V92A Mutation Altered the Folding Propensity of Chicken Apocytochrome *c* and Its Interaction with Phospholipids†

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ABSTRACT: Chicken apocytochrome *c* has been shown to possess a much stronger tendency to fold spontaneously in aqueous solution than the equivalent enzyme from other species. In the present work, the amino acid that determines its folding ability was elucidated by site-directed mutagenesis. Wild-type chicken apocytochrome *c* and three mutants V92A, S103A, and V92A/S103A were expressed in *Escherichia coli*. The wild-type apoprotein and S103A exhibited the same folding property during dialysis renaturation processes as that chemically prepared from chicken cytochrome *c*, while those containing V92A mutation did not. Quantitative studies by 2,2,2-trifluoroethanol (TFE) and sodium perchlorate ($NaClO_4$) titration demonstrated that the V92A mutation decreased the helix content that could be induced and confirmed that valine 92 is the major determinant of the folding propensity of chicken apocytochrome *c*. Furthermore, CD spectra, turbidity measurements, and a translocation assay on a model membrane system showed that the V92A mutation also drastically altered the conformation of apocytochrome *c* after being incorporated into lipid bilayer and decreased the aggregation of phospholipid vesicles after association of the apoprotein, thus rendering the molecule more competent for translocation across the membrane. Our results showed that a single amino acid substitution could radically alter the folding propensity of an unfolded polypeptide chain and thus influence the conformation following its insertion into phospholipid bilayer.

Apocytochrome *c* is the heme-free precursor of cytochrome c, an electron carrier in the respiratory chain located on the outer surface of mitochondrial inner membrane. It is synthesized in the cytosol and posttranslationally imported into mitochondria by following a quite unique pathway compared to other mitochondrial precursor proteins (Hartl et al., 1989). It does not possess a cleavable N-terminal presequence, and neither a membrane potential nor ATP is required for its import. Further, no proteinaceous component responsible for its import has ever been identified. Although much effort has been made to uncover the mystery of its import (Nicholson et al., 1988; Hakvoort et al., 1990; Dumont et al., 1991, 1993; Mayer et al., 1995), a conclusive mechanism remains ambiguous. De Kruijff and co-workers (Snel et al., 1994; de Jongh & de Kruijff, 1990; de Jongh et al., 1992, 1994) studied in detail the interaction of horse heart apocytochrome *c* with phospholipids and proposed that direct interaction between apocytochrome *c* and phospholipids of the outer mitochondrial membrane should play an important role at least in some steps of the import process.

We are interested in the conformational change of apocytochrome *c* following its interaction with phospholipids and have tried to study the relationship between its folding propensity and its conformation in lipid bilayer. It is well known that the folding state of apocytochrome *c* is quite sensitive to environment. Though it was generally believed to be in typical random coil state in aqueous solution (Fisher et al., 1973; de Jongh & de Kruijff, 1990), recent studies have revealed the presence of some residual structure. It is significantly compact at neutral pH, even though lacking in secondary structure [C state (Hamada et al., 1993)], and adopts compact conformation with significant secondary structure (A state) under conditions of high ionic strength at both acidic and neutral pH, which resembles the "Molten Globule" state of acid-denatured cytochrome *c* at high ionic strength (Goto et al., 1990).

We have reported that chicken apocytochrome *c* has much stronger tendency to fold spontaneously than those from other species (Yang et al., 1993; Wang et al., 1994), with the partially folded state possessing some characteristics of folding intermediate (Tong et al., 1994; Tong & Yang, 1994). It contains a significant fraction of secondary structure, with decreased surface hydrophobicity and a highly dynamic hydrophobic core. In addition, the partially folded state tends to aggregate. Hydrophobic chromatography suggested that the stronger folding ability of chicken apocytochrome *c* could be ascribed to its relatively stronger hydrophobicity (Tong et al., 1995a). However, the exact sequence responsible has not been determined.

Chicken apocytochrome *c* gene has been successfully cloned and expressed at high levels in *Escherichia coli* in our laboratory (Tong et al., 1995b). Here, we report the results of studies by site-directed mutagenesis. Valine 92 was found to be the major determinant of the folding propensity of chicken apocytochrome *c*. Further, V92A mutation also drastically altered the apoprotein's conformation after being incorporated into lipid bilayer. The results are discussed in the light of folding of apocytochrome *c* and its interaction with phospholipids.

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MATERIALS AND METHODS

Production and Purification of Chicken Apocytochrome c and Its Mutants. The entire coding sequence of chicken apocytochrome *c* has been cloned into pUC19 (pUCC1; Tong et al., 1995b). For site-directed mutagenesis, the *Eco*RI−*Bam*HI fragment from pUCC1 containing the coding sequence was first cloned into phagemid pALTER-1, and oligonucleotide-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-stranded DNA using dideoxy chain termination method. Consistent mutation frequency of 70−80% was observed. In order to facilitate cloning the gene into the expression plasmid pET-3d, a *Nco*I site at the start codon was first introduced by altering one base from A to C (the mutagenic oligonucleotide primer used was GTCTAGTACTGAC**C**ATGGGAGATATT, the underlined is *Nco*I site), and subsequent mutations were constructed on the *Nco*I-containing plasmid pSTCN. Primers for V92A and S103A mutation were GTCTGAGAGAG**C**AGACTTAATAGC and GATGCCACT**G**CAAAGTAAAAG, respectively. The total sequences for V92A, S103A, and double mutation V92A/S103A were reconfirmed.

Wild-type chicken apocytochrome *c* and mutants V92A, S103A, V92A/S103A were expressed in *E. coli* strain Bl21-(DE3) containing the plasmid pET-3d-C (Tong et al., 1995b) or an equivalent substituted version. These plasmids are derivatives of pET-3d (Studier et al., 1986; Rosenberg et al., 1987) and contain the entire coding sequence of chicken apocytochrome *c* or the mutated versions cloned into the *Nco*I−*Bam*HI sites of this vector. Preparation of inclusion bodies and purification of the apoproteins were carried out as reported (Tong et al., 1995b), and purity of the proteins was at least 95% as shown by SDS−PAGE.[1] The purified proteins were lyophilized.

