Evaluation of Electrostatic and Hydrophobic Effects on the Interaction of Mitochondrial Signal Sequences with Phospholipid Bilayers†

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ABSTRACT: The information that directs a nuclear-coded protein to be imported into mitochondria resides in an N-terminal extension, called a signal sequence. The primary sequences of all known ones differ. The only common feature is their ability to theoretically form an amphiphilic, positively charged, α-helix. We previously showed that a short stable helical segment was required for a peptide to be functional in import [Wang, Y., & Weiner, H. (1993) *J. Biol. Chem. 268*, 4759–4765]. Here we investigate the interaction of three altered signal sequences with phospholipid membranes containing cardiolipin to ascertain the importance of electrostatic and hydrophobic interactions with the membrane. The three already described peptides were derivatives of the signal sequence from aldehyde dehydrogenase, which is composed of three segments, two helices separated by a linker. ANCN had the C-helix replaced by the N-helix of the signal sequence of cytochrome *c* oxidase subunit IV, ANCC had the C-terminal helix replaced by the C-terminal random coil of cytochrome oxidase subunit IV, and linker deleted had the linker region deleted. ANCC, which functioned poorly as a signal sequence, had a very low affinity for binding to the negatively charged membranes. In contrast, both ANCN and linker deleted showed a relatively high affinity for the membranes and were capable of functioning as a good leader sequence. It appears that linker deleted possessed a stronger hydrophobic effect with membranes while ANCN had a higher electrostatic interaction. On the basic of these studies, a model was proposed to describe the interaction of mitochondrial signal sequences with negatively charged phospholipid membranes involving electrostatic interaction for initial binding and hydrophobic interaction for insertion. If the peptide does not have the ability to form a stable helix, it will not be inserted into the membrane. Though not proven, it is tempting to postulate that there is an initial interaction between the signal sequence and the mitochondrial membrane prior to binding to the protein translocation apparatus.

Mitochondrial protein import is directed by an extension of amino acids located at the N-terminal end of proteins that are coded by nuclear genes. The various known mitochondrial signal sequences share very low sequence homology. The only common feature found so far among all signal sequences is that they have the potential to form positively charged amphiphilic secondary structures (von Heijne, 1986; Epand *et al.*, 1986; Roise *et al.*, 1986, 1988; Endo *et al.*, 1989; Karslake *et al.*, 1990; Bruch & Hoyt, 1992, Thornton *et al.*, 1993; Hammen *et al.*, 1994). It has been demonstrated that this amphiphilic secondary structure is directly related to the targeting function of the signal sequence (Roise *et al.*, 1988; Bedwell *et al.*, 1989; Lemire *et al.*, 1989; Wang & Weiner, 1993). Since the signal sequence contains all the information necessary for mitochondrial protein import (Horwich *et al.*, 1985), this peptide must interact with the mitochondria. Import receptors and other protein components of the important apparatus in the mitochondrial membranes have been identified (Söllner *et al.*, 1992; Maarse *et al.*, 1992; Horst *et al.*, 1993; Ramage *et al.*, 1993; Kassenbrock *et al.*, 1993; Emtage & Jensen, 1993; Kiebler *et al.*, 1993; Moczko *et al.*, 1993). It has been found that MOM22, one of the components of import receptor complex, has a region rich in negatively charged amino acid residues (Kiebler *et al.*, 1993). This region is speculated to be responsible for binding of the receptor to positive charged signal sequence.

It is reasonable to assume, however, that the precursor proteins could bind to the mitochondria membranes at an early stage of the import process. Because of the positively charged amphiphilic property, signal sequences are well-suited for interaction with mitochondrial membranes. Studies with a variety of signal sequences showed that they are highly surface-active and they can bind to lipid bilayers containing negatively charged lipids (Tamm, 1986; Roise *et al.*, 1986; Skerjac *et al.*, 1987; Jordi *et al.*, 1989; Frey & Tamm, 1990; Hoyt *et al.*, 1991; de Kroon *et al.*, 1991; Roise, 1992; Swansom & Roise, 1992; Zardeneta & Horwitz, 1992).

Recently, 2D NMR techniques have been employed to study the conformation of mitochondrial leader peptides in a micelluar environment (Endo *et al.*, 1989; Karslake *et al.*, 1990; Bruch & Hoyt, 1992; Thornton *et al.*, 1993; Hammen *et al.*, 1994). It was shown that the peptide from rat liver aldehyde dehydrogenase contained an N-Helix and a C-helix separated by a flexible linker region (Karslake *et al.*, 1990). Different from this helix–linker–helix conformation, the signal sequence of yeast cytochrome *c* oxidase subunit IV (COX IV)[1] had only one helix, located at its N-termini (Endo *et al.*,

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[1] Abbreviations: ALDH, aldehyde dehydrogenase; COX IV, cytochrome *c* oxidase subunit IV; linker deleted, linker region (RGP) deleted from the ALDH signal sequence; CD, circular dichroism; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine, CL, cardiolipin; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle.

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1989). We previously reported that two chimeric peptides, ANCN and ANCC, displayed different efficiencies for leading ALDH protein into mitochondria (Wang & Weiner, 1993). ANCN, containing the N-terminal helix of the ALDH leader peptide, the linker, and the N-terminal helix of COX IV, had a similar import efficiency as did the native ALDH signal sequence. ANCC, in which the C-terminal helix was replaced by the random coil of COX IV C-terminal portion, showed weaker import capability. We also showed that the linker deleted ALDH signal sequence, which formed a long helix, could lead import of ALDH protein into mitochondria as well as did the native ALDH signal sequence, although the linker deleted signal sequence could no longer be proteolytically processed after import (Thornton *et al.*, 1993). It was not clear at which step of the import process did the inhibition of ANCC import occur. It is possible that ANCC could not bind well to phospholipids of mitochondrial membranes since forming a helical conformation is required for this binding (Roise *et al.*, 1986; Eilers *et al.*, 1989).

