Temperature Dependence of Polypeptide Partitioning between Water and Phospholipid Bilayers†

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ABSTRACT: Various thermodynamic forces (e.g., the hydrophobic effect, electrostatic interactions, peptide immobilization, peptide conformational changes, "bilayer effects," and van der Waals dispersion forces) can participate in the transfer of polypeptides from aqueous solution into lipid bilayers. To investigate the contributions of these forces to peptide−membrane thermodynamics, we have studied the temperature dependence of the water−bilayer partitioning of 4 polypeptides derived from the first 25 amino acid residues in the presequence of subunit IV of yeast cytochrome *c* oxidase (Cox IVp) using electron paramagnetic resonance spectroscopy. The partitioning of the Cox IVp peptides into phospholipid bilayers increases as the temperature is increased from 3 to 40 °C. The contribution of bilayer surface expansion to the temperature-dependent partitioning is estimated to be relatively small and to contribute minimally to the increased bilayer binding of the peptides with increasing temperature. Thermodynamic analysis of the data shows that the transfer of the peptides from water into bilayers at 298 K is driven by the entropic term ($-T\Delta S_{tr}$) with values ranging from −6.7 to −10 kcal mol^{-1}, opposed by the enthalpic term (ΔH_{tr}) by approximately 4 kcal mol^{-1}, and accompanied by a change in heat capacity (ΔC_p) ranging from −117 to −208 cal K^{-1} mol^{-1}. Our results indicate that while a variety of forces do, in fact, contribute to the transfer free energies (ΔG_{tr}), the major driving force for the water-to-bilayer transfer is the hydrophobic effect.

Understanding biochemical processes such as membrane protein folding, protein-mediated membrane fusion, transmembrane signal transduction, and protein translocation across membranes requires quantifying the forces governing protein insertion into and stability within lipid bilayers. Dissecting these forces can be simplified by studying the thermodynamics of polypeptide partitioning between water and phospholipid bilayers. Such minimalist studies allow a separate, controlled treatment of electrostatic and hydrophobic forces and enable the determination of the changes in enthalpy (ΔH_{tr}), entropy (ΔS_{tr}), and heat capacity (ΔC_p) associated with the transfer of polypeptides from water into bilayers. While previous studies of the water-to-bilayer transfer of small polypeptides (three to eight amino acid residues) (Beschiaschvili & Seelig, 1992; Jacobs & White, 1989; Jain *et al.*, 1985; Turner *et al.*, 1995) and other small solutes (De Young & Dill, 1988, 1990; Seelig & Ganz, 1991; Wimley & White, 1993) have advanced the current understanding of protein−membrane interactions, the contributions of various thermodynamic forces to the incorporation of polypeptides into phospholipid bilayers are still unclear.

Water-to-bilayer transfer can be modeled simply as hydrophobically driven removal of solutes from bulk water into bulk nonpolar solvent (Tanford, 1980). Nevertheless, several experiments (Seelig & Seelig, 1977; Wiener & White, 1992) and theory (Marqusee & Dill, 1986) suggest that bilayers are better viewed as an interfacial phase which is chemically heterogeneous. This complication does not preclude the hydrophobic effect, often characterized by the relative dominance of ΔS_{tr} near room temperature, from being the major driving force for the bilayer incorporation of polypeptides. The hydrophobic effect is also characterized by a $\Delta H_{tr} \approx 0$ at room temperature; however, several experimental studies, showing a major contribution of ΔH_{tr} at room temperature, suggest that a "nonclassical" hydrophobic effect drives small amphipathic solutes and peptides from water into bilayers (Beschiaschvili & Seelig, 1992; Huang & Charlton, 1972; Seelig & Ganz, 1991). The debate centers on the relative contributions of ΔS_{tr} and ΔH_{tr} to the ΔG_{tr} (transfer free energy) and on the interpretation of thermodynamic variables in terms of hydrophobic and bilayer−solute interactions (Wimley & White, 1993). In addition, the transfer of larger polypeptides (>20 amino acid residues) into bilayers has not been extensively studied, and the relative contributions of ΔS_{tr} and ΔH_{tr} to the ΔG_{tr} values are not known.