Sample Preparation. The lyophilized apoproteins were dissolved in 8 M urea (ultrapure, Sigma) and 50 mM DTT (Sigma) with protein concentration about 10 mg/mL. After half an hour at 37 °C, they were used directly in the TFE and $NaClO_4$ titration experiments and also in the interaction with phospholipids. Protein concentration was determined by adsorption at 276 nm, with the extinction coefficient E (1%) = 10.1 (Damaschun et al., 1991). To monitor the conformational change during dialysis, proteins about 2 mg/mL in 8 M urea and 50 mM DTT were dialyzed at 4 °C against PIPES buffer (10 mM PIPES, pH 7.0, 50 mM NaCl) containing 0.01% mercaptoethanol (Yang et al., 1993). The dialysis buffer was changed twice at 8 and 22 h from the very beginning. Samples were drawn at 8, 22, and 72 h and were frozen immediately in liquid nitrogen. They were kept at −40 °C and used immediately after thawing. Protein concentration was determined according to Lowry et al. (1951).

Circular Dichroism Measurements. CD spectra for the dialyzed samples were collected on a Jasco J-500C spectropolarimeter in PIPES buffer at room temperature with path length 1 mm and protein concentration 10 μM. The final spectra were the average of four scans with time constant 4 s and scan speed 50 nm/min and corrected with appropriate background. All other CD spectra were recorded on a Jasco J-720 spectropolarimeter at 25 °C with time constant 1 s and scan speed 100 nm/min. After correction for appropriate background, the spectra were smoothed by the fast Fourier transform (FFT) algorithm supplied with the machine. Spectra for TFE and $NaClO_4$ titrations were the average of four scans with path length 1 mm and protein concentration 10 μM. Spectra for apocytochrome *c* after interaction with SUVs were the average of eight scans with path length 0.1 mm and protein concentration 100 μM. All the spectra were converted to mean residue ellipticity.

TFE Titration and Data Analysis. TFE (Sigma) titration of the apoproteins was monitored by CD according to Jasanoff and Fersht (1994). Briefly, the proteins in 8 M urea and 50 mM DTT at about 10 mg/mL were diluted into 0−99% TFE (volume of TFE added/total volume added) and ddH_2O to protein concentration 10 μM, and their CD spectra were recorded. Final concentration of urea was lower than 100 mM. Titration data were shown as dependence of $[\theta]_{222}$ on percentage of TFE (v/v), and were analyzed by unweighted least-squares fitting to the theoretical curves and will be detailed in the results.

$NaClO_4$ Titration and Data Analysis. $NaClO_4$ was prepared as 8 M stock solution, and titrations from 0 to 5 M $NaClO_4$ were also monitored by CD. Proteins in 8 M urea and 50 mM DTT at about 10 mg/mL were diluted into 5 mM sodium phosphate buffer (pH 7.0) containing the desired concentration of $NaClO_4$ to protein concentration 10 μM, and their CD spectra were recorded. Titration data were shown as dependence of $[\theta]_{222}$ on concentration of $NaClO_4$ and were analyzed by unweighted least-squares fitting to the theoretical curves and will be detailed in the results.

Preparation of Vesicles. Trypsin-enclosed soybean phospholipid LUVs used in the following translocation assay were prepared by the reverse-phase evaporation method of Szoka and Papahajopolous (1978) as previously reported (Yang et al., 1993). Generally, the trapped volume was more than 30 μL/μmol of P_i. Soybean phospholipid SUVs used in the CD and turbidity study were prepared by sonication in PIPES buffer on the MSE sonicator at 0 °C under nitrogen purge. After sonication, the solution was centrifuged at 100000*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 destruction (Rouser et al., 1970).

Translocation Assay. Translocation of the apoproteins across lipid bilayer of the trypsin-enclosed soybean phospholipid LUVs was assayed essentially as described by Rietveld et al. (1986). One microgram of soybean trypsin inhibitor (SBTI) per nmol of lipid phosphate was added externally to inhibit any residual trypsin activity outside. The assay was initiated by adding 80 μg of protein (about 10 mg/mL in 8 M urea and 50 mM DTT) to the vesicles (108 nmol of P_i) at 0 °C in a total volume of 150 μL (PIPES buffer). Samples were drawn immediately after mixing the vesicles with the proteins (t = 0 min) and after incubation at 30 °C for 5, 10, 20, and 30 min, respectively. The reaction was stopped immediately by mixing with equal volume of 2× SDS−PAGE sample buffer and boiling at 100 °C for 5 min. SDS−PAGE was performed on Pharmacia Multiphor

[1] Abbreviations: CCHL, cytochrome *c* heme lyase; CD, circular dichroism; DTT, dithiothreitol; LUVs, large unilamellar vesicles; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; SUVs, small unilamellar vesicles; TFE, 2,2,2-trifluoroethanol.

	3	89	92	100	103
Chicken	..I.....S.....V.....D.....S....				
Pigeon	..I.....A.....A.....Q.....A....				
Duck	..V.....S.....A.....D.....A....				

FIGURE 1: Sequence differences among chicken, pigeon, and Peking duck apocytochrome *c*. The sites to be mutated were underlined. The data were taken from *Atlas of Protein Sequence and Structure*, Vol. 5, M. O. Dayhoff, Ed., 1972, National Biomedical Research Foundation, Georgetown University, Washington, D.C.

II electrophoresis unit by using 15% gel, and protein bands were detected by Coomassie blue R-250 stain.

SUVs Titration. The conformational change of the apoproteins following their interaction with soybean phospholipid SUVs was detected by CD. The molar ratio of lipid to protein ranged from 100/1 to 250/1.

Turbidity Measurements. The aggregation of SUVs after binding of apocytochrome *c* was monitored spectrophotometrically at 600 nm in a cuvette with 1 cm path length on a Shimadzu UV-2101PC spectrophotometer. Aliquots of apocytochrome *c* were added successively to suspension of SUVs (lipid concentration, 1 mM) in PIPES buffer. The molar ratio of lipid to protein ranged from 200/1 to 25/1.

RESULTS

Determination of the Sites To Be Mutated. To elucidate by site-directed mutagenesis which amino acid(s) may determine the folding behavior of chicken apocytochrome *c*, we compared its amino acid sequence with two closely related sequences of Peking duck and pigeon, which do not have spontaneous folding ability (unpublished results). The sequence differences among them are shown in Figure 1. It can be seen that valine 92 and serine 103 are unique for the chicken apoprotein. Further comparison with sequences from other species (Hampsey et al., 1988) showed that residue 92 is among the most variable, but valine is unique for chicken apocytochrome *c* and is conspicuous for its higher hydrophobicity. Residue 103 is also a variable one, but serine is not unique for it and residue 103 is near C-terminus of the polypeptide. Considering the above-mentioned circumstances, we tried to mutate Val92 or Ser103 to alanine. Wild-type chicken apocytochrome *c* and three mutants V92A, S103A, V92A/S103A were purified according to the previous procedure (Tong et al., 1995b), and their folding properties were studied.