In order to further understand the functional difference among the three signal sequences mentioned above, ANCN, ANCC, and linker deleted, we studied the interaction of the peptides with lipid bilayers which is assumed to be the initial step for precursor proteins to interact with mitochondria. We present data to show that failure to interact with the lipid bilayer accounts for, at least partially, the poor import ability of ANCC and propose a mechanism to explain the poor binding.

MATERIALS AND METHODS

Peptide Synthesis and Purification. Peptides were synthesized by the Purdue University Macromolecular Structure Laboratory. The purification was carried out by using a Vydac semipreparative C_{18} reverse phase HPLC column monitored at 215 nm. Typically, 0.5–1.5 mg of peptide was injected for each run. The peptide was eluted with a nonlinear gradient of water–acetonitrile with a flow rate of 2 mL/min. The purity of the peptides was verified by amino acid sequence, mass spectroscopy (performed in the Biochemistry Department of Purdue University), and amino acid analysis (performed in the Chemistry Department of Purdue University).

Determination of Peptide Concentration. The concentrations of peptides were determined from their molar extinction coefficients at 272 nm. These parameters were obtained by quantitative amino acid analysis of the peptides and are 4000 $(\text{cm M})^{-1}$ for ANCN, 1970 $(\text{cm M})^{-1}$ for ANCC, and 1340 $(\text{cm M})^{-1}$ for linker deleted.

Vesicle Preparation. Small unilamellar vesicles were prepared by mixing phosphatidylcholine (Avanti) and cardiolipin (Sigma), which were separately dissolved in a 16 mL glass test tube in chloroform–methanol (3:1), and then mixed. Nitrogen gas was blown into the tube containing about 0.5 mL of the lipid mixture to evaporate the solvent. The tube was strongly shaken with a vortex mixer to evenly dispense the lipids onto the wall of the glass tube. Then, 0.5–1.5 mL of 20 mM phosphate buffer (pH 7.3) containing 0.5 mM EDTA was added. The tube was filled with nitrogen gas and sealed with several layers of parafilm, and the lipid was resuspended by vortexing. This lipid solution was then sonicated in a water bath sonicator until the lipid suspension became clear (5–30 min). Large unilamellar vesicles were made according to the freeze–thaw/extrusion method (Hope *et al.*, 1985). Briefly, the SUVs made as above were freeze–thawed 2–5 times in a dry ice–acetone bath and then extruded 5–10 times through two layers of 0.22 μm pore polycarbonate filters under nitrogen pressure until the suspension became clear.

Measurement of the Steady-State Fluorescence of the Tyrosine Residue of the Peptide. The measurements were performed on a Hitachi F-2000 fluorescence spectrophotometer at 25 °C. The bandwidths of both excitation and emission were 10 nm. Data were collected and transferred to an IBM personal computer to be analyzed on the Spectra Calc program (Galactic Corp.). For measuring of the binding of peptides to vesicles, excitation and emission wavelengths were set at 272 and 304 nm, respectively. SUV stock solution, at a phospholipid concentration of 10 mg/mL, was added to the cuvette containing 2 mL of 10 mM sodium phosphate, pH 7.3. Peptide concentrations were 0.5–5 μM, depending on the phospholipid composition of vesicles. To allow equilibrium to be reached, the solution was constantly stirred for 3 min before starting to record data. Fluorescence intensity was recorded for two 5 s intervals and averaged. Each experiment was repeated at least 3 times.

Vesicle Disruption Experiment. LUVs were prepared as above except that 100 mM carboxyfluorescein (Molecular Probe) was included in the 10 mM sodium phosphate, pH 7.3. These LUVs were passed through a Sephadex G-25 column equilibrated with buffer containing 100 mM NaCl to separate untrapped dye from the vesicles. Peptide-induced dye leakage from the vesicles as a function of time was measured by recording the fluorescence increase at 519 nm upon excitation at 470 nm (Hoyt *et al.*, 1991). NaCl (100 mM) was included in the phosphate buffer.

Measurement of Fluorescence Polarization. Measurements were obtained on an SLM 8000 fluorescence spectrophotometer at 25 °C in 10 mM sodium phosphate, pH 7.3. Bandwidths of excitation and emission were 2 and 4 nm, respectively. The excitation wavelength was 272 nm; the emission wavelength was 304 nm. Samples were continuously stirred. Vesicles in the absence of peptide were used as a control. Polarization (P) was calculated as

$$P = [I_{0/0} - I_{0/90}(I_{90/0}/I_{90/90})]/[I_{0/0} + I_{0/90}(I_{90/0}/I_{90/90})]$$

where I is the fluorescence intensity measured when the relative rotational degrees between excitation and emission polarizers were set as indicated by the subscripts.

Circular Dichroism. Spectra were recorded at room temperature in 10 mM sodium phosphate, pH 7.3, on a Jasco-600 CD/ORD instrument interfaced with an IBM PS/2 computer. The cell path length was 0.1 cm. Vesicles in the absence of peptide were used for base-line corrections.

Stopped Flow Measurement. The experiment was performed at the Indiana University School of Medicine on a HiTech Scientific SF-51 UV absorption/fluorescence spectrophotometer with the instrument set in the fluorescence mode. The instrument was coupled to a stopped flow mixing chamber which had a deadtime of 2 ms. Both injectors were charged with 1.5 mL of solution, one containing peptide and the other containing vesicles. Excitation was of 272 nm, and the window of the emission wavelength was from 300 nm to 320 nm. Experiments were performed in 10 mM sodium phosphate, pH 7.3, at 25 °C.