After synthesis in the cytosol, many proteins must cross one or more membranes in order to reach their final destination (Pfanner & Neupert, 1990; Randall *et al.*, 1987; von Heijne, 1988; Wickner, 1988). Many of these proteins contain amphipathic signal sequences, mostly located at the

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[1] Abbreviations: CD, circular dichroism; Cox IVp, cytochrome *c* oxidase subunit IV presequence; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; HPLC, high-performance liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MTSSL, *S*-(1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate spin-label; NH_4OAc, ammonium acetate; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; τ_c, rotational correlation time; ΔG_{tr}, nonelectrostatic contribution to the change in Gibbs free energy upon water-to-bilayer transfer; ΔH_{tr}, total change in enthalpy due to water-to-bilayer transfer; ΔS_{tr}, total change in entropy due to water-to-bilayer transfer.

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N-terminus, which are necessary and often sufficient for the protein to target the appropriate membrane for insertion and possibly translocation (von Heijne, 1994). Several studies have demonstrated that the precise identity of the sequences is not crucial for targeting the correct membrane; instead, specificity is achieved by a fine balance of hydrophobic and electrostatic interactions (Allison & Schatz, 1988; Baker & Schatz, 1989; Izard *et al.*, 1995; Roise & Schatz, 1988). Moreover, it has been shown that the hydrophobic effect and electrostatic interactions determine the membrane topology, orientation, and immersion of synthetic peptides derived from amphipathic signal sequences (Tamm, 1991). Model studies on the water-to-bilayer transfer of synthetic peptides based on signal sequences, therefore, are also potentially useful in understanding how protein trafficking is controlled.

The polypeptides used in the present study are derivatives of the first 25 amino acid residues in the mitochondrial presequence of yeast cytochrome *c* oxidase subunit IV (Cox IVp).[1] One particular advantage to using the Cox IVp model system is that it is one of the most extensively characterized signal sequences, both genetically and biophysically. Cox IVp synthetic peptides bind to mitochondria and model membranes (Roise *et al.*, 1986; Tamm, 1986) and compete with protein translocation (Gillespie *et al.*, 1985; Ito *et al.*, 1992). The peptides have helical conformations in lipid vesicles with a molar negative charge greater than 20% and adopt an extended conformation in lipid vesicles with lower negative charge (Tamm & Bartoldus, 1990). In addition, Cox IVp peptides bind reversibly to lipid vesicles (a prerequisite to partitioning studies), and the electrostatic contribution to the free energy of transfer of Cox IVp peptides has been determined (Thorgeirsson *et al.*, 1995).

To investigate the thermodynamics of polypeptide partitioning between aqueous solution and phospholipid bilayers, we have measured the temperature dependence of the partitioning of four synthetic Cox IVp polypeptide variants between water and POPG/POPC lipid vesicles using EPR spectroscopy. Our results indicate that incorporation of the peptides into bilayers increases as temperature increases to an extent larger than anticipated if it were only due to bilayer surface-expansion effects. Fitting the data to a thermodynamic model shows that transfer of the peptides from water into bilayers is accompanied by a decrease in heat capacity and by an increase in both entropy and enthalpy. Combined with the recent determination of the isothermal affinites of individual amino acid side chains for bilayers (Thorgeirsson *et al.*, 1996), the present work is consistent with the hydrophobic effect being a major driving force for the incorporation of these polypeptides into phopholipid bilayers.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) were obtained from Avanti Polar Lipids (Alabaster, AL). 4-Carboxy-TEMPO and *S*-(1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate (MTSSL) spin labels were obtained from Aldrich Chemical Co. and Reanal (Hungary), respectively.