Conformational Changes during the Dialysis Process. The spontaneous partial folding of chicken apocytochrome *c* was first observed (Yang et al., 1993) in a dialysis renaturation process according to Hennig and Neupert (1983). To obtain a qualitative view of the folding behavior of the expressed apoproteins, the same procedure was carried out, and the typical results are given in Figure 2. The expressed wild-type apoprotein showed properties (Figure 2A) quite similar

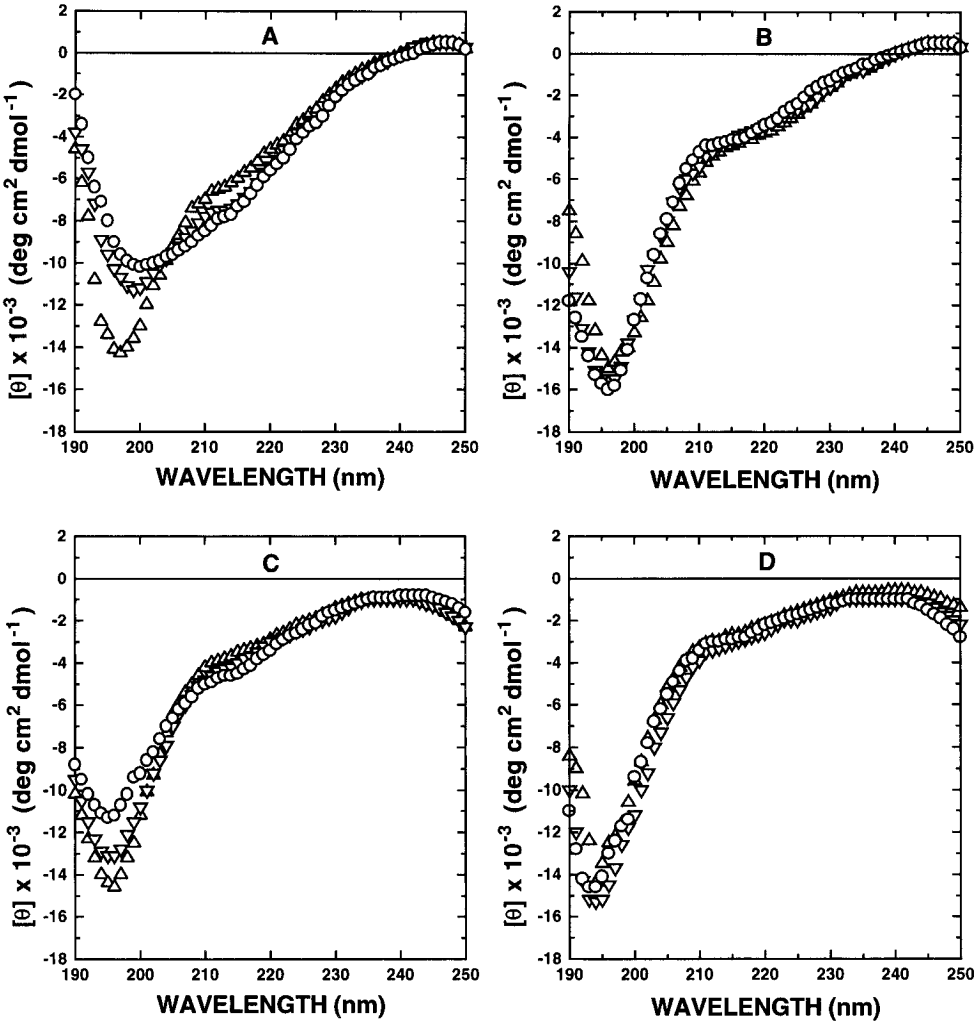

FIGURE 2: CD spectra of the wild-type chicken apocytochrome *c* (A) and the mutants V92A (B), S103A (C), and V92A/S103A (D) during prolonged dialysis renaturation process. The spectra after 8 h (△), 22 h (▽), and 72 h (○) of dialysis are shown.

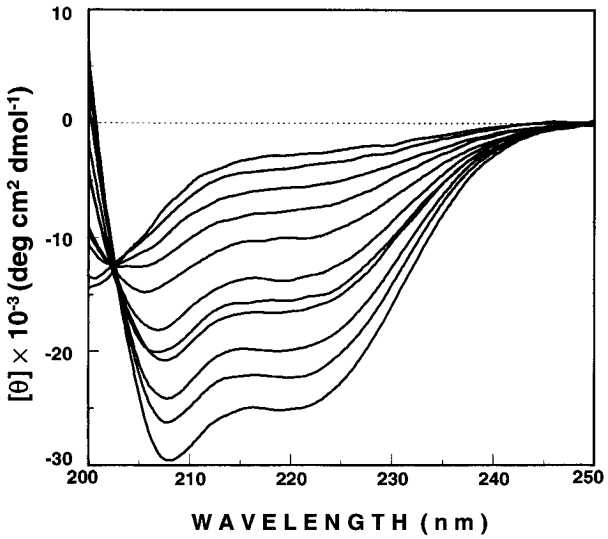

FIGURE 3: CD spectra of the wild-type chicken apocytochrome *c* at different concentrations of TFE. From top to bottom, the TFE concentrations (%) were 0, 5, 10, 15, 20, 30, 40, 60, 80, 90, and 99, respectively. There is an isodichroic point at about 203 nm.

to those of the chemically prepared apoprotein (Yang et al., 1993). During prolonged dialysis, the ellipticity around 210 to 220 nm was increasing while that around 200 nm was decreasing significantly, which indicates an increase of ordered structure and a decrease of random coil. Mutation S103A did not eliminate the partial folding of chicken apocytochrome *c* (Figure 2C), as revealed in the significant decrease of the ellipticity around 200 nm, from the typical value about −15 000 deg cm^2 dmol^{-1} for largely unordered apocytochrome *c* to −11 000 deg cm^2 dmol^{-1}. We noted that the extent of change for S103A is smaller than that for the wild type, notably around 210−220 nm. The decrease in ellipticity around 200 nm did not lead to the increase around 210−220 nm. We do not exactly know the cause, but some aggregation present in samples for the wild type (Tong et al., 1994) and S103A may result in differential scattering and adsorption flattening during measurements and lead to deformation of the spectra. So, qualitatively, S103A possesses the same folding tendency as the wild type. However, V92A mutation completely eliminated the spontaneous partial folding, as shown in spectra for V92A (Figure 2B) and V92A/S103A (Figure 2D). During prolonged dialysis, there was almost no change in the CD spectra, a result quite similar to that for apocytochrome *c* from other species we have studied. The results of intrinsic tryptophan fluorescence measurements also showed the same tendency (data not shown). So, it seems that valine 92 determines the relatively stronger folding propensity of chicken apocytochrome *c*. To further assess the obtained results, quantitative titrations by TFE and $NaClO_4$ were carried out.

