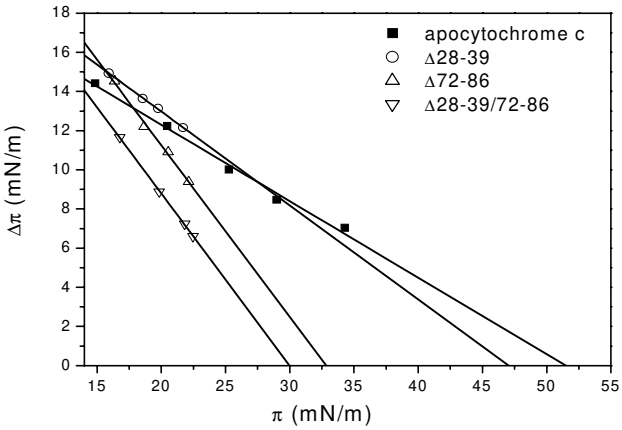

Fig. 2. Relations between the initial surface pressure and the surface pressure increase. Displayed is the π-Δπ plots of surface pressure changes after injection of apocytochrome c or its deleted mutants (0.2 μmol/L) underneath soybean phospholipid monolayer at different initial surface pressure. The limiting pressures of apocytochrome c, Δ28–39, Δ72–86 and Δ28–39/72–86 were 51.50, 47.04, 32.84 and 29.99 mN/m, respectively.

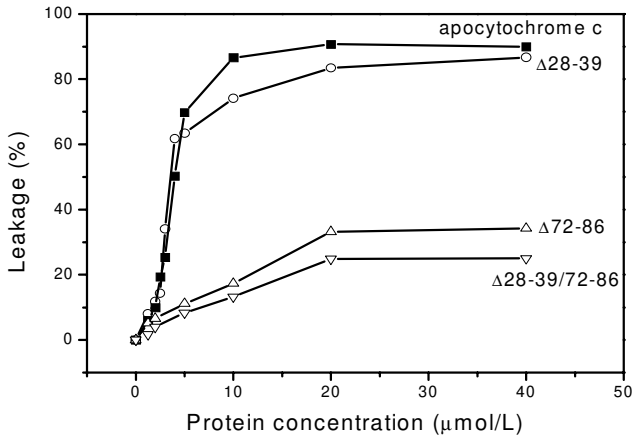

Fig. 3. Comparison of leakage of entrapped FS induced by apocytochrome c or its deleted mutants from soybean phospholipid LUVs. Increasing amounts of apocytochrome c or its deleted mutants were incubated with 500 μmol/L of soybean phospholipid LUVs. FS was excited at 490 nm and emission at 520 nm was recorded with the bandwidths of 1.5 and 3 nm for excitation and emission, respectively. Each value is an average of 3 experiments with three different vesicle preparations of the same liposome formulation.

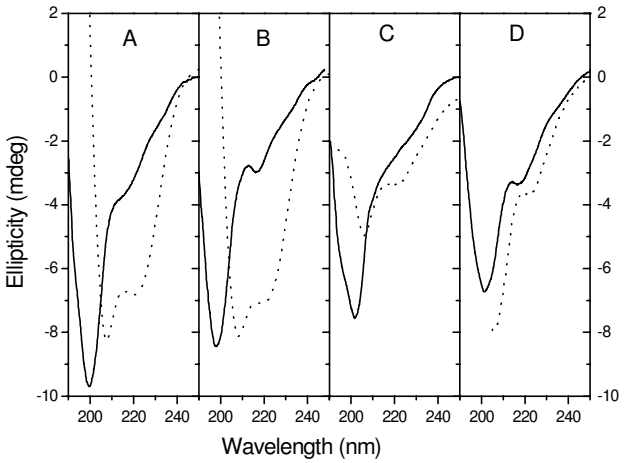

Fig. 4. Circular dichroism spectra of apocytochrome c or its deleted mutants in aqueous solution (solid line) and in the presence of 0.5% SDS (dot line). The concentration of proteins was 100 μmol/L. The concentration of SDS was 0.5% (w/v). The spectra scanned from 250 to 190 nm and 8 scans were averaged. Measurements were carried out at 30°C using cells with pathlength of 0.1 mm. All spectra were acquired with a resolution of 0.5 nm. (A) apocytochrome c; (B) Δ28–39; (C) Δ72–86; (D) Δ28–39/72–86.