Lipid Vesicle Preparation. To obtain the desired molar ratios of negatively charged lipid, appropriate amounts of POPC and POPG were dissolved in chloroform. Excess chloroform solvent was removed by passing a stream of nitrogen gas through the samples, and the dry lipid mixtures were placed in high vacuum overnight to remove trace amounts of chloroform. The lipids were resuspended at 20 °C in 2 mM MOPS buffer (pH 7.0, 150 mM NH_4OAc, 0.2 mM EDTA) to yield a total lipid concentration of 60 mM. After 7 cycles of freezing and thawing the lipid solutions, unilamellar vesicles were prepared by extrusion through 100 nm pore size polycarbonate membranes using a LiposoFast extruder (Avestine, Canada).

Peptide Synthesis and Spin-Labeling. The amino acid sequences for the peptides used in this study were (NH_3^+)-MLSCRQXIRFFKPATRTLSSSRYLL(COO^-), where X is a guest site at position 7. The sequences are derivatives of the first 25 amino acid residues in the mitochondrial presequence of yeast cytochrome *c* oxidase subunit IV (Cox IVp) in which the native 4th residue, leucine, was replaced by cysteine as an attachment site for the spin-label; the native 19th residue, cysteine, was replaced by serine; and the native 7th residue, serine, was replaced by the guest amino acids glycine, alanine, valine, and leucine. Peptides were synthesized by solid-phase peptide synthesis using (9-fluorenylmethoxy)carbonyl (Fmoc) amino acids and a Wang resin and, subsequently, were purified using a Vydac C_{18} HPLC column as described previously (Yu *et al.*, 1994b). The identities of the products from peptide synthesis were confirmed by electrospray-ionization mass spectrometry.

The purified peptides were dissolved in 10 mM MOPS buffer (pH 7.0). Small volumes of a concentrated stock solution of the thiol-specific spin-label MTSSL in acetonitrile were added to the peptide solution until the concentration of MTSSL was approximately in 2-fold molar excess of the peptide. The spin-labeling reaction was allowed to proceed for 1 h before HPLC purification using a 20−45% water/acetonitrile gradient containing 0.1% trifluoroacetic acid. Fractions containing the spin-labeled peptides were collected as single peaks, lyopholized, and stored dry at −20 °C. The identities of the spin-labeled peptides were confirmed by electrospray-ionization mass spectrometry (purities >96%).

Temperature-Controlled Electron Paramagnetic Resonance (EPR) Measurements. EPR spectra were measured using a Bruker ESP300 EPR spectrometer (Bruker, Germany) equipped with a low-noise microwave amplifier (Mitech) and a loop-gap resonator (Medical Advances). The loop-gap resonator was inserted into a homemade quartz vacuum Dewar, and the temperature was controlled by a Eurotherm B-VT 2000 thermostat to within 0.5 °C of reported values. After introduction into the EPR cavity, samples were allowed to thermally equilibriate for at least 10 min before spectra were collected. The concentrations of Cox IVp peptide variants (between 80 and 110 μM) were determined by comparing their integrated spectral areas to that for a 100 μM sample of the standard 4-hydroxy-TEMPO. The concentrations of bilayer-bound and free peptide fractions (P_b and P_f, respectively) in the lipid-containing samples were determined by EPR spectral subtraction. To ensure high accuracy, the partitioning ratio (P_b/P_f) should be between 0.1 and 10. In this work, the surface potentials of the lipid vesicles were varied by using lipid vesicles containing molar negative charges of 1−3.5%, and the resulting range of P_b/P_f was between ∼0.15 for Cox IVp 7-glycine at low temperature and ∼4.2 for Cox IVp 7-leucine at high temperature.