RESULTS

Fluorescence Enhancement To Assess Binding. The three peptide analogs of the ALDH leader peptide to be employed in this study contained a tyrosine residue at the position two amino acids from their C-terminal ends (Figure 1). The

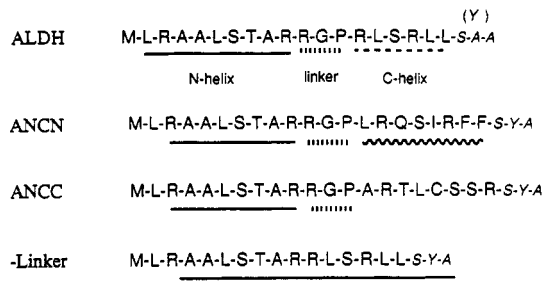

FIGURE 1: Amino acid sequences of ANCN, ANCC, linker delted (–Linker), and native ALDH signal sequences. AN, the N-terminal portion of the ALDH signal sequences; CN, the N-terminal portion of the COX IV signal sequence; CC, the C-terminal portion of the COX IV signal sequence. Amino acids in italics are from the mature part of ALDH where the tyrosine is an alanine.

tyrosine fluorescence spectra of the three peptides recorded in aqueous solution were quite similar, with emission peaks at 304 nm when excited at 272 nm. When vesicles containing cardiolipin, which has a negatively charged head group, were added to the peptide solution, the fluorescence intensity of ANCN and of linker deleted was greatly enhanced, indicating that both peptides interacted with the lipid bilayers (Figure 2). In contrast, little change could be observed in the tyrosine fluorescence spectrum of the ANCC peptide in the presence of the vesicles. The spectrum of *N*-acetyltyrosine amide also did not change in the presence of the vesicles. Because the tyrosine group in the peptide was located at the C-terminal end, it was possible that the ANCC peptide actually interacted with the vesicles through its N-terminal part without involving the tyrosine residue. If this were the case, little fluorescence change would be obtained. In order to test for this possibility, the fluorescence polarization technique was used to examine the binding of the ANCN and ANCC peptides to vesicles. Since fluorescence polarization depends upon the overall rate of rotational motion of the fluorescent chromophore, the binding of a peptide to a vesicle which has a slow rotational rate, due to its large size, should increase the fluorescence polarization of the peptide. Results presented in Figure 3 show that the fluorescence polarization of ANCN gradually increased with increasing concentration of negatively charged vesicles. Fluorescence polarization of ANCN barely changed in the presence of vesicles made from pure PC, indicating that the change of the polarization indeed resulted from the binding of ANCN to the negatively charged membrane. In contrast, the fluorescence polarization of ANCC barely increased in the presence of the negatively charged vesicles. The results of fluorescence polarization experiments also indicate that ANCC had a weaker binding affinity to the membranes than did ANCN.

To further verify that the differences between ANCN and ANCC found in the fluorescence experiments were caused by their different affinity to phospholipid bilayers, vesicle disruption experiments were performed. Figure 4 presents the time course for the leakage of carboxyfluorescein dye from vesicles after adding peptides. In this experiment, the dye at a concentration of 100 mM was initially enclosed in the vesicles. The fluorescence of the dye was quenched at this concentration. The increase of carboxyfluorescein fluorescence upon adding the peptides indicated that an interaction of the peptides with the vesicles occurred which caused leakage of the dye from the vesicles to bulk solution. It is shown in Figure 4 that at the same concentration of peptides, ANCC caused less leakage of the fluorescence dye compared to that caused by ANCN, indicating that ANCC can perturb lipid bilayer less effectively than can ANCN. Combining all these results together, it can be concluded that the smaller increase of the tyrosine fluorescence intensity of the ANCC peptide (Figure 2) reflected its low affinity to membranes compared with those of ANCN and linker deleted.

Partition Constant for ANCN and Linker Deleted. The enhancement of the tyrosine fluorescence intensity of ANCN and linker deleted upon binding to vesicles (Figure 2) provides a sensitive way to measure incorporation of the peptides into lipid bilayers. The fluorescence intensity of the peptides measured as a function of lipid concentration can be used to fit a partition model which has been shown to be an excellent model for interpreting incorporation of the isotherm of small peptides into lipid bilayers (Frey & Tamm, 1990; Roise, 1992). The partition constant, K_p, which is defined as

$$K_p = [P_b]/[P_f][L] \quad (1)$$

can be calculated by this method. Here $[P_b]$ and $[P_f]$ are the molar concentrations of bound and free peptides, respectively, and [L] is the lipid concentration in moles per liter. The fluorescence intensity of a solution containing the peptides is