TFE Titration. TFE has been used to quantitatively determine the helical propensity of short peptides (Jasanoff & Fersht, 1994). Here, it was used to quantitatively determine the helical propensity of apocytochrome c, a largely unordered polypeptide chain at neutral pH and low ionic strength, and the effect of the mutations. As the concentration of TFE was increased, apocytochrome *c* gradually assumed more and more helical in structure, as can be seen by the typical double negative maximum at about 208 and 222 nm in CD spectra of the wild-type apoprotein (Figure 3). An isodichroic point near 203 nm was observed,

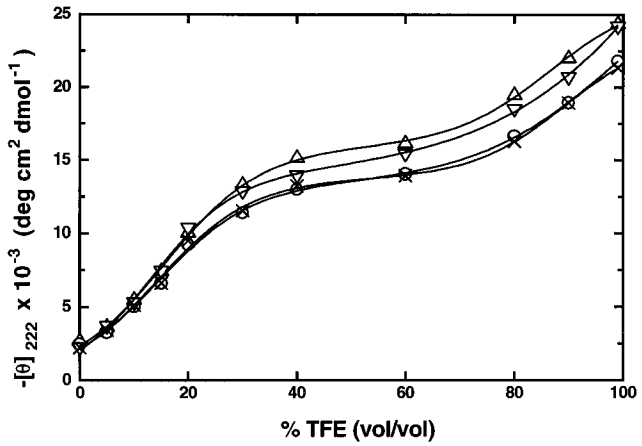

FIGURE 4: TFE titration curves for wild-type chicken apocytochrome *c* (△) and the mutants V92A (○), S103A (▽), and V92A/S103A (×). Fitting curves are derived from the TFE binding model (eq 6).

Table 1: Data of Conformational Transition for Apocytochrome *c* and the Mutants Induced by TFE

	$[\theta]_{222}$[a] in 0% TFE	$[\theta]_{222}$[a] in 60% TFE	$[\theta]_{222}$[a] in 99% TFE	$K_I°$ [b]	m_I[c] [kcal mol^{-1} (% TFE)$^{-1}$]
wild	2640	16000	24300	0.172 ± 0.003	0.066 ± 0.002
S103A	2270	15400	24200	0.173 ± 0.002	0.072 ± 0.005
V92A	2450	14000	21800	0.190 ± 0.002	0.065 ± 0.004
V92AS103A	2250	13900	21300	0.185 ± 0.003	0.072 ± 0.003

[a] The unit for $[\theta]$ is deg cm^2 dmol^{-1}. [b] $K_I°$ is the equilibrium constant for intermediate formation in water. [b] $K_I°$ and [c] m_I were determined from titration curve by fitting to eq 6.

which indicates the presence of just two conformations for each residue: helix and random chain. Due to the presence of some urea from the stock samples (about 100 mM), the spectra are only given to 200 nm. Similar transitions for mutants V92A, S103A, and V92A/S103A were also observed (spectra not shown). The titration curves for them are shown in Figure 4. It was quite unexpected that all the curves are biphasic, suggesting the presence of one stable folding intermediate in the TFE titration process. However, as apocytochrome *c* is a 104-residue-long polypeptide chain, it is reasonable that the single cooperative transition for short peptides is not applicable. Native cytochrome *c* contains three major and two minor helical fragments (Bushnell et al., 1990), amounting to about 43% helix. Since the N-terminal helix is broken by heme ligation to cysteine 14 and 17, the predicted helical content that could be induced for apocytochrome *c* can amount to about 50% and is consistent with the value of $[\theta]_{222}$ (15 000−16 000 deg cm^2 dmol^{-1}, Table 1) of the intermediate at 40−60% TFE. These strong helix-forming fragments could be induced to fold at relatively lower concentration of TFE and form a stable intermediate, while the rest could be further induced to fold when [TFE] was increased further.

Qualitatively, the wild-type and mutant S103A are quite similar in titration behavior. Both of them can obtain more helical structure than mutants V92A and V92A/S103A at the same TFE concentration. If we assume the direct proportionality of $[\theta]_{222}$ to helical content and $[\theta]_{222}$ for 100% helix about −33 000 deg cm^2 dmol^{-1}, the difference (Table 1) means that, at 60% TFE, about four to six more residues are present as helical conformation in the wild type or S103A than in the mutants containing V92A mutation.

To obtain a quantitative measure of the helical propensity of the apoproteins, we tried to estimate the folding equilibrium from the titration curves by assuming that there is one stable folding intermediate for apocytochrome *c* when titrated by TFE. The process is represented by an equilibrium among an unfolded form (U), an intermediate (I), and a folded form (N) as shown below.

$$\mathrm{U} \overset{K_\mathrm{I}}{\longleftrightarrow} \mathrm{I} \overset{K_\mathrm{IN}}{\longleftrightarrow} \mathrm{N} \qquad (1)$$

$$\text{set} \quad K_\mathrm{N} = K_\mathrm{I} K_\mathrm{IN}$$

However, the analysis was complicated by the absence of a linear baseline at low TFE concentration and no saturation near 100% TFE. For the first problem, we assume the direct proportionality of helicity to $[\theta]_{222}$ according to Jasanoff and Fersht (1994). The latter can be accommodated by suitable model of TFE−peptide interaction. There are two models for analyzing TFE−peptide interaction, one is weak-binding model (eq 2), the other is exchange model (eq 3), and the latter one was shown to fit the titration data better than the former by Jasanoff and Fersht (1994).

$$\Delta G^{[\mathrm{TFE}]} = \Delta G^\circ - m[\mathrm{TFE}] \qquad (2)$$

$$\Delta G^{[\mathrm{TFE}]} = \Delta G^\circ - m[\mathrm{TFE}]/[\mathrm{H_2O}] \qquad (3)$$