Circular dichroism spectra

Interaction of apocytochrome c with vesicles containing negatively charged phospholipid induces a partially α-helix conformation [11, 25]. To assess the effect of deletions on the conformational changes of apocytochrome c following its interaction with lipids, CD spectra of apocytochrome c and its deleted mutants were measured, and the results were shown in Fig. 4. Here a quantitative interpretation of the CD spectra virtually was impossible, due to vesicle aggregation induced by apoprotein, so the detergent SDS was used instead of negatively charged phospholipid. From Fig. 4 it can be seen the CD spectrum of Δ28–39 showed characteristics quite similar to that of apocytochrome c, which indicated that the deletion of segment 28–39 did not affect the conformational changes of apocytochrome c induced by mimic phospholipid molecule SDS. However, under the same condition the content of α-helix following the interaction of Δ72–86 or Δ28–39/72–86 with SDS was lower than that of apocytochrome c, as revealed in the decrease of the ellipticity around 222 nm. Thus, the deletion of segment 72–86 has significant effect on the conformational changes of apocytochrome c, following its interaction with phospholipid.

Intrinsic fluorescence measurement

Apocytochrome c or its deleted mutants have one tryptophan residue at position 59. Using an excitation wavelength of 295 nm, the fluorescence emission spectrum is dominated by the Trp fluorescence, whereas tyrosine and phenylalanine residues make small contributions to the overall intrinsic protein fluorescence. Fluorescence emission spectra of apocytochrome c and its deleted mutants following interaction with soybean phospholipid SUVs were shown in Fig. 5. Association of apocytochrome c with membrane will result in a blue shift of peak (λ_{max}) of fluorescence intensity, which indicates that Trp residue is shifted to a more hydrophobic environment. A similar blue shift of Δ28–39 to that of apocytochrome c was observed, which may indicate that the deletion of segment 28–39 did not obviously affect the interaction of apocytochrome c with lipids. However, in the case of Δ72–86 or Δ28–39/72–86 no such changes in peak of fluorescence intensity could be detected.

Quenching of the intrinsic fluorescence by KI, acrylamide or HB

In order to compare the membrane-integrated states of apocytochrome c and its deleted mutants following interaction with soybean phospholipid SUVs, the quenching of the intrinsic fluorescence by KI, acrylamide or HB was measured. KI and acrylamide were used as aqueous quenchers. Hydrophobic quenching was accomplished by HB. It has been reported [26] that the partition coefficient of HB in erythrocyte membrane was about 10^4 and this molecule could act as a very efficient collision quencher of fluorophores embedded in the hydrophobic domain of membrane proteins.

Figure 6 shows the Trp fluorescence quenching of apocytochrome c or its deleted mutants by KI, acrylamide or HB. 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 quenching efficiency of intrinsic

fluorescence of Δ72–86 or Δ28–39/72–86 by aqueous quencher KI or acrylamide was higher than that of apocytochrome c or Δ28–39. However, the results of quenching by hydrophobic quencher HB were contrary to that of KI or acrylamide. This may mean that the penetration of apocytochrome c or Δ28–39 into the hydrophobic core of lipid bilayer is more efficient than those of Δ72–86 and Δ28–39/72–86. Here, the obtained results further showed that the C-terminal hydrophobic segment 72–86 plays an important role in the conformational changes of apocytochrome c, inducing by its interaction with phospholipid.

