Peptide 68-88 of apocytochrome c plays a crucial role in its insertion into membrane and binding to mitochondria

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Received September 25, 2001

Abstract Apocytochrome c (Apocyt. c) is the precursor of cytochrome c. It is synthesized in the cytosol and posttranslationally imported into mitochondria. In order to determine the crucial sequence in apocyt. c translocation, deleted mutant and chemically synthesized peptides with different length were used. Obtained results showed that sequence 68-88 of apocyt. c plays a critical role in its insertion into membrane and binding to mitochondria.

Keywords: apocyt. c, crucial peptide, insertion into membrane, binding to mitochondria.

Apocytochrome c (Apocyt. 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, and neither a membrane potential nor ATP is required for its import[2]. Further, no proteinaceous component responsible for its import has ever been identified[3,4]. In the intermembrane space apocyt. c is converted into holocytochrome c by cytochrome c heme lyase (CCHL).

Further, model membrane studies have demonstrated that apocyt. c binds with high affinity, preferentially to the negatively charged lipid component of the outer mitochondrial membrane, followed by penetration and translocation of apocyt. c into liposomes[5]. So, it was proposed by Neupert et al. that instead of receptor system apocyt. c might directly insert into the mitochondrial membrane system by its interaction with phospholipid[6]. Thus, it is interesting to identify which sequence in apocyt. c is crucial for its penetration and translocation across membrane.

The main difficulty of such study is to prepare different peptides with proper chain length, and only limited and undesirable peptides could be acquired if usual chemical and enzymatic cleavage method was used. Margoliash et al. found that only a carboxyl-terminal segment composed of residues 66-104 was able to compete with apocyt.c import into mitochondria[7]. However, this result has not been approved by model membrane studies. On the other hand, 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[8]. The interaction of fragment 66-80 with lipids is even much weaker. Sherman et al. have investigated the amino acid sequences of cytochrome c critical

for mitochondrial import by using a series of mutants, each of which peptide composed of ten amino acid residues was deleted. However, no mutants which are incapable of binding to mitochondria have been identified[9].

Since apocyt. c-phospholipid interaction is involved in its translocation into mitochondria, the direct interaction between hydrophobic amino acids of apocyt. c with the fatty acid chains of the mitochondrial outer membrane should be considered in its insertion into membrane. So, hydrophobic amino acid residues or sequence rich in hydrophobic amino acid residues were suggested to be a candidate with insertion ability. To address this hypothesis, a series of deletion mutants were obtained by site-directed mutagenesis, in which segments rich in hydrophobic amino acids 28-39, 61-66, 72-86 and 28-39/72-86 were deleted. After expression in *E. coli* and purification, highly purified deleted mutants were obtained. It was found that only the deletion of residues 72-86 could remarkably affect the translocation of apocyt. c[1)], indicating its critical role in apocyt. c translocation across lipid bilayer. To confirm the important role of this sequence, three C-terminal peptides related to 72-86 and a N-terminal peptide N19 were synthesized. The insertion of these synthesized peptides into monolayer, their binding to liposomes and competitive inhibition of ^{35}S-apocyt. c binding to mitochondria were determined and compared. The results will be reported in the present paper.

1 Materials and methods

1.1 Materials

Three or four-week chickens were purchased from Beijing Merial Viral Laboratory Animal Technology Co., Ltd and collagase II from Gibco. PMSF, aprotinin, benzamidine, percoll, kynuramine, glucose-6-phosphate, horse apocyt. c, PNP, PNPP, PIPES (piperazine-N, N-bis (2-ethanesulfonic acid)) and FS (fluorescein sulfonate) were obtained from Sigma. Restriction endonucleases, modification enzymes and TNT^{TM} coupled reticulocyte lysate systems were purchased from Promega. ^{35}S-cysteine was from Dupont NEN Inc. Fluorescent probe-pyrene was obtained from Molecular Probe Inc. Soybean phospholipids were obtained from Sigma, and were purified prior to use. Plasmid pSTCN containing the entire coding sequence of chicken apocyt.c has been constructed by our laboratory. Other chemical reagents were of the best quality commercially available. HMSB buffer used in mitochondria isolation consisted of 5 mmol/L hepes-KOH, pH 7.4, 220 mmol/L mannitol, 70 mmol/L sucrose, 0.2% BSA. Accordingly, HMSBI buffer consisted of HMSB and inhibitors (2 mmol/L EGTA, 1 mmol/L PMSF, 1 μg/μL aprotinin and 1 mmol/L benzamidine). PIPES buffer used in liposomes preparation and related experiments 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.