Thermodynamics of Water−Bilayer Partitioning Equilibria. The incorporation of amphipathic polypeptides into phospholipid bilayers can be described by the partition model (Tamm, 1991; Thorgeirsson *et al.*, 1995):

$$K_{app}' = X_b/P_f \quad (1)$$

where K_{app}' is the apparent partition constant in units of liters per mole, X_b is the moles of bilayer-bound peptide per mole of peptide-accessible lipid, and P_f is the bulk peptide concentration in the aqueous phase. In this work, the concentrations of peptide residing in bilayers (P_b) and peptide existing free in solution (P_f) were determined using EPR spectroscopy, and K_{app} was calculated by the expression:

$$K_{app} = \frac{X_b}{X_f} = \frac{P_b \quad C_w}{(5/9)C_L P_f} \quad (2)$$

where C_w is the concentration of bulk water (55.5 M) and C_L is the total lipid concentration. The term C_w is included so that transfer free energies calculated from K_{app} are expressed in unitary units (Kauzmann, 1959; Tanford, 1980). The term C_L is multiplied by $^5/_9$ since the Cox IVp peptide variants reside in the outer leaflet of the bilayer in the absence of a transmembrane potential (Roise, 1992) and the outer leaflet contains $^5/_9$ of the total lipid for vesicles with a diameter equal to 100 nm.

In the limit of a low membrane surface potential ($<0.5RT/F$), the apparent partition constant for the partitioning of a peptide with a formal charge of z_p into a membrane with an electrostatic surface potential Ψ is expressed as

$$K_{app} = K_0 \exp(-z_p F\Psi/RT) \quad (3)$$

where K_0 is the partition constant in the absence of surface potential and F is Faraday's constant. Ψ is calculated using Gouy−Chapman−Stern theory as described previously, and $z_p = 5$ for the Cox IVp peptides (Thorgeirsson *et al.*, 1995). For ideal solutions, the nonelectrostatic contribution to the change in Gibbs free energy (ΔG_{tr}) upon transfer of the peptides from the aqueous phase into the lipid bilayer phase can then be calculated by the expression:

$$\Delta G_{tr} = -RT \ln(K_0) = -RT \ln(K_{app}) - zF\Psi \quad (4)$$

where $-zF\Psi$ accounts for the electrostatic contribution to ΔG_{tr} and K_{app} is calculated according to eq 2.

By definition, the change in Gibbs free energy upon transfer of a solute from water into bilayers at constant temperature is

$$\Delta G_{tr} = \Delta H_{tr} - T\Delta S_{tr} \quad (5)$$

where ΔH_{tr} and ΔS_{tr} are the total changes in enthalpy and entropy, respectively, accompanying the water-to-bilayer transfer. The thermodynamic variables ΔH_{tr} and ΔS_{tr} can be experimentally determined by fitting the temperature dependence of ΔG_{tr} according to eq 5. A nonlinear temperature dependence of ΔG_{tr}, however, suggests a change in the heat capacity (ΔC_p) of the system upon transfer of the solute from water to bilayers. Assuming the ΔC_p is constant in the temperature range studied, one can use the following equation for the temperature dependence of ΔG_{tr} (Baldwin, 1986):

$$\Delta G_{tr} = \Delta H° + \Delta C_p(T_{obs} - T_{ref}) - T_{obs}\Delta S° - T_{obs}\Delta C_p \ln(T_{obs}/T_{ref}) \quad (6)$$

where T_{obs} is the observed temperature, T_{ref} is the standard-state reference temperature (298 K in this study), and $\Delta H°$ and $\Delta S°$ are the standard-state changes in enthalpy and entropy, respectively, upon solute transfer at 298 K. The temperature dependences of the ΔG_{tr} values of the Cox IVp peptides were nonlinear in the temperature range examined, and the variables $\Delta H°$, $\Delta S°$, and ΔC_p were estimated by fitting the ΔG_{tr} values versus absolute temperature to eq 6 by the Levenberg−Marquardt method of nonlinear least squares.