Table 1. Effective Stern-Volmer quenching constants (Ksv) for tryptophan fluorescence of apocytochrome c or its deleted mutants by I^-, acrylamide and HB in the presence of SUVs

	Ksv[a]		
	KI (M^{-1})	Acrylamide (M^{-1})	HB (M^{-1})
Apocytochrome c	1.28	1.24	1.04×10^5
Δ28–39	1.23	1.40	1.09×10^5
Δ72–86	1.65	1.90	0.64×10^5
Δ28–39/72–86	1.69	2.08	0.64×10^5

[a]The Stern-Volmer quenching constants (Ksv) of 3 experiments were calculated as described in 'Materials and methods'.

Comparison between translocation of apocytochrome c and its deleted mutants across LUVs

The influence of deletions could be more clearly revealed by the translocation assay on trypsin-enclosed LUVs. This system was put forward by Rietveld *et al*. [27], and we have used it to compare the ability of apocytochrome c from different species to be incorporated into the vesicles [22, 28]. Here, it was used to assess the effect of the deletions on the translocation of apocytochrome c.

The time-dependent digestion of apocytochrome c or its deleted mutants by enclosed trypsin was shown in Fig. 7. The difference between Δ28–39 and wild-type apoprotein was not significant. But the effect of segment 72–86 deletion was quite astonishing. The rate of apocytochrome c or Δ28–39 being digested in soybean phospholipid LUVs is about 3–4 times as high as that of Δ72–86 or Δ28–39/72–86.

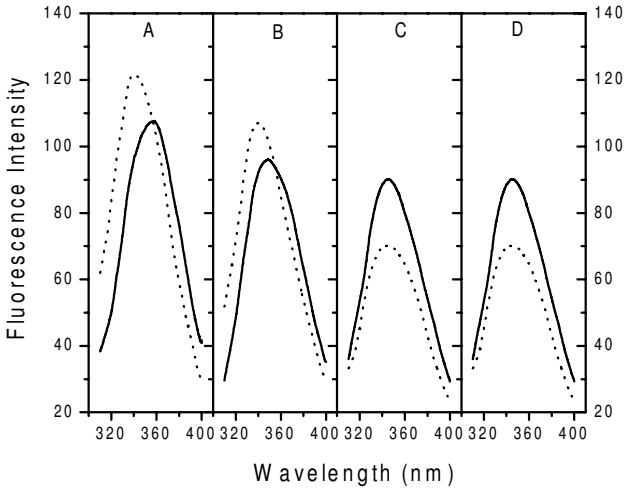

Fig. 5. Comparison of the fluorescence emission spectra of apocytochrome c or its deleted mutants following its interaction with soybean phospholipid SUVs. Two μmol/L of protein was incubated with 200 μmol/L of soybean phospholipid SUVs in PIPES buffer at 30°C. Excitation wavelength was set at 295 nm, and the emission spectra were recorded from 310–400 nm with bandwidths of 5 nm for both excitation and emission. (A) apocytochrome c; (B) Δ28–39; (C) Δ72–86; (D) Δ28–39/72–86. The solid lines represent the fluorescence emission spectra of apocytochrome c or its deleted mutants in aqueous solution, and the dot lines represent those following the interaction with soybean phospholipid SUVs.

Discussion

Until now, the molecular mechanism of apocytochrome c translocation remains elusive. Since no proteinaceous component responsible for its import has ever been identified [5, 6], apocytochrome c may translocate across mitochondrial membrane through its direct interaction with lipids [12, 29, 30]. It is generally accepted that both electrostatic and hydrophobic interactions are involved during apocytochrome c translocation [22]. The former was studied in detail by De Kruijff *et al*. [31]. Comparatively, the study on the hydrophobic interaction is much less. In the present paper, the effect of hydrophobicity on the translocation of apocytochrome c has been studied by using different mutants in which one or two hydrophobic segments have been deleted. Another objective of this study was to seek which hydrophobic segment plays a critical role in the translocation of apocytochrome c.