1) Jia, S. T., Effects of hydrophobic region deletion on the translocation of apocytochrome c, Master thesis, Institute of Biophysics, the Chinese Academy of Sciences, July, 1997.

1.2 Synthesis and modification of the peptides

Peptides were synthesized by solid-phase method and purified by reversed phase HPLC. The synthesized products were identified by mass spectrum. The C-terminal of peptides was labeled by pyrene, and the peptide-pyrene conjugate was purified by reversed phase HPLC.

1.3 Transcription, translation *in vitro* and purification of apocyt. c

The *Nco* I-*Bam*H I fragment from pSTCN containing the coding sequence of chicken apocyt.c was cloned into the plasmid pET-3d under the control of promotor and ϕ10 terminal code, linked by T4 ligase, and then transformed into JM109. The transformant was identified by dual digestion. The chicken apocyt. c mRNA transcribed from the linearized recombinant plasmid by T7 RNA polymerase was translated with a nuclease-treated rabbit reticulocyte lysate. *In vitro* translated apocyt. c was radiolabeled by ^{35}S-cysteine. The free ^{35}S-cysteine was removed by dialysis. The translation product was identified by SDS-PAGE and fluorography.

1.4 Preparation and purification of mitochondria

Chicken heart mitochondria were isolated according to the method established by our lab[10]. The entire procedure was carried out at 0 to 4℃. Activities of monoamine oxidase, cytochrome c oxidase, adenylate kinase, acid phosphatase and glucose-6-phosphatase were assayed to check the purity of mitochondria. Mitochondria pellets were gently suspended in HMSB buffer containing 10% DMSO, frozen in liquid nitrogen and stored in aliquots at −70℃. Protein was assayed by the method of amino black using bovine serum albumin as a standard. The intactness of mitochondrial outer membrane was assayed by the latency of cytochrome c oxidase[11], which was defined as $\text{Latency}=\left(1-\frac{A_-}{A_+}\right)\times 100\%$, where A_- and A_+ represent the rate of cytochrome c oxidized by cytochrome c oxidase with or without 0.2% lubrol, respectively.

1.5 Preparation of liposomes

The purified soybean phospholipids (in chloroform/methanol (3∶1)) were taken to dryness under a stream of nitrogen, and lyophilized overnight. Large unilamellar vesicles (LUVs) were prepared by the reverse-phase evaporation[12]. The phospholipid concentration was determined by perchloric acid digestion.

1.6 Monolayer experiments

Insertion of peptides into phospholipid monolayer was performed on HAN-2000 membrane balance, which is designed by Dr. Han in our laboratory. The experiments were performed as described previously[13].

1.7 FS leakage experiments

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 apocyt. c or

peptides with 50 nmol LUVs in 100 μL of PIPES buffer at 30℃ for 30 min, the vesicles were sedimented by centrifugation (35000 *g*, 25 min, 4℃). The control followed the same procedure except for the addition of buffer instead of apocyt. c or peptides. The leakage of FS was quantified according to the standard curve of fluorescence versus [FS]. The intensity of fluorescence of FS was measured using spectrofluorometry (Ex. 490 nm; Em. 520 nm) with the bandwidth of 1.5 and 3 nm for excitation and emission, respectively.

1.8 Competitive inhibition

The binding of apocyt. c to mitochondria was determined by incubating 100 μg mitochondrial protein with ^{35}S-cysteine labeled apocyt. c. To detect the effect of peptides on the binding of apocyt. c to mitochondria, increasing amounts of synthesized peptides were preincubated with mitochondria at 25℃ for 15 min. Then the binding reaction was started by the addition of ^{35}S-apocyt. c. These mixtures were incubated for 15 min at 25℃, followed by centrifugation (10000 *g*, 4 min, 4℃) to separate bound and nonbound proteins. The pellet was resuspended in HMSE buffer, washed twice, lysed with 10 μL 10% SDS and subjected to cellulose acetate filter membrane. The dried filter membrane was put into 5 mL dimethylbenzene solution. The amount of ^{35}S-apocyt. c binding to mitochondria was determined by scintillation counting.