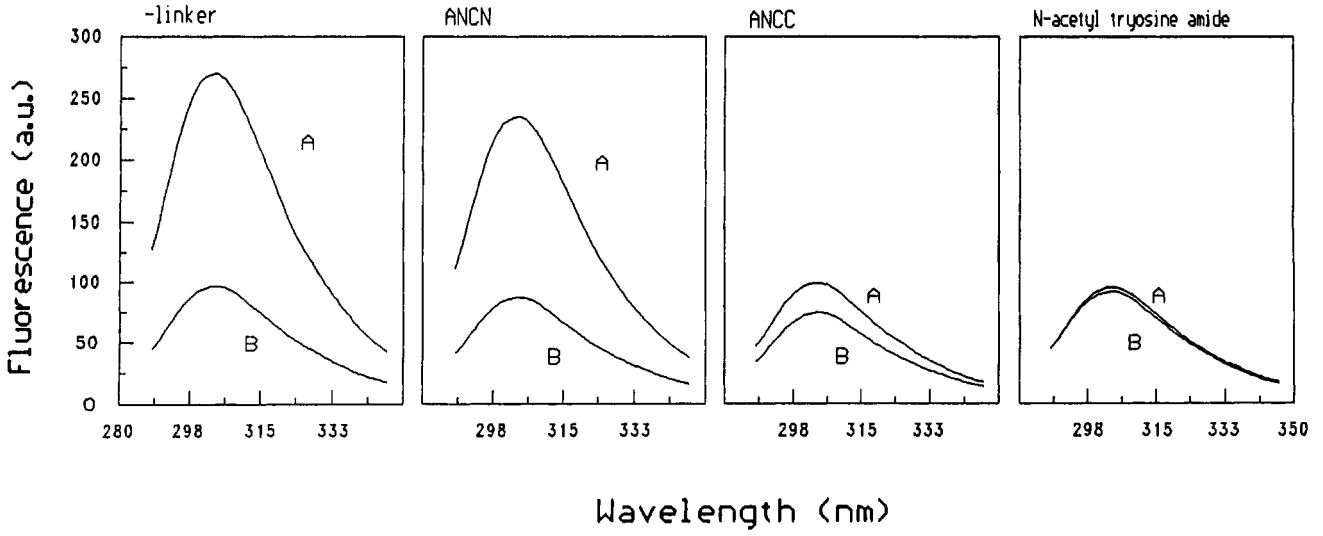

FIGURE 2: Tyrosine fluorescence spectra of the signal sequences. The concentration of the peptides was 8 μM for linker deleted (–linker), 5 μM for ANCN, 20 μm for ANCC, and 7 μM for *N*-acetyltyrosine amide. A, with PC vesicles containing 15% (mole) CL (60 μM lipid); B, without any vesicles. Fluorescence intensity presented in arbitrary units.

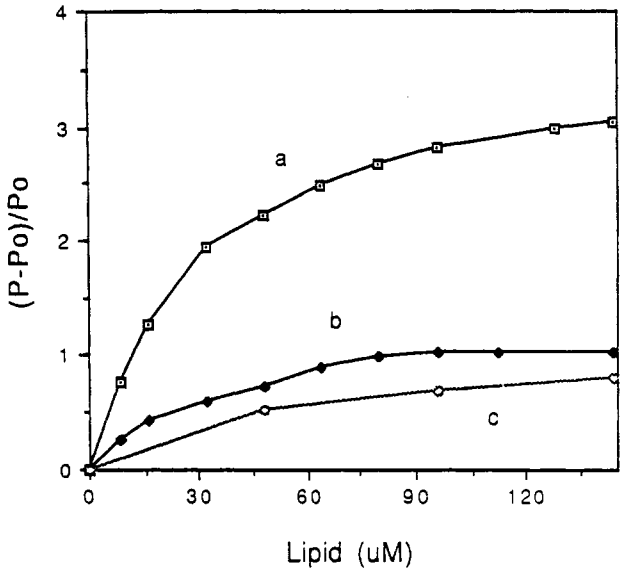

FIGURE 3: Change of fluorescence polarization of ANCN and ANCC as a function of lipid concentration. P_0, polarization of the peptides in the absence of vesicles, was 0.089 for ANCN and 0.090 for ANNC. P, polarization of the peptides in the presence of vesicles. The concentration of the peptides was 8.5 μM for all measurements. a, ANCN in the presence of PC vesicles containing 8% (mole) CL; b, ANCC in the presence of PC vesicles containing 8% (mole) CL; c, ANCN in the presence of PC vesicles containing no CL.

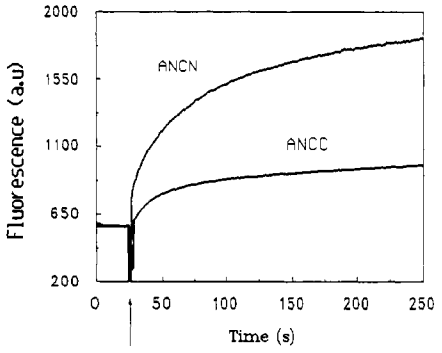

FIGURE 4: Time course of carboxyfluorescein fluorescence increase from vesicles after adding peptides. Peptides were added at the time indicated by the arrow at a final concentration of 1.5 μM. LUVs were composed of PC and CL at a ratio of PC:CL = 85:15 (mol/mol).

given by

$$F = [F_f[P_f]/([P_f] + [P_b])] + [F_b[P_b]/([P_f] + [P_b])] \quad (2)$$

where F is the measured fluorescence intensity, F_f is the fluorescence intensity of the peptide in the absence of lipid membrane, and F_b is the fluorescence intensity of membrane-bound peptide. Combining eqs 1 and 2 leads to