RESULTS

Peptide System Used for Water−Bilayer Partitioning Experiments. The 4 peptides used in this study are derivatives of the first 25 amino acid residues of the presequence of yeast cytochrome *c* oxidase subunit IV (Cox IVp) in which glycine, alanine, valine, and leucine are substituted in the guest site at position 7. The Cox IVp peptide variants bind reversibly to lipid bilayers. Previous CD studies have shown that the Cox IVp peptides in solution have an extended conformation, containing little or no α-helix; moreover, substitions of glycine, alanine, valine, and leucine into the guest site do not alter the secondary structure significantly (Thorgeirsson *et al.*, 1996). Various studies indicate that the bilayer-incorporated peptides also have an extended conformation and bind parallel to the surface of the bilayer with the peptide backbones residing near the interface of the head-group and acyl-chain regions. This model is based on studies of the depths of spin-labels attached to various positions near the amino-terminal region of the peptide (Yu *et al.*, 1994b), the electrostatics of bilayer binding (Thorgeirsson *et al.*, 1995), and energetic considerations (Thorgeirsson *et al.*, 1996).

EPR Spectroscopic Measurements of Peptide Partitioning. The EPR spectra of the spin-labeled peptides in aqueous lipid vesicle suspensions contain two components, one from the peptides tumbling rapidly in solution and the other from the peptides tumbling slower within bilayers. Figure 1a shows such an EPR spectrum for the Cox IVp 7-valine[2] peptide variant. The sharp spectral component corresponding to the peptide fraction in solution is identical to a spectrum taken of the peptide in the absence of lipid vesicles (Figure 1c) and is characteristic of a fast-motional EPR spectrum for a nitroxide spin-label attached to a short peptide in solution with a rotational correlation time (τ_c) of ∼0.5 ns (Schneider & Freed, 1989; Todd & Millhauser, 1991). The spectral component corresponding to the peptide fraction incorporated into the bilayers is resolved by subtracting the solution spectrum from the two-component spectrum. The resulting bilayer-incorporated component (Figure 1b) shows motional broadening with a τ_c of ∼3−4 ns and is indicative of the interaction of the nitroxide with the more viscous lipid bilayer phase (Altenbach & Hubbell, 1988; Yu *et al.*, 1994a).

[2] The four Cox IVp peptide variants are abbreviated as Cox IVp 7-glycine, Cox IVp 7-alanine, Cox IVp 7-valine, and Cox IVp 7-leucine for the polypeptides which have the guest amino acid residues glycine, alanine, valine, and leucine, respectively, substituted at position 7 of the host polypeptide (cf. *Peptide Synthesis and Spin-Labeling* section under Materials and Methods for the amino acid sequence).

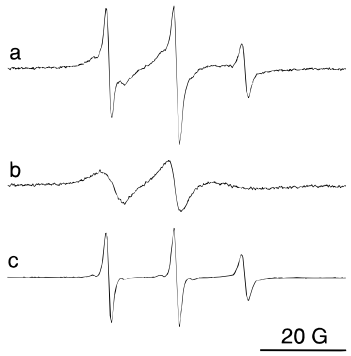

FIGURE 1: (a) EPR spectrum for Cox IVp 7-valine in the presence of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) lipid vesicles containing 3.0 mol % 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) and buffered by 2 mM MOPS (pH 7.0, 100 mM NH_4-OAc, 0.2 mM EDTA). The total peptide concentration is 90 μM, the lipid concentration is 40 mM, and the temperature is 20 °C. The broad component of the spectrum corresponding to the bilayer-incorporated fraction of the peptide (b) remains after subtracting the sharp solution spectrum of Cox IVp 7-valine (c) from the two-component spectrum (a).

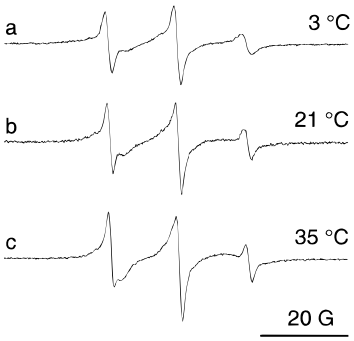

FIGURE 2: EPR spectra for Cox IVp 7-leucine in the presence of POPC lipid vesicles containing 1.0 mol % POPG and buffered by 2 mM MOPS (pH 7.0, 100 mM NH_4OAc, 0.2 mM EDTA). The temperatures at which the spectra were taken are (a) 3 °C, (b) 21 °C, and (c) 35 °C. As temperature increases, both membrane-incorporated and solution components of the spectra sharpen due to faster motion of the nitroxides. The fraction of bilayer-incorporated peptide increases as a function of temperature, as can be seen by the relative increase in the intensity of the broad bilayer-incorporated component at the expense of the relative intensity of the sharp solution component (cf. Figure 1b and 1c).