1.9 Instruments

UV-visible scanning spectrophotometer is Shimadzu UV-2101 PC. Fluorescence spectrophometer is Hitachi F-4010. Liquid scintillation counter is Beckman LS-9800. Centrifuges are Beckman Avanti J25-I and Beckman L8-80.

2 Results

2.1 Synthesis, purification and identification of proper segments of apocyt.c

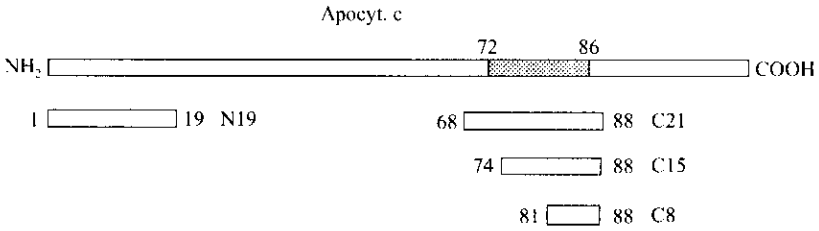
It was found that only the deletion of sequence 72-86 could remarkably affect the translocation of apocyt. c[1)], which implicates its important role in apocyt. c translocation across lipid bilayer. A series of peptides, C8, C15 and C21, corresponding to the sequences 81-88, 74-88 and 68-88, respectively, were synthesized in order to confirm the exact length of this important sequence (fig. 1). In order to label with pyrene, an additional cysteine residue was introduced into the C-terminus. The overall yield of the peptide was >95% as shown by integration of the HPLC peak areas. The expected molecular mass (m/z = 2611.85) of C21 was confirmed by mass spectrum (observed mass m/z = 2612). All synthesized peptides have a featureless CD spectrum, typical for a largely disordered structure.

2.2 Interaction of four synthesized peptides with lipids

The interaction of synthesized peptides with lipids was compared by measuring the peptide

1) See the footnote on page 19.

binding to lipid monolayer and liposomes, its ability to insert into lipid monolayer as well as to perturb the bilayer structure.



Sequence of N19: NH_2-GDIEKGKKIFVQKCSQCHT
Sequence of C8: NH_2-IFAGIKKK
Sequence of C15: NH_2-YIPGTKM IFAGIKKK
Sequence of C21: NH_2-LENPKKYIPGTKM IFAGIKKK

Fig.1. Structure and abbreviation of synthesized peptides. The shadow denotes the deleted region.

2.2.1 Synthesized peptides binding to lipids. (1) Binding to lipid monolayer. The binding of synthesized peptides to the lipid monolayer using home-made membrane balance has been carried out. The surface pressure increased with the concentration of peptides in the subphase at the same initial surface pressure and reached equilibrium after a short period. The dissociation constant of peptide binding to membranes was defined as the sample concentration in the subphase when the surface pressure increased to 50% of the maximum, and it can be used to describe the binding ability of peptides to lipid membrane quantitatively. The binding curves of C21, C15, C8 and N19 to soybean phospholipids monolayer at 20 mN/m are shown in fig. 2. Their dissociation constants calculated from binding curves are 160, 1200, 1500 and 1400 nmol/L, respectively, indicating that C21 has the highest affinity to lipid membranes. The binding ability of horse heart and tuna heart apocyt.c to soybean phospholipid monolayer was also determined and the dissociation constant of them is 120 and 160 nmol/L[13], respectively. In other words, the binding ability of C21 to lipid monolayer is as high as that of the intact apocyt. c. In contrast, other C-terminal peptides and N19 exhibit low affinity to membranes. So, it is clear from the present results that the sequence of 68-88 plays an important role in the binding of apocyt. c to lipids.

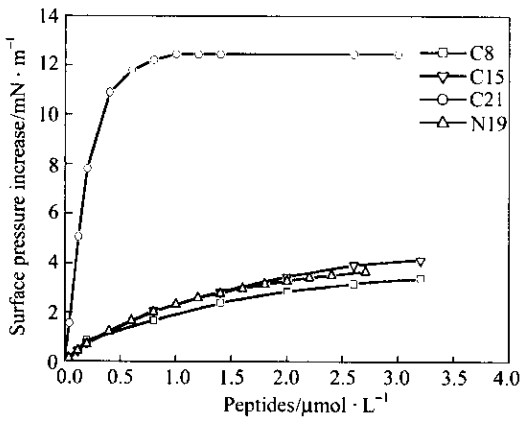


Fig. 2. Binding of synthesized peptides C8, C15, C21, N19 to soybean phospholipid monolayer.