It was postulated that through hydrophobic-hydrophobic interaction apocytochrome c can insert into the lipid bilayer with its concomitant conformational changes, which are thought to be the driving force for apoprotein transfer from an aqueous compartment into membrane [24]. This was supported by the comparative translocation studies across model membrane between wild type apocytochrome c and its hydrophobic fragment deleted mutants. Obtained results in the present paper clearly indicated that the hydrophobicity of apocytochrome c (particularly segment 72–86) plays an important role in the different steps of translocation: insertion into monolayer, destabilization of lipid bilayer structure, inducing conformational changes of apoprotein and translocation across model membrane.

Another question we are interested in is which segment plays more important role in the translocation of apocytochrome c. Although considerable studies have been made, a conclusive

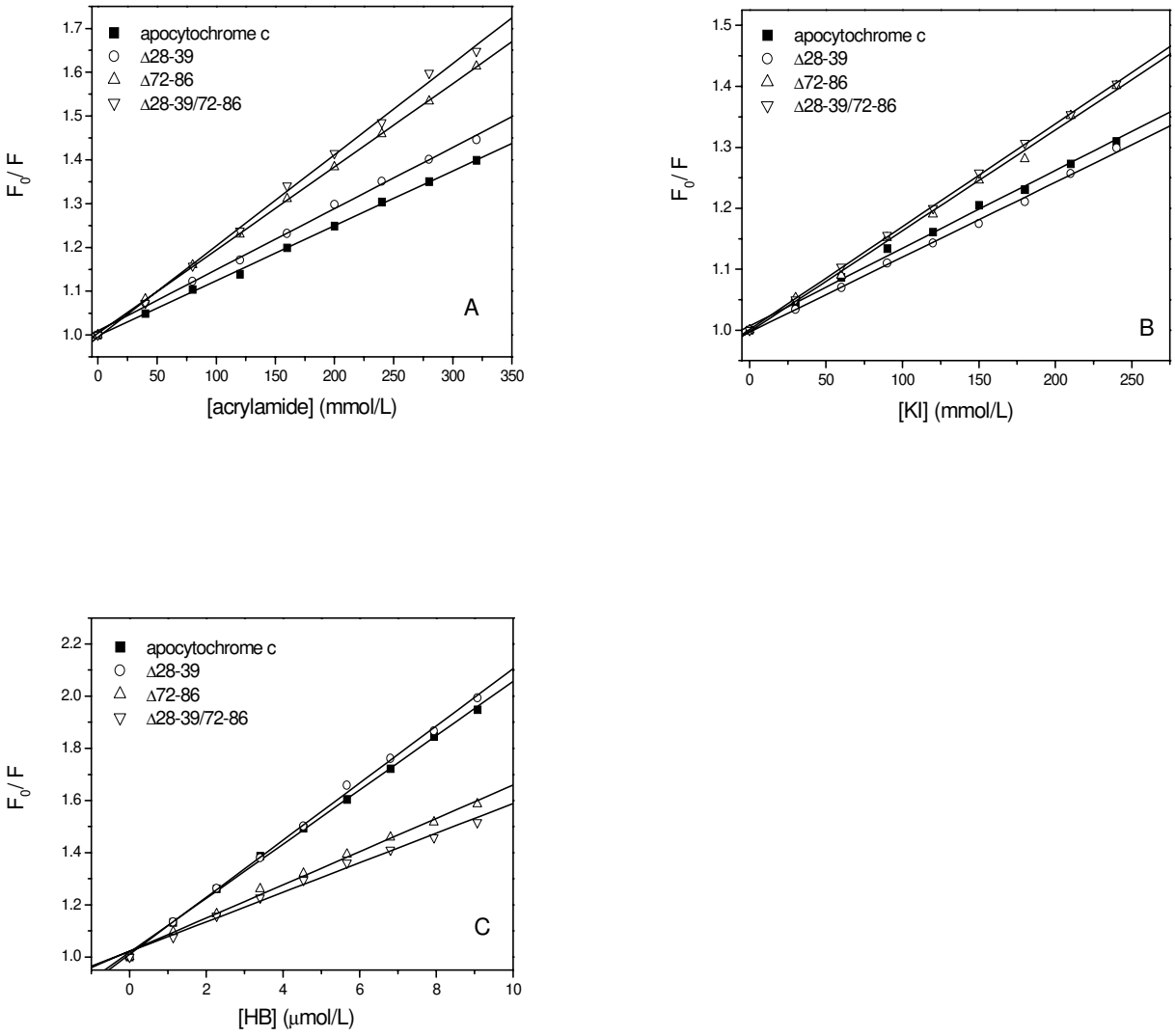

Fig. 6. Stern-Volmer plots of quenching of intrinsic fluorescence of apocytochrome c or its deleted mutants by acrylamide (A), I^- (B), and HB (C) in the presence of soybean phospholipid SUVs. Proteins (2 μmol/L) were incubated with soybean phospholipid SUVs (200 μmol/L) in PIPES buffer at 30°C. Spectra were recorded at 30°C. Spectra bandwidths were 5 nm for both excitation and emission.

result remains ambiguous. Model membrane studies with peptides derived from apocytochrome c had demonstrated that the N-terminal fragments 1–38, 1–59, and 1–65 were able to translocate, at least partially, across the bilayer [32]. By using various constructs of *Drosophila melanogaster* apocytochrome c, it was also found that the N-terminal sequence was much more critical for apocytochrome c association with mitochondria [33]. On the other hand, considerable