To examine the effect of varying temperature on the distribution of the polypeptides between the aqueous and lipid bilayer phases, EPR spectra were collected for the Cox IVp variants at temperatures ranging from 3 to 40 °C. The EPR spectra indicated that peptide binding to bilayers increases as the temperature is increased. For example, Figure 2 shows the EPR spectra of Cox IVp 7-leucine at 3, 21, and 35 °C and in the presence of POPC lipid vesicles containing 1 mol % POPG. Both bilayer-incorporated and solution components of the spectra narrow as the temperature increases due to faster motion of the nitroxides. With increasing temperature, the bilayer-incorporated component (cf. Figure 1b) comprises a larger fraction of the two-component spectra, indicating that the peptide incorporates increasingly more into bilayers as the temperature is increased. The concentrations of peptide bound to bilayers (P_b) and free in solution (P_f) were determined using EPR spectral subtraction. The partitioning ratios (P_b/P_f) of Cox IVp 7-leucine were 1.6, 3.1, and 4.2 at temperatures of 3, 21, and 35 °C, respectively.

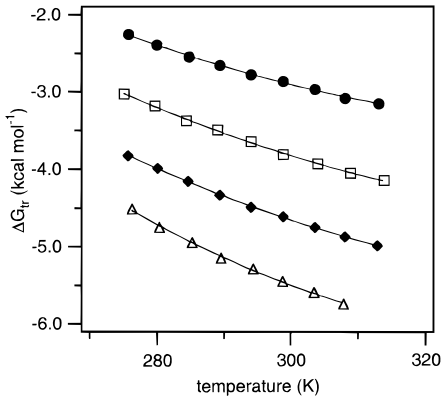

FIGURE 3: Temperature dependence of the Gibbs free energies of transfer (ΔG_{tr}) from solution to bilayers for Cox IVp 7-glycine (●), 7-alanine (□), 7-valine (♦), and 7-leucine (△). Increasing the temperature from 3 to 40 °C favors the bilayer-incorporated-peptide state by ~1 kcal mol^{-1}. The solid lines represent fits of the data to eq 6 by nonlinear least squares. The absolute error on each data point is estimated to be ±0.15 kcal mol^{-1}.

Table 1: Thermodynamic Parameters for the Partitioning of Cox IVp Polypeptides from Buffered Solution into Phospholipid Bilayers at 298 K

Cox IVp peptide[2]	ΔC_p (cal K^{-1} mol^{-1})	ΔG_{tr} (kcal mol^{-1})	ΔH_{tr} (kcal mol^{-1})	$-T\Delta S_{tr}$ (kcal mol^{-1})
7-glycine	−117 ± 22	−2.85 ± 0.21	+3.88 ± 0.15	−6.73 ± 0.15
7-alanine	−129 ± 20	−3.77 ± 0.21	+4.45 ± 0.14	−8.22 ± 0.15
7-valine	−139 ± 14	−4.60 ± 0.14	+4.16 ± 0.10	−8.76 ± 0.10
7-leucine	−208 ± 46	−5.43 ± 0.49	+4.56 ± 0.35	−9.99 ± 0.35