(2) Binding to liposomes. Besides phospholipid monolayer, the binding of synthesized peptides to liposomes was also studied. The C-terminal cysteine residue of peptides was labeled with fluorescent probe-pyrene. The binding of peptides to liposomes was monitored by measuring the

change of the fluorescence intensity of pyrene following its interaction with liposomes. There are two excitation bands of pyrene, one at 330 nm and the other at 340 nm. Here the fluorescence of pyrene was measured by excitation at 340 nm. The obtained results showed that the emission spectra of pyrene labeled C8, C15 and N19 were little affected following incubation with liposomes. Whereas a remarkable change (about 5 fold increase) in emission spectra of pyrene labeled C21 was observed, as shown in fig. 3. Since the spectra characteristics of pyrene are very sensitive to the environment, the increase of fluorescence intensity may indicate that the labeled pyrene was moved from hydrophilic into hydrophobic region. These findings implicated that following binding C21 was located in the hydrophobic region of the lipid bilayer. The less change of the emission spectra of pyrene labeled C8, C15 and N19 indicates their weaker binding to liposomes. So, the obtained results further showed that C21 has the highest binding ability to liposomes among the four synthesized peptides.

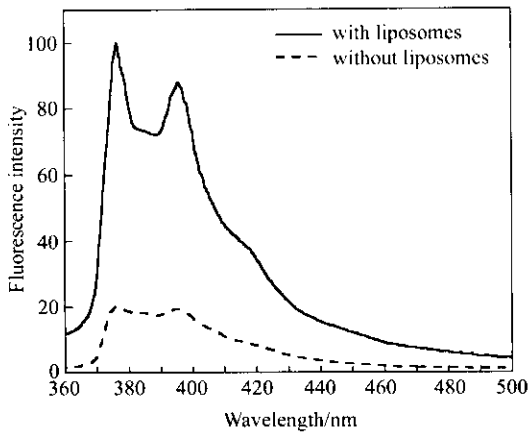


Fig. 3. Changes in pyrene emission spectra as a consequence of C21 binding to liposomes.

2.2.2 Insertion into phospholipid monolayer. To get further insight into the peptide-lipid interaction, we compared the penetration of these four peptides into lipid monolayer. Firstly, a monolayer of soybean phospholipid was spread at the air-water interface. After the initial surface pressure reached equilibrium, the tested peptides were injected into the subphase, and the changes of surface pressure were recorded. The maximum pressure increase induced by peptides was measured and compared at an initial surface pressure of 25 mN/m. It can be seen from fig. 4 that C8, C15 or N19 only induces a very low surface pressure increase (about 2 mN/m), indicating that the interaction of these peptides with lipid is quite weak, while C21 has the highest penetration ability, and the increase of surface pressure is as high as 9 mN/m. Under the same condition, the increase of surface pressure induced by apocyt. c is about 10 mN/m, while that of deleted mutant Δ72-86 is much lower than those of C21 and apocyt. c. Thus the insertion ability of apocyt. c into phospholipid monolayer was almost

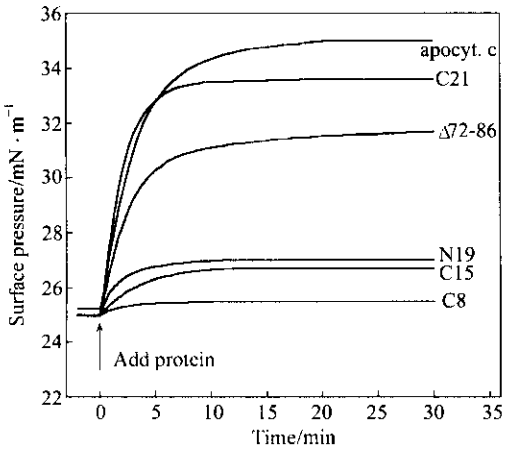


Fig. 4. Interaction of synthesized peptides or deleted mutant of apocyt. c with phospholipid monolayer.