In our analysis, the exchange model does fit better than the binding model for data of the first transition (0−60% TFE). However, the exchange model could not predict the titration behavior near 100% TFE, where no saturation was observed; while the binding model could. So, binding model was used in our analysis. At any [TFE],

$$[\theta]_{222} = A_\mathrm{I} f_\mathrm{I} + A_\mathrm{N} f_\mathrm{N} = A_\mathrm{I} K_\mathrm{I} f_\mathrm{U} + A_\mathrm{N} K_\mathrm{N} f_\mathrm{U} \qquad (4)$$

$$f_\mathrm{U} + f_\mathrm{I} + f_\mathrm{N} = f_\mathrm{U} + K_\mathrm{I} f_\mathrm{U} + K_\mathrm{N} f_\mathrm{U} = 1 \qquad (5)$$

where f_U, f_I, and f_N are fraction of molecules in U, I, or N form, respectively, and A_I and A_N are constants. So, we can obtain the expression

$$[\theta]_{222} = (A_\mathrm{I} K_\mathrm{I} + A_\mathrm{N} K_\mathrm{N})/(1 + K_\mathrm{I} + K_\mathrm{N}) \qquad (6)$$

and

$$-RT \ln K_\mathrm{I} = \Delta G_\mathrm{I}^{[\mathrm{TFE}]} = \Delta G_\mathrm{I}^\circ - m_\mathrm{I}[\mathrm{TFE}] \qquad (7)$$

$$-RT \ln K_\mathrm{N} = \Delta G_\mathrm{N}^{[\mathrm{TFE}]} = \Delta G_\mathrm{N}^\circ - m_\mathrm{N}[\mathrm{TFE}] \qquad (8)$$

By fitting the titration data to eq 6, we can obtain m_I and K_I°, which estimates the equilibrium constant for intermediate formation in water. The fitting results are given in Table 1. It should be noted that the accurate fitting data for K_N° and m_N could not be obtained because transition of the second phase did not end, so they are not given in Table 1 (the values for K_N° are in the range of 10^{-5}−10^{-6}, and m_N is about 2-fold of m_I). From Table 1, K_I° for V92A or V92A/S103A is larger than that for the wild type or S103A, while the differences in m_I among the four proteins are not significant. According to Jasanoff and Fersht (1994), $m = nRT(K^{\mathrm{helix}} - K^{\mathrm{coil}})$, where n is the number of interacting sites in both helix and coil, K^{helix} and K^{coil} are the binding constants for TFE to the helix and coil states, respectively. As V92A or S103A mutation may not change the value for n, K^{helix}, or K^{coil}, m_I is same for the four proteins. $\Delta\Delta G_\mathrm{I}^\circ$ for V92A mutation is about −0.05 kcal/mol, which means that V92A mutation slightly stabilized the intermediate state, though it decreased the helical content. The value is in the range of that reported by Jasanoff and Fersht (1994) but is much smaller than the difference in transfer free energy between valine and alanine ($\Delta\Delta G = -2.23$ kcal/mol for cyclohexane to water; $\Delta\Delta G = -1.24$ kcal/mol for octanol to water) (Rose & Wolfenden, 1993). This may be because apocytochrome *c* is in largely unfolded state in water.

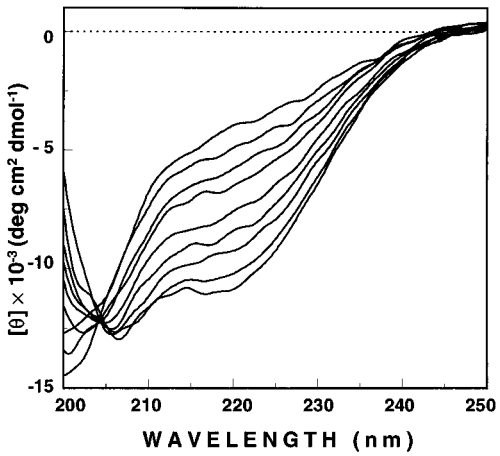

FIGURE 5: CD spectra of the wild-type chicken apocytochrome *c* at different concentrations of $NaClO_4$. From top to bottom, the concentrations of $NaClO_4$ (M) were 0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, and 5.0, respectively. There is an isodichroic point at about 204 nm.

$NaClO_4$ Titration. $NaClO_4$ is a strong reagent to induce A state of proteins at acidic pH (Goto et al., 1990) and horse apocytochrome *c* at neutral pH (Hamada et al., 1993). Here, we carried out $NaClO_4$ titration at pH 7.0 to quantitatively determine the influence of V92A mutation on the A state formation of chicken apocytochrome *c*.

As the concentration of $NaClO_4$ was increased, the CD spectra for apocytochrome *c* showed a transition similar to those in TFE titration except that the extent of change was smaller and the transition was leveled off at about 3 M $NaClO_4$. Figure 5 shows the CD spectra for the wild-type apoprotein (those for the mutants V92A, S103A, and V92A/S103A showed a similar transition). An isodichroic point around 204 nm was observed, which indicates that the transition from C state to A state is also a two-state process (Hamada et al., 1993). These titration curves are given in Figure 6. They are different from those of TFE titration in that they are monophasic because of the different mechanism of interaction. However, the differences among the four proteins for $NaClO_4$ titration showed the same tendency as those for TFE titration. S103A is quite similar to the wild type, while mutants containing V92A mutation show relatively lower contents of helical structure at high concentration of $NaClO_4$, with $[\theta]_{222}$ about 1000 deg cm^2 dmol^{-1} lower, corresponding to about three residues.

To obtain a quantitative assessment of the A state formation of the apoproteins upon $NaClO_4$ titration, analysis of the data by assuming a two-state mechanism and on the basis of the anion binding-induced transition model (Goto et al., 1990; Hamada et al., 1993) was carried out. The model assumes that the free energy of A-state formation is linear

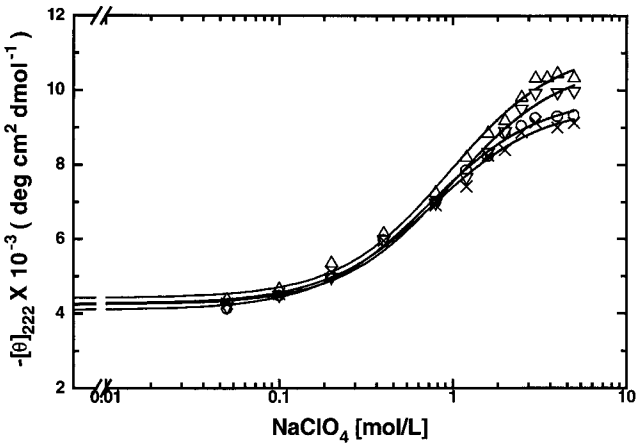

FIGURE 6: $NaClO_4$ titration curves for wild-type chicken apocytochrome *c* (△) and the mutants V92A (○), S103A (▽), and V92A/S103A (×). Fitting curves are derived from the anion binding-induced transition model (eq 9).