evidence has proved the important role of the C-terminal sequence. Experiments by E. Margoliash [34] suggested that the C-terminal fragment consisting of residues 66–104 was able to inhibit the import of apocytochrome c into mitochondria. Further, Stuart *et al*. found that an apoprotein in which the 8 C-terminal residues were replaced by a 27-residue non-cytochrome c sequence became association with mitochondria to a small extent [35]. In the present paper the importance of the C-terminal hydrophobic segment 72–86 to the translocation of apocytochrome c across liposomes, as compared to the relative insignificance of the N-terminal region from 28–39, correlates well with the observations of E. Margoliash [34] and Stuart [35].

Taken together, it seems that a conclusion can be drawn that the C-terminal sequence 72–86 plays a crucial role in the insertion and translocation of apocytochrome c across mitochondrial membrane. This was further supported by our recent experiments using a series of synthesized peptides related to hydrophobic segment 72–86 (unpublished results).

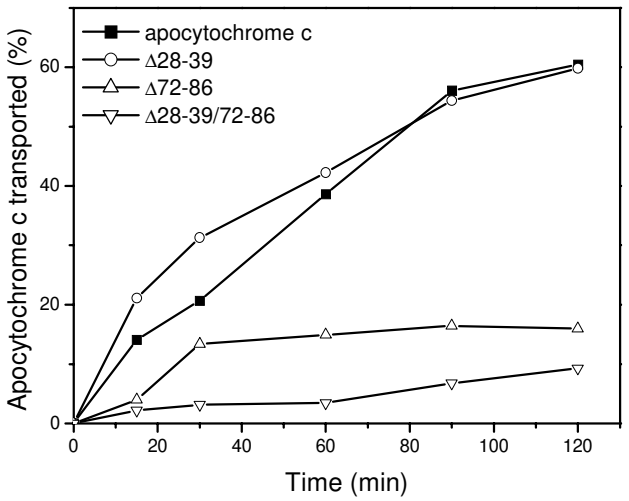

Fig. 7. Comparison of translocation of apocytochrome c and its deleted mutants across soybean phospholipid LUVs. Trypsin-containing vesicles (108 nmol of Pi) of soybean phospholipid were incubated with 40 μg of protein. Samples were drawn immediately after mixing the vesicles with the proteins (t = 0 min) and after incubation at 30°C for 15, 30, 60 and 120 min, respectively. The reaction was stopped immediately by mixing with equal volume of SDS-PAGE sample buffer and boiling at 100°C for 5 min. Then SDS-PAGE was performed and protein bands were detected by Coomassie blue R-250 stain. The mean translocation of 3 experiments is shown (S.D. ± 5–10% of percentages).

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