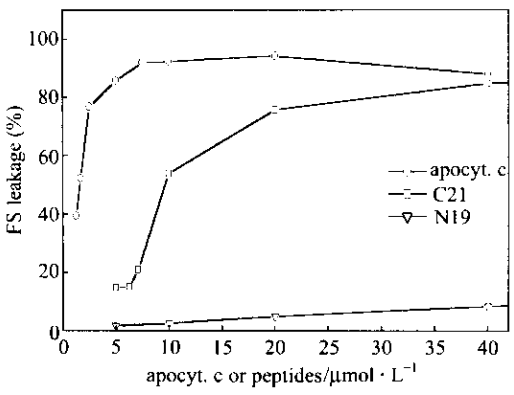


Fig. 5. Comparison of entrapped-FS leakage from soybean phospholipid LUVs induced by apocyt. c and synthesized peptides.

lost by the deletion of 72-86. Therefore, from these findings, the important role of sequence 68-88 in the insertion of apocyt.c into membrane was further confirmed.

2.2.3 Entrapped FS leakage induced by synthesized peptides. The entrapped-FS release experiments were carried out to investigate the change of barrier properties of vesicles following peptides binding. As shown in fig. 5 apocyt. c and C21 can induce FS leakage of 91.8% (at a concentration of 7.5 μmol/L), and 84.7% (at 40 μmol/L), respectively. These results indicated that the perturbation of lipid bilayer structure caused by 40 μmol/L C21 was similar to that of apocyt. c at 7.5 μmol/L. However, in the case of C8, C15 and N19 no obvious effects could be observed under the same conditions.

2.3 Competitive inhibition of ^{35}S-apocyt.c binding to mitochondria by synthesized peptides

As above-mentioned, C21 plays a crucial role in the interaction of apocyt. c with lipid monolayer or liposomes. We have therefore sought to study whether it would also be important in the translocation of apocyt. c across mitochondria. The effect of synthesized peptides on the binding of apocyt. c to mitochondria was thus studied and the competitive inhibition of ^{35}S-apocyt. c binding to mitochondria by synthesized peptides was compared. First of all, radiolabeled apocyt. c was prepared by transcription and translation *in vitro* with a nuclease-treated rabbit reticulocyte lysate in the presence of ^{35}S-cysteine. In order to remove the free ^{35}S-cysteine several methods including active-charcoal adsorption have been tried. Finally, we found that dialysis method is an optimal one.

The results of competitive inhibition of ^{35}S-apocyt. c binding to mitochondria by apocyt. c or synthesized peptides are shown in fig. 6. It can be seen from fig. 6 that the binding of ^{35}S- apocyt. c was very weakly inhibited by unlabeled apocyt. c at 1000 fmol/ì L. It began to decrease as the concentration of apocyt. c was over 1000 fmol/ì L and decreased 65% at 10000 fmol/ì L. Since the affinity of chemically synthesized unlabeled apocyt. c to binding sites is lower than that of translated products, the binding of ^{35}S-

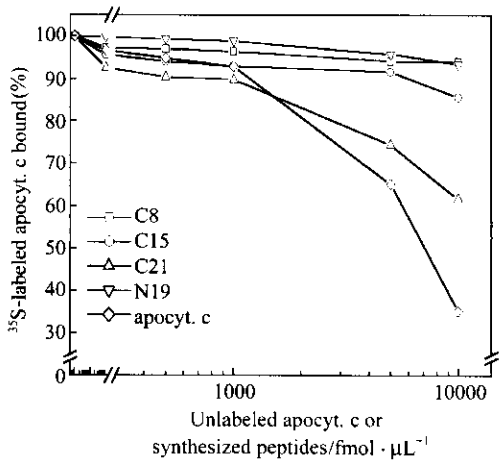


Fig. 6. Competitive inhibition of ^{35}S -apocyt. c binding to mitochondria by unlabeled apocyt. c or synthesized peptides.

apocyt. c is not completely inhibited even at high concentration of unlabeled apocyt. c. The competitive inhibition curve of ^{35}S-apocyt. c binding by C21 was similar to that by intact apocyt. c, i.e. the binding of ^{35}S-apocyt. c declined 40% at 10000 fmol/μL of C21. Under the same conditions, the binding of ^{35}S-apocyt. c was very weakly inhibited by other three peptides. So it may deduce that among four peptides only C21 can compete with apocyt. c for the binding sites in mitochondria.