$$F = (F_f + F_bK_p[L])/(1 + K_p[L]) \quad (3)$$

Fluorescence Corrections. A correct estimation of F_b is required for the determination of the partition constant, K_p. Unfortunately, it was noted that the presence of vesicles could reduce transmitted light. A commonly used control, vesicles in the absence of peptides, can only correct the fluorescence background caused by light scattering. If the binding of peptides induced aggregation or fusion of vesicles, the error caused by the transmitted light reduction could be even more serious. This possibility was indicated by the observation that the fluorescence intensity of 10 μM peptide started to decrease when the ratio of lipid to peptide reached a value of approximately 20 for vesicles containing 20% (mole) CL. To correct for this error, *N*-acetyltyrosine amide at the same concentration as that of the peptide was used as control. It was possible to correct the fluorescence decrease caused by transmitted light reduction and the background caused by light scattering using this control only if the binding of the peptides did not cause vesicle fusion or aggregation. To prevent aggregation or fusion resulting from the binding of the peptides, lipid vesicles containing less than 20% (mole) cardiolipin, which consequentially had relative low affinity to peptide, were used for the titration. Using low concentrations of peptide, 1–5 μM, for the titration can also reduce the fusion/aggregation of vesicles. To detect possible vesicle fusion/aggregation during the titration, the change of the size of the vesicles was measured by setting both the emission and the excitation wavelengths at 350 nm immediately after each fluorescence data point was collected. The light intensity of 350 nm/350 nm is proportional to the size of the vesicle (Gibson & Strauss, 1984). The values obtained from the peptide–vesicle solution with the 350 nm/350 nm setting were compared to data from vesicle solutions in the absence of peptides. An increase of the ratio was considered to be caused by the fusion/aggregation of the vesicles. The titration was terminated when the ratio value increased by 10%. The titration data thus obtained for phospholipid vesicles with different composition were fitted to eq 3 to obtain F_b and K_p for the two peptides.

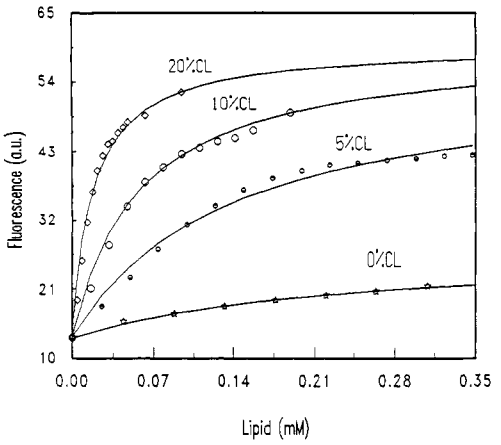

FIGURE 5: Titration of linker deleted tyrosine fluorescence intensity by PC vesicles containing different amounts of CL. Solid lines represent the best fit of fluorescence titration data to eq 3 using $F_b = 4.9F_f$. Light scattering was also recorded for each titration by setting both the excitation and the emission wavelength at 350 nm. Only the data with a light scattering increase smaller than 10% when comparing vesicle solutions in the presence and absence of peptide were used. The correlation coefficient was greater than 0.98 for all the curves.

Calculation of the Partition Constant. The calculated F_b values varied as a function of the amount of cardiolipin in the vesicles. For linker deleted binding to PC vesicles containing 0%, 5%, 10%, and 20% (mole) CL, they were 3.7 ± 0.5, 4.1 ± 0.8, 4.8 ± 0.4, and 4.9 ± 0.4 times the F_f value, respectively. Since the binding of peptide was very weak to vesicles containing 0 and 5% (mole) CL, it was necessary to add high concentrations of vesicles to obtain a fluorescence increase with a sufficient signal/noise ratio. This could have caused an artifact in the fluorescence measurement. In order to obtain a more accurate K_p value, the experimental data were refitted to eq 3 using an F_b value of the peptide bound to PC vesicles containing 20% (mole) CL. The fitted line and experimental data are shown in Figure 5. A similar treatment was also applied to the ANCN peptide. The partition constants for

Table 1: Partition Constants of ANCN and Linker Deleted to PC Vesicles Containing Various Amounts of CL

	$K_p \times 10^3$ (M^{-1})			
	ANCN		linker deleted	
CL% (mole)	buffer[a]	0.3 M NaCl	buffer[a]	0.3 M NaCl
0			0.7 ± 0.3	1.4 ± 0.3
2.5	0.43 ● 0.05			
5	5 ± 0.5		9.1 ± 2.9	3.0 ± 0.9
10	11 ± 3	0.8 ± 0.3	18 ± 3	3.9 ± 1.1
20	87 ± 23	1.8 ± 0.6	61 ± 3	17 ± 5

[a] 10 mM sodium phosphate, pH 7.3.

the two signal sequences thus obtained are summarized in Table 1.

Electrostatic and Hydrophobic Effect. As expected, both peptides had an increased affinity to membranes which contained higher amounts of CL, confirming the fact that an electrostatic interaction is important for the binding of mitochondrial signal sequences or other positively charged amphiphilic peptides to the lipid bilayer. By comparing the partition constants of the two peptides bound to different membranes, it was noted that the affinity of ANCN to the vesicles containing 20% CL was higher than that of linker deleted. Its binding, however, was more sensitive to the changes in cardiolipin in the vesicles. The higher sensitivity of ANCN to the surface charge of the lipid bilayers suggests that electrostatic forces play a more important role for its binding than they possibly do for the binding of linker deleted. This could be related to the fact that ANCN has five arginines while linker deleted has four. The partition constant of ANCN to membranes containing no cardiolipin was too low to be measured with any accuracy, while to this membrane the partition constant of linker deleted peptide was determined to be $(7 \pm 3) \times 10^2$ M^{-1}. For the binding of an amphiphilic peptide to a membrane containing no charged phospholipid, the hydrophobic effect should be the major driving force. Therefore, the higher affinity of the linker deleted peptide for CL-free vesicles suggested that this peptide possessed a stronger hydrophobic interaction with lipid bilayers than did ANCN. To further test for this possibility, the partition constants of the two peptides were measured in the presence of a high concentration of salt. Salt usually reduces electrostatic interactions and enhances hydrophobic effects. It was found that the partition constants for ANCN showed a pronounced decrease in the presence of 300 mM NaCl compared to that of linker deleted. To the vesicles containing no CL, where the binding is dominated by hydrophobic effects, linker deleted showed an increased partition constant in the presence of the salt, indicating that the loss of electrostatic force in the presence of salt had been overcome by the increase of hydrophobic effect generated by the salt.