Table 2: Data of Conformational Transition for Chicken Apocytochrome *c* and the Mutants Induced by $NaClO_4$

	$[\theta]_{222}$[a] in 0 M $NaClO_4$	$[\theta]_{222}$[a] in 5 M $NaClO_4$	C_m[b] (M)	Δn[c]	K_1[d]
wild	3820	10300	0.73	1.32	0.00018
S103A	3720	9900	0.76	1.37	0.00018
V92A	3790	9300	0.60	1.51	0.00027
V92AS103A	3900	9100	0.62	1.37	0.00029

[a] The unit for $[\theta]$ is deg cm^2 $dmol^{-1}$. [b] C_m is the midpoint concentration of the transition determined by $\Delta G_{app} = 0$. [c] Δn is the difference in the number of anions bound between the A and C states determined by equation $\Delta n = d \ln K_{app}/d \ln [NaClO_4]$. [d] K_1 is the equilibrium constant in the absence of $NaClO_4$ determined by fitting to eqs 9 and 10.

with the logarithm of the concentration of $NaClO_4$, i.e.,

$$\Delta G_{app} = \Delta G_1 - \Delta nRT \ln(1 + K_b[NaClO_4]) \quad (9)$$

where Δn is the difference in the number of anions (ClO_4^-) bound between A and C state, and K_b is the intrinsic binding constant for the Δn sites of A state. $K_1 = \exp(-\Delta G_1/RT)$ is the equilibrium constant in the absence of $NaClO_4$, and the observable equilibrium constant

$$K_{app} = \exp(-\Delta G_{app}/RT) = (\theta - \theta_C)/(\theta_A - \theta) \quad (10)$$

where θ is the observed value of the signal, and θ_C and θ_A are the corresponding values for C and A state, respectively.

The value of Δn can be determined from $\Delta n = d \ln K_{app}/d \ln [NaClO_4]$ by plots of $\ln K_{app}$ against $\ln [NaClO_4]$ for the four proteins, and the fitting results are given in Table 2. The midpoint concentration of the transition (C_m) can also be calculated from the plots by $\ln K_{app} = 0$. It is reasonable that Δn values for the four proteins, averaged 1.39, are the same, because the mutations do not alter the charge of apocytochrome *c*, and the C to A state transition induced by $NaClO_4$ was assumed to arise from binding of ClO_4^- anion to the positively charged residues (Goto et al., 1990). On the other hand, the value of Δn is relatively lower than that obtained by Goto et al. (1990) for cytochrome *c* at acidic pH (about 2.4). This is the result of the difference in pH, since, at neutral pH, the net charge of the protein is much lower, and thus its ability to bind anions is drastically decreased, which was also reflected in the much higher

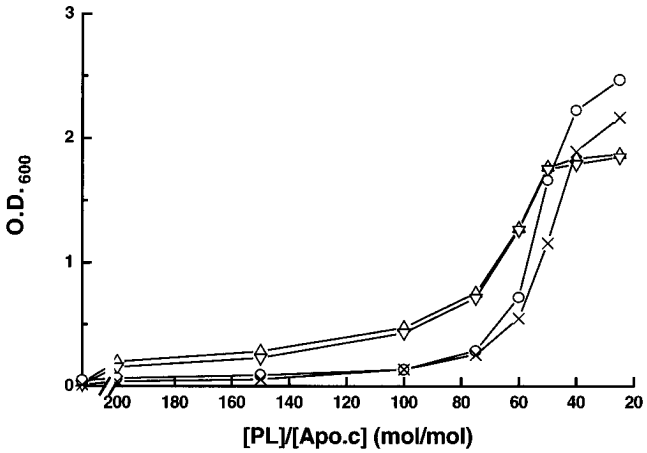

FIGURE 7: Aggregation of soybean phospholipid SUVs after association with the wild-type chicken apocytochrome *c* (△) or the mutants V92A (○), S103A (▽), or V92A/S103A (×) monitored spectrophotometrically at 600 nm.

concentration of $NaClO_4$ required to induce C to A state transition of apocytochrome *c* at neutral pH (Hamada et al., 1993). So, the Δn value of 1.39 was used in the following fittings. In addition, it is reasonable to assume that the value of K_b for the four proteins are essentially the same due to the same reason as stated above. So, we estimated the values of K_b and K_1 that gave fit to all the data for the four proteins by fitting to eq 10 (Figure 6). The value of K_b obtained was 500, which is much lower than that for cytochrome *c* at acidic pH [1400 (Goto et al., 1990)], and is consistent with the above mentioned reason. The values of K_1 obtained for the four proteins are also given in Table 2. K_1 for the wild-type apoprotein and S103A are of the same value about 1.8×10^{-4}, and those for the V92A and V92A/S103A are about 2.8×10^{-4}. These correspond to free energies of 5.17 and 4.91 kcal/mol, respectively, and $\Delta\Delta G°$ for V92A mutation is about −0.26 kcal/mol (stabilizing). The influence of V92A mutation on K_1 is consistent with that on C_m (Table 2). So, similar to the results of TFE titration, V92A mutation slightly stabilized the A state of chicken apocytochrome *c*, though it decreased the helical content.

Conformation after Interaction with Soybean Phospholipid Vesicles. We have previously reported that chicken apocytochrome *c* with different unfolded states assumes different conformation upon interaction with soybean phospholipids and proposed that a more flexible structure of apocytochrome *c* following its insertion into phospholipid bilayer is required for its efficient translocation across the membrane (Yang et al., 1993). Here, we carried out a similar procedure to assess the effect of the mutations on the conformation of chicken apocytochrome *c* following its interaction with soybean phospholipid vesicles, except that the proteins were first dissolved in 8 M urea and 50 mM DTT in order to ensure that they were in the same largely unfolded state before the interaction. Since binding of apocytochrome *c* to the vesicles induced strong vesicle aggregation, we first measured the critical molar ratio of lipid to protein by measuring the turbidity at 600 nm, and the results are given in Figure 7. The wild-type apoprotein and S103A always caused stronger vesicle aggregation than those containing V92A mutation, and the critical molar ratio of lipid to protein was about 100 for the former, while about 80 for the latter. In our following CD experiments, the molar ratio of lipid to protein was kept higher than 100, and titrations with the molar ratio from 100