3 Discussion

The usual approaches for the study on protein translocation across membrane are not suitable for the import research of apocyt. c into mitochondria, because it is very difficult to distinctly divide the whole process into stages of binding, translocation, wholly imported and mature protein formation. Apocyt. c-lipid interaction and its penetration into liposomes indicated that the spontaneous membrane insertion activity may be an important feature of the apocyt. c import into mitochondria[6]. It has been reported that the association constant, K_a, of binding sites in mitochondria for apocyt. c is $10^7 M^{-1}$. In other words, the number of bound apocyt. c was 90 pmol per mg of mitochondrial protein, which is 10-fold higher than those of other preproteins[14]. Such high density of binding sites can only be afforded by the lipids of mitochondrial outer membrane. Moreover, the association constant, K_a of acidic phospholipids for apocyt. c is just $10^7 M^{-1}$[15]. These results raise the possibility that apocyt. c might translocate across mitochondrial membrane through its direct interaction with lipids. By genetic manipulation, Neupert et al. have constructed a fusion protein (pSc_1-c), which is composed of the matrix-targeting domain of the cytochrome c_1 presequence and the amino terminus of apocyt. c with the exception of the initial 6 amino acids[6]. Such fusion protein could be imported into matrix via the cytochrome c_1 route in the presence of a membrane potential (Δy) across the inner mitochondrial membrane. But such translocation was independent of both the cytochrome c_1 receptor and the general insertion pore (GIP), both of which are essential for cytochrome c_1 import. Thus, it was suggested that during translocation the spontaneous insertion ability of apocyt. c can substitute for a receptor/GIP system, which is essential for the import of other preproteins. The next two questions to be answered are: how to measure the insertion ability of apocyt. c and which sequence is more essential for insertion? Inasmuch as the insertion of apocyt. c into membrane cannot be measured on mitochondria, the combination of its insertion into model membrane with its binding or import into mitochondria might be a more suitable approach.

In the present work, the critical role of the C-terminal sequence 68-88 in the interaction of apocyt. c with lipids was demonstrated for the first time by studying the binding of synthesized peptides to lipids, i.e. its insertion into lipid monolayer and perturbation of phospholipid bilayer. The ability of C21 to insert into lipid monolayer is similar to that of intact apocyt. c, and the absence of this sequence caused by the deletion of 72-86 is associated with the remarkable decrease of apocyt. c insertion into membrane. Thus, the data point to the possibility that 68-88 might be

the critical sequence. Further, the essential role of C21 (sequence 68-88) in apocyt. c import into mitochondria was also studied. Our findings showed that among the four synthesized peptides, only C21 can competitively inhibit the binding of apocyt. c to mitochondria.

Previously, Margoliash et al. found that only peptide 66-104 can competitively inhibit apocyt. c import into mitochondria[7], basically our results appear to be consistent with such view and it seems that apocyt. c import into mitochondria would not be introduced by the N-terminal sequence. The function of cytochrome c heme lyase (CCHL) during the conversion of apocyt. c to cyt. c was attaching heme group to cysteines 14 and 17 of the N-terminal, which was proposed to provide binding sites with high affinity and hence to play an important role in apocyt. c association to mitochondria[16]. However, replacing the two cysteines with either alanines or serines, no influence on the binding to mitochondria could be observed[17]. It has also been found by Dr. Junchao Tong that the high affinity binding of apocyt. c to mitochondria was not directly related to holocytochrome c formation[18]. Our results presented here also showed that synthesized N-terminal peptide N19 was incapable of insertion into membrane and could not inhibit the binding of apocyt. c to mitochondria. Therefore, it may indicate that N-terminus alone is incapable of affording high affinity for apocyt.c binding. It is also possible that there is some interaction between N- and C-terminal, which may play a certain role in the translocation of apocyt. c across membrane. Related studies are still in progress.

Taken together, in accord with aforementioned results, a conclusion can be drawn that sequence 68-88 of C-terminal plays a crucial role in the binding and translocation of apocyt. c across mitochondrial membrane.

Acknowledgements We thank Ms. Zhou Yanrong (Institute of Biotechnology, Academy of Military Medical Sciences) for her help in the synthesis of peptides. This work was supported by the National Natural Science Foundation of China (Grant No. 39730130) and the Chinese Academy of Sciences.

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