Kinetics of Binding. The kinetics of the binding of ANCN were studied by measuring the time course of the fluorescence intensity change using a stopped flow mixing chamber coupled to a fluorescence instrument. Upon mixing the ANCN peptide and vesicles containing 10% CL (mole), the voltage on the photomultiplier decreased exponentially with time (Figure 6), indicating increased fluorescence. Equilibrium was reached in 10–15 s. Assuming reversible binding and that the lipid concentration was higher than that of the peptide, the initial rate constant for association of the peptide to the membrane can be estimated to be 1.3×10^5 s M^{-1} by fitting the experimental data to the first-order reaction kinetic equation (Fersht, 1985). The rate constant for dissociation was calculated to be 7 s^{-1}.

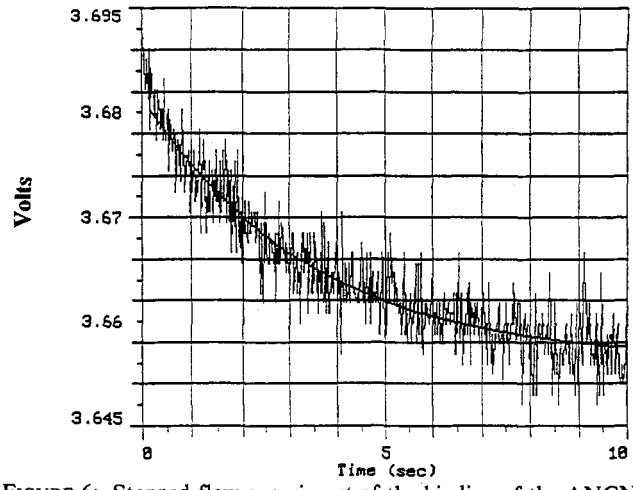

FIGURE 6: Stopped-flow experiment of the binding of the ANCN peptide to vesicles containing 10% (mole) CL. The final concentrations of the vesicle and the peptide were 50 and 4 μM, respectively.

DISCUSSION

As with the natural signal sequence of liver aldehyde dehydrogenase, both ANCN and the linker deleted analog of native ALDH functioned well in their ability to direct the import of ALDH into mitochondria. In contrast, ANCC showed a much lower import efficiency (Wang & Weiner, 1993). The three peptides were shown to have distinctive structural features when they were in a membrane-mimicking environment (Wang & Weiner, 1993; Thornton *et al.*, 1993). ANCN, like the ALDH signal sequence peptide, formed a helix–linker–helix conformation. Linker deleted contained a long helix, spanning almost the entire length of the peptide. ANCC had a very low helical content. These results coupled with other studies lead us to suggest that a short stable N-terminal helix is important for a signal sequence to function in mitochondrial protein import (Wang & Weiner, 1993). Here we presented data to show that the three peptides have different binding affinities to negatively charged phospholipid membranes. ANCC, which poorly directed import, had a very low affinity for the membranes based upon steady-state and fluorescence polarization data. In contrast, both ANCN and linker deleted bound to the liposome containing CL. The apparent correlation between membrane binding affinity and import efficiency of the three signal sequences indicates that initial binding to mitochondrial membrane could be an essential step for the signal peptide to direct protein import into mitochondria.

It was not possible to determine directly why ANCC did not bind well to the membrane. However, by studying the factors which contributed to the binding of ANCN and linker deleted peptide, it was possible to conclude that the poor binding of ANCC most likely was related to its inability to form a stable amphiphilic helix.

Electrostatic and hydrophobic effects are the major forces that allow binding of the amphiphilic mitochondrial signal sequence (P) to a negatively charged membrane (M). These two effects may act sequentially, for all studies show the peptides are void of structure when in solution. If this is the case, the binding can be described by a two step model:

$$P + M \underset{}{\overset{k_1}{\rightleftharpoons}} P^*M \overset{k_2}{\rightleftharpoons} PM$$

In this model, the first step is the absorption of the signal sequence onto the surface of the membrane, driven mainly by an electrostatic force. The concentration of peptides directly

adjacent to the membrane would be greatly increased by the electrostatic interaction. The kinetics of the first step can be considered as a diffusion-limited process. Therefore, the rate constant, k_1, can be estimated by the Smoluchowski–Debye theory (Riggs *et al.*, 1970) which gives the rate constant of a diffusion-limited reaction between two charged particles, *a* and *b*, as

$$k_1 = 4\pi N R_{ab}(D_a + D_b)E_r/(\exp E_r - 1) \quad (4)$$

where N is Avogadro's number, R_{ab} is the reaction radius of the two particles, D_a and D_b are the diffusion constants for *a* and *b*, respectively, and E_r is the ratio of the electrostatic energy between two particles at a distance R_{ab} to the thermal energy K_BT where T is the absolute temperature and K_B is Boltzmann's constant. In this model, the negatively charged vesicle cannot be considered as a point charge. Therefore, the electrostatic energy, E_e, which was described by Coulomb's law in eq 4, is replaced by

$$E_e = -z_p e\Phi_0 \quad (5)$$

where z_p is the number of charges on the signal sequence, e is the electronic charge, and Φ_0 is the surface potential of the membrane. Φ_0 can be described by the Gouy–Chapman theory which gives the relation between the surface potential and the distance from a charged surface in solution (Jones, 1975; McLaughlin, 1977). When the peptide is immediately adjacent to the surface of the membrane, the distance can be considered to be zero. This leads to