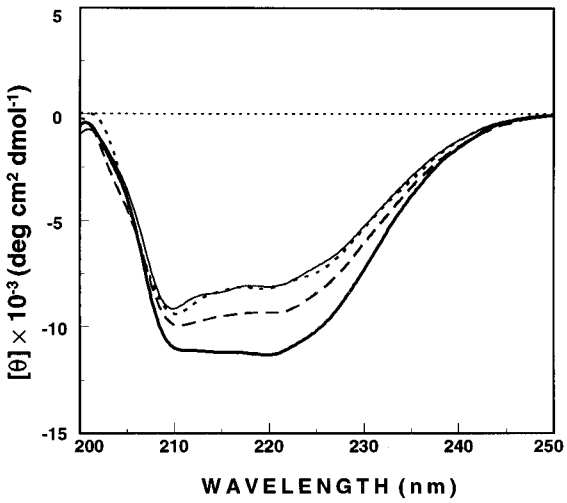

FIGURE 8: CD spectra of the wild-type chicken apocytochrome *c* (—) and the mutants V92A (−), S103A (- - -), and V92A/S103A (···) after association with soybean phospholipid SUVs. The molar ratio of lipid to protein was 150/1.

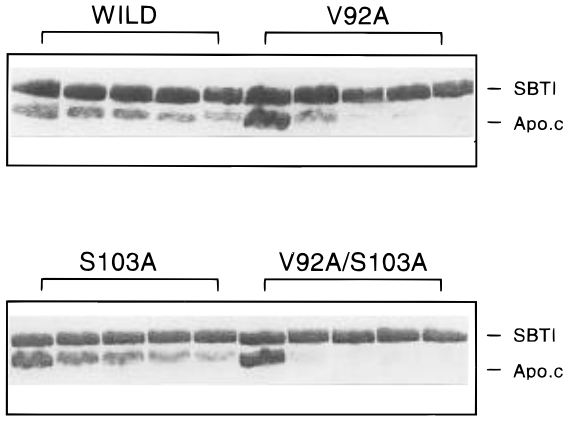

FIGURE 9: Translocation asssay of the wild-type chicken apocytochrome *c* and the mutants V92A, S103A, and V92A/S103A. Samples were drawn at 0, 5, 10, 20, and 30 min (corresponding to the bands from left to right for each protein) after incubation of the proteins with trypsin-enclosed LUVs. SBTI was used to inhibit the residual trypsin activity outside of the LUVs and was also an internal reference in electrophoresis.

to 250 were carried out. To minimize the influence of background scattering on CD measurement, a cuvette with 0.1 mm path length was used. The typical spectra at molar ratio of lipid to protein 150/1 are shown in Figure 8. Association of apocytochrome *c* with lipid vesicles induced a drastic conformational change, from the largely unfolded state to that containing significant secondary structure. Quantitative analysis of the spectra was difficult because the spectra could only be recorded to 200 nm due to the presence of some urea. Qualitatively, as judged from the larger ellipticity around 215 nm, the spectrum for the wild type or S103A seemed to show more features of β-structure than that for V92A and V92A/S103A, which have almost the same spectrum. Besides, this is also consistent with their stronger ability to induce vesicle aggregation, since intermolecular β-structure is generally believed to be the cause of aggregation (Muga et al., 1991). We do not exactly know the cause of the difference between S103A and the wild type, perhaps the stronger aggregation resulted in larger error due to the same reason as in Figure 2. Though greater aggregation may lead to larger effect of adsorption flattening, the ellipticity around 210−220 nm for the wild type and S103A was always higher than that for the mutants containing V92A mutation. So, V92A mutation altered the interaction of apocytochrome *c* with phospholipids to a larger extent.

Translocation Assay. The influence of V92A mutation on the conformation of chicken apocytochrome *c* after interaction with phospholipids could be more clearly revealed by the translocation assay on trypsin-enclosed LUVs. This system was put forward by Rietveld et al. (1986), and we have used it to study the different ability of apocytochrome *c* from different species to be incorporated into the vesicles (Wang & Yang, 1993; Wang et al., 1994). Here, we used it to assess the effects of the mutations on the efficiency of translocation across lipid bilayer of chicken apocytochrome *c*. The difference between S103A and the wild-type apoprotein was not significant, nor was that between V92A/S103A and V92A. But the effect of V92A was quite astonishing. The photograph for electrophoresis of one experiment is given in Figure 9. V92A mutation altered the conformation of chicken apocytochrome *c* after being incorporated into lipid bilayer to such an extent that almost all the proteins added were digested within 10 min of incubation, while significant amounts of the wild type or S103A were still present following 30 min of incubation. The translocation efficiency of V92A or V92A/S103A is similar to that of pigeon or duck apocytochrome *c* (unpublished results). It should be mentioned that the enclosed trypsin is sufficient to digest all the apoproteins added within 2 min, and the externally added SBTI is sufficient to inhibit the total activity of enclosed trypsin.

DISCUSSION

In summary, the major finding of the present study is that valine 92 is the major determinant of the folding propensity of chicken apocytochrome c, and V92A mutation also drastically altered its conformation after being incorporated into lipid bilayer.

Folding of Chicken Apocytochrome c. Folding and unfolding of cytochrome *c* have been extensively studied, while there are far fewer studies on apocytochrome c, a largely unfolded polypeptide chain. The unfolded state and partially unfolded state are the focus of current studies on protein folding (Dill & Shortle, 1991; Dobson, 1992; Shortle, 1993) because the unfolded state of protein was found to possess a significant amount of residual structure, which may serve as initiation site of protein folding, while the partially folded state may be intermediate in protein folding pathway. In fact, study of the folding of apocytochrome *c* is of more physiological value since, *in vivo*, apocytochrome *c* has to cross the outer mitochondrial membrane to access its destination. Its conformation in aqueous solution, at the interface of water and membrane, or after being incorporated into lipid bilayer, is thus of great importance. Chicken apocytochrome c, due to its unique folding property, is quite useful for investigating these conformational processes.

The folding state of apocytochrome *c* and in particular of the chicken apoprotein is highly dynamic and unstable in aqueous solution. We used TFE and $NaClO_4$ titration to measure its potential to form helix structure and the effect of the mutations. It is remarkable that a single amino acid substitution could change the folding property of apocytochrome *c* to such a significant extent, since valine to alanine substitution is generally believed to be conservative. From

the quantitative study, one can conclude that V92A mutation reduced the helical content of the protein that could be induced, while also increasing the equilibrium constant in water for the coil to helix transition or the C state to A state transition (Hamada et al., 1993). Although the mechanism of TFE and $NaClO_4$ induced folding is different (compare eqs 2 and 9)—the former is the result of $K^{helix} > K^{coil}$ (Jasanoff & Fersht, 1994) and the latter is the result of preferential binding of anions to the A state, i.e., Δn (Goto et al., 1990)—V92A mutation did not alter the second item in the expression of ΔG, i.e., m[TFE] in TFE titration (eq 2) or ΔnRT ln $(1 + K_b[NaClO_4])$ in $NaClO_4$ titration (eq 9), but altered the first one, i.e., the free energy in water. Though the absolute value of $\Delta\Delta G°$ for $NaClO_4$ titration is larger than that for TFE titration, which may reflect the difference between A state and the intermediate state in TFE titration, the tendency for V92A mutation is the same.