$$\sinh(e\Phi_0/2K_BT) = \sigma/(8N\epsilon_0\epsilon_r K_B T c_i)^{1/2} \quad (6)$$

where σ is the surface charge density, ϵ_0 is the permittivity of vacuum, ϵ_r is the dielectric constant of water, and c_i is the concentration of electrolyte in bulk solution. The σ term can be calculated by

$$\sigma = e_t/A \quad (7)$$

where e_t is the total net charge of the membrane and A is the total surface area of the membrane. Combining eqs 4–7, assuming that the average diameter of an SUV is 300 Å (Qiu *et al.*, 1993), the head group area of cardiolipin is 140 Å^2, which is twice that of PC (Sargent *et al.*, 1988), and the diffusion constant of the peptide, D_a, is 10^{-6} cm^2/s (Riggs *et al.*, 1970), which is much longer than the diffusion constant of the vesicle, then the rate constant of this step is estimated to be 6.6×10^7 M^{-1} s^{-1}.

The second step of the reaction in this model, the insertion of the hydrophobic face of the peptide into the lipid bilayers, should be mainly driven by hydrophobic effects. It appears that this step (k_2) can be the rate-limiting step because the observed reaction constant estimated from the stopped flow experiment described in Figure 6 is less than 5×10^5 M^{-1} s^{-1}, which is much smaller than that calculated for step 1.

The reaction kinetics and thermodynamics of step 1 for the three peptides should be similar to each other because the simple electrostatic interaction between the membrane and peptides, which is the major driving force for step 1, is independent of the conformation of the peptide. The Gouy–Chapman theory (Jones, 1975; McLaughlin, 1977) can be used to describe step 1 for the evaluation of the concentration of peptides adjacent to the surface of a membrane, [P_s], which should obey the Boltzmann relation (Tamm, 1990; Swanson & Roise, 1992):

$$[P_s] = [P_f] \exp(-z_p e\Phi_0/K_BT) \quad (8)$$

where [P_f] is the concentration of free peptides. The other symbols have the same meaning as before. In the application of this theory to the peptide binding model, peptides are treated as point changes. No conformational property of the peptide is included in the analysis. If this treatment is valid, it implies that forming the α-helical structure is not necessary for step 1. The results obtained for the binding of the COX IV signal sequence to membranes showed a good agreement between experimental data and this theoretical analysis (Swanson & Roise, 1992; Frey & Tamm, 1990). A similar result was obtained here when using the same approach to analyze the partition constants, summarized in Table 1. Figure 7 shows that the observed partition constant, K_p, is related to the surface charge density. This relationship can be described by Gouy–Chapman theory, indicated by the excellent fit of the experimental data to eq 9 (see legend to Figure 7). The best-fit analysis gave values of the intrinsic partition constant, K_p', which is the partition constant of peptides to membranes with zero surface potential, of 300 ± 80 M^{-1} for linker deleted and 25 ± 10 M^{-1} for ANCN. The effective charges of the two peptides were reduced in the best-fit analysis to 1.4 and 2.6 for linker deleted and ANCN, respectively. A similar decrease in net charge has also been observed for the COX IV signal sequence (Swanson & Roise, 1992; Frey & Tamm, 1990).

On the basis of the above analysis, it appears that forming amphiphilic α-helices is required only for the second step of the reaction, the insertion of the peptide into the acyl chain region of the lipid bilayer. Hydrogen exchange experiments performed in a solution containing micelles showed that the exchange of amide protons from the residues on the hydrophobic face of the linker deleted peptide was protected, implying that those residues were indeed inserted into micelles (Thornton *et al.*, 1993). Studies employing different techniques also indicated that the mitochondrial signal sequences were oriented parallel to the surface of the lipid bilayer (Frey & Tamm, 1990; Hoyt *et al.*, 1991). This suggests that the hydrophobic face of the amphiphilic helix is buried in the membrane. By insertion of the hydrophobic face of an α-helix into the membrane, the hydrophobic effect can be greatly enhanced. The ANCC signal sequence may fail to form a hydrophobic face due to its low α-helical propensity; this would make it difficult for the peptide to be inserted into the acyl region of the membrane. For ANCN, preliminary NMR studies (data not shown) indicate that it forms two helices separated by the linker, similar to that of the ALDH signal sequence. The mean residue hydrophobic moments (Eisenberg *et al.*, 1984a,b) of the AN-helix and the CN-helix are 0.64 and 0.68, respectively, which are almost identical to that of the linker deleted value of 0.67. However, in 50% trifluoroethanol, ANCN peptides showed a slightly lower helical content than did linker deleted, as judged by their ellipticity at 222 nm (Wang & Weiner, 1993; Thornton *et al.*, 1993), suggesting that ANCN could have a lower helix content when bound to membranes. This could decrease the peptide's ability to insert into the membrane. The lower hydrophobic interaction displayed by ANCN supports the observation that the relative phase of the two helices in the helix–linker–helix conformation shifted from each other. This was initially indicated by NMR experiments which showed that ALDH and linker deleted had different hydrogen exchange patterns upon binding to micelles (Karslake *et al.*, 1990; Thornton *et al.*, 1993).