As valine is a strong β-sheet former while alanine a strong α-helix former (Chou & Fasman, 1978; O'Neil & DeGrado, 1990; Padmanabhan et al., 1990; Kim & Berg, 1993), we should have expected that V92A mutation would enhance the helical structure in chicken apocytochrome *c* induced by TFE or $NaClO_4$. However, the results showed that the helical content was reduced. Since apocytochrome *c* is a 104-residue-long polypeptide, our previous expectation, which may apply to short peptide, may not be suitable here due to some nonlocal interaction, notably the hydrophobic interaction. Hydrophobic interaction is the major force in specifying and stabilizing protein structure and also in stabilizing the protein folding intermediate—"molten globule" state (Goto & Nishokiori, 1991) and the compact hydrophobic collapsed state of apocytochrome *c* [C state (Hamada et al., 1993)]. Though helix propensity is an intrinsic property of an amino acid, stable presence of helix needs the assistance of other forces, such as capping effect or long distance hydrophobic interaction. We know that valine is among the most hydrophobic amino acid residues, while alanine is a weak one (Rose & Wolfenden, 1993), and residue 92 is in the C-terminal helix (87−102) of cytochrome *c*. For chicken apocytochrome c, the stronger hydrophobicity of valine 92 may strengthen the nonlocal intramolecular hydrophobic interaction and enhance its helical propensity. According to the argument of Shortle and Meek (1986) and the results of Flanagan et al. (1993) on SNaseΔ and its mutants, V92A mutation should be a m+ substitution (increase the size of the unfolded state). So, the C state of the wild-type chicken apocytochrome *c* or S103A should be more compact than that of V92A or V92A/S103A. Our recent studies by fluorescence energy transfer support such a point (unpublished results). In case of the prolonged dialysis process, valine 92 may enhance not only the intramolecular hydrophobic interaction but also the intermolecular one, which leads to some aggregation.

Interaction of Chicken Apocytochrome c with Phospholipids. Apocytochrome *c* strongly interacts with negatively charged phospholipids due to its high content of positively charged residues, highly dynamic structure, and high surface activity. The positively charged residues are mainly distributed in the N- and C-terminal helices. Such amphiphilic helix, like the presequence of mitochondrial precursor proteins (Tamm, 1991), can be strongly inserted into negatively charged phospholipid bilayer and lead to exposure of at least part of the molecule to the other side of the membrane, which can then be digested by enclosed trypsin as in the model membrane translocation assay system (Rietveld et al., 1986). In the present paper, we studied and compared the conformation of wild-type chicken apocytochrome *c* and the mutants S103A, V92A, and V92A/S103A after being incorporated into soybean phospholipid bilayer by CD and by the model membrane translocation assay system. Obtained results showed that there is a correlation between the folding propensity of apocytochrome *c* and its conformation in the phospholipid bilayer. V92A mutation was found to drastically influence the interaction of chicken apocytochrome *c* with the membrane. First, V92A mutation decreased the aggregation of vesicles induced by association of apocytochrome *c*. Second, the secondary structure of chicken apocytochrome *c* after being incorporated into lipid bilayer was altered by V92A mutation. From the results, it appears that V92A mutation decreased the content of β-structure, notably the intermolecular one. Finally, the conformational differences were further confirmed by the translocation assay on trypsin-enclosed LUVs.

By using TFE titration to assess the helical propensity of apocytochrome *c* and $NaClO_4$ titration to assess its A state formation, we could not only determine the effect of the mutations on the folding property of chicken apocytochrome *c*, but also relate the helix-forming propensity or the A state-forming propensity to its interaction with phospholipids. Helix is the major conformation of proteins spanning lipid bilayer and is well known to be important in the function of signal sequence or targeting sequence (Tamm, 1991). On the other hand, the "molten globule" state has been implied to be the intermediate in the insertion of protein into lipid bilayer (Bychkova et al., 1988). It has been shown that, upon membrane insertion, transition of colicin A from its native state to a "molten globule" state at the membrane surface occurred (van der Goot et al., 1991). Considering that apocytochrome *c* is in a highly dynamic state at the interface of lipid and water (de Jongh et al., 1992), it is suggested that V92A mutation may influence the "intermediate" state of chicken apocytochrome *c* at the interface, just as its effect on the folding induced by TFE or $NaClO_4$. The initial electrostatic interaction between the positively charged apocytochrome *c* and the negatively charged phospholipids results in the shielding of charge and exposition of the hydrophobic face and hence enhances intermolecular interaction, which leads to vesicle aggregation. With higher hydrophobicity and β-structure tendency, located in the C-terminal helix, valine 92 may greatly strengthen the intermolecular interaction (intermolecular β-structure), as can be seen in the enhanced vesicle aggregation and in the enhanced β-structure feature in the CD spectra, which may attenuate the formation and insertion of C-terminal helix and thus drastically decrease the translocation efficiency of chicken apocytochrome *c* across lipid bilayer. After valine 92 was mutated to alanine, a strong helix-forming residue with weak hydrophobicity, the translocation efficiency was greatly increased.

The C-terminal helix of apocytochrome *c* is conspicuous for its higher hydrophobicity and helix-forming ability compared with the N-terminal one (Jordi et al., 1989; de Jongh & de Kruijff, 1990). The former was shown (de Jongh et al., 1994) to be oriented preferentially perpendicular to the membrane surface, i.e., in the transmembrane configuration, and may act as a loose anchor that is important in

the translocation of apocytochrome *c* across lipid bilayer. Our results appear to be consistent with such a model. Though the C-terminal fragment could not translocate across lipid bilayer itself while the N-terminal one could (Jordi et al., 1989), the former, as an anchor, may facilitate the translocation of the latter, and thus of the whole molecule. We are now proceeding to studying the exact mechanism of the effect of valine 92 and wish to provide more detail about the interaction of apocytochrome *c* with phospholipids. In addition, we are going to extend our study from a model membrane system to mitochondria. The related studies are still in progress.

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