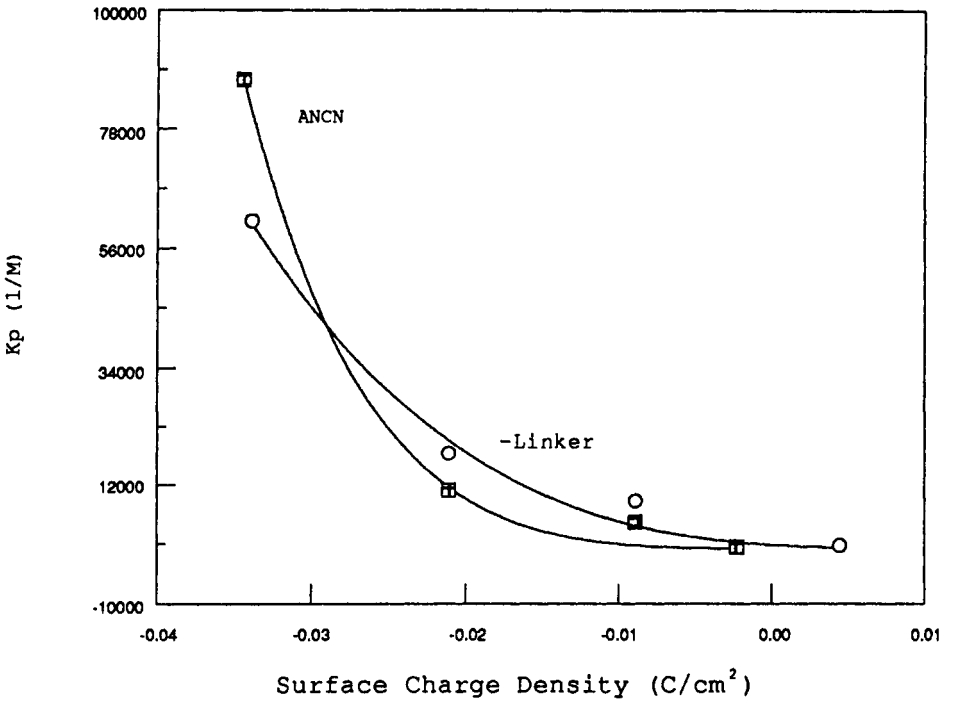

FIGURE 7: Effect of surface charge density on the binding of peptides to membranes. K_p for linker deleted to PC vesicles containing 20%, 10%, 5%, and 0% (mole) CL (0% vesicle was replaced by 2.5% vesicle for ANCN) is presented. The values of the surface charge density of the vesicles were calculated by eq 7. The final form of the equation used to fit the experimental data was $K_p = K_p'[x + x^2 + 1)^{1/2}] \exp(2z_p)$ where $x = \sigma/(8N\epsilon_0\epsilon_r K_b T c_i)^{1/2}$ and K_p' is the intrinsic partition constant which does not depend on the surface charge of the membrane. The best fit gave values of $K_p' = (3 \pm 0.8) \times 10^2$ M^{-1} and $z_p = 1.4 \pm 0.3$ for linker deleted, $K_p' = 25 \pm 10$ M^{-1} and $z_p = 2.6 \pm 0.5$ for ANCN.

The importance of electrostatic forces in the binding of peptides and proteins to the phospholipid membrane has long been recognized (Kimelberg & Papahadjopoulos, 1971; Tamm, 1986; Roise, 1992; Bergers *et al.*, 1993). The electrostatic force is mainly involved in the initial interaction between the peptide and the membrane. The insertion of the peptide into the bilayer is driven by hydrophobic effects. Increasing either effect could increase the apparent binding affinity of the peptide to the membrane. Deletion of the linker from the ALDH signal sequence removed one positively charged residue from the peptide. The decrease of the electrostatic interaction could have been compensated by a subsequential increase of the hydrophobic effect.

We have previously suggested that the N-terminal helical stability of the ALDH signal sequence can be affected by the stability of the C-terminal helix (Wang & Weiner, 1993). For ANCC, its N-terminal helical stability is greatly reduced because of the random coil conformation of the C-terminal portion. The partition constant of ANCC is estimated to be at least 100-fold lower than that of ANCN. ANCN and ANCC have the same number of positively charged residues and identical sequences in their N-terminal portions, but this 100-fold lower partition constant can be explained by the model described above. That is, its N-terminal portion cannot form a stable helix which allows the peptide to be inserted into the membrane.

It can be concluded from the data presented in this study that for a peptide to be inserted into a membrane, it must have both a positively charged face as well as the ability to form a stable α helix. The latter is of paramount importance for the peptide to be inserted into the membrane bilayer. Is this model relevant for import of proteins into mitochondria? It is known that protein receptors exist on the membrane (Söllner *et al.*, 1989; 1990; Hines & Schatz, 1993) and that their concentration may be low. We suggest that it could be advantageous for the peptide to initially bind to the mitochondrial membrane rather than directly to the import apparatus. This would increase the chance of collision between the signal sequence and receptor by increasing the concentration of precursor proteins on the membrane surface as well as changing the diffusion from three-dimensional space into two-dimensional surface. This alone would increase the rate of collision by a factor of perhaps 10^8 if one assumes that the concentration of precursors were in a concentrated volume the size of one protein molecule attached to the surface of the mitochondria. Why do the precursors not bind to other membranes if the initial binding is to a membrane surface and not directly to a translocator? We, like others, suggest that the high concentration of cardiolipin in mitochondria may be responsible. Others have demonstrated that mitochondrial signal sequences will not bind to synthetic bilayers void of cardiolipin (Ou *et al.*, 1988; Leenhouts *et al.*, 1993). Thus, the actual presence of the negative head groups may be responsible for allowing the helix to be inserted. Once inserted, it may be able to diffuse laterally until it interacts with a component of the translocator.

The model for binding we presented can be used to rationalize why there is such diversity in the sequence of the various mitochondrial signal sequences. For binding, both electrostatic and hydrophobic interactions are important. If one is strong, the other could be weak. Thus, a balance between the interactions is necessary, so exceptions to the "helical wheel" can be expected, as demonstrated by the analysis of hydrophobic moments and helical wheel projections (von Heijine, 1986).

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