Thermodynamic Analysis of Peptide Partitioning. A two-state model in which a change in temperature changes the peptides' equilibrium distribution between solution and bilayers but not their conformation or bilayer-incorporated topology is assumed in the analysis of the partitioning equilibrium. The nonelectrostatic component of the change in Gibbs free energy due to transfer of the peptides from solution to bilayers (ΔG_{tr}) was calculated according to eq 4 using the EPR-determined partitioning data. Figure 3 shows the resulting ΔG_{tr} values vs temperature. The data show that an increase in temperature from 3 to 40 °C favors bilayer incorporation of the peptides by ~1 kcal mol^{-1} (P_b/P_f is ~2.5 times larger). The negative slope of the ΔG_{tr} vs T plots becomes somewhat less negative with increased temperature, indicating a nonlinear temperature dependence of ΔG_{tr} and suggesting a decrease in the heat capacity of the system upon bilayer incorporation of the polypeptides. The temperature dependences of the ΔG_{tr} values for the four peptide variants were fit to eq 6, and the resulting thermodynamic parameters ΔC_p, ΔH_{tr}, and $-T\Delta S_{tr}$ at 298 K are included in Table 1.

Estimation of Bilayer Surface Expansion Effects. The expansion of lipid bilayers due to increasing temperature can increase the partitioning of polypeptides and other solutes into bilayers. Therefore, we estimated the contribution of bilayer expansion to the temperature dependence of the bilayer incorporation of the Cox IVp peptides. Using the ^{2}H-NMR data of Seelig and Seelig (1977), we estimated the order parameter, S_{mol}, of the phospholipid vesicles as a function of temperature. The normalized bilayer surface density (σ) was then calculated by the following equation (De Young & Dill, 1988):

$$\sigma = \frac{2}{3}S_{mol} + \frac{1}{3} \quad (7)$$

The bilayer surface density, σ, is normalized to the density of the crystalline state of the bilayer so that a σ value of 1 corresponds to the crystalline state and σ decreases as the bilayer surface becomes less dense. The Cox IVp partition constants were corrected for bilayer surface expansion by multiplying them by $K(\sigma_{298})/K(\sigma_{T_{obs}})$, where $K(\sigma_{298})$ is the partition constant of hexane at a lipid surface density corresponding to 298 K and $K(\sigma_{T_{obs}})$ is the partition constant of hexane at a lipid surface density corresponding to T_{obs} (De Young & Dill, 1990). Figure 4 shows the ΔG_{tr} values for Cox IVp 7-alanine calculated from partition constants both corrected and uncorrected for the estimated bilayer surface expansion effects. For all of the Cox IVp peptides, it is found that surface expansion effects alone are not sufficient to account for the increased incorporation of the peptides into the bilayers as temperature is increased. Fitting the ΔG_{tr} values calculated using partition constants corrected to the bilayer surface density at 298 K to eq 6 yields ΔC_p values of -108, -122, -131, and -200 cal K^{-1} mol^{-1} for Cox IVp 7-glycine, 7-alanine, 7-valine, and 7-leucine, respectively. The ΔH_{tr} values are decreased by $\sim$1 kcal mol^{-1} from the reported values in Table 1, with a corresponding increase in the $-T\Delta S_{tr}$ values so that the ΔG_{tr} values are unchanged. While the estimated contribution of bilayer surface expansion to the partitioning of the peptides decreases the ΔC_p and ΔH_{tr} values and increases the $-T\Delta S_{tr}$ values, its contribution is relatively small and is not a major determinant in the temperature dependence of the Cox IVp partitioning into bilayers.

DISCUSSION

The observed temperature dependence of polypeptide partitioning between water and phospholipid bilayers is qualitatitively consistent with the hydrophobic effect. First, as the temperature is increased from 3 to 40 °C, a larger fraction of the Cox IVp peptides resides in bilayers and a smaller fraction remains in aqueous solution. This temperature-dependent partitioning of the peptides into bilayers is consistent with the water-to-bilayer transfer being entropically favored. Second, Figure 3 shows that the transfer free energies (ΔG_{tr} values) are decreased by $\sim$1 kcal mol^{-1} by the temperature increase, with the differences between ΔG_{tr} at high and low temperatures $[\Delta G_{tr}(T_{high}) - \Delta G_{tr}(T_{low})]$ equal to -0.89, -1.12, -1.15, and -1.23 kcal mol^{-1} for Cox IVp 7-glycine, 7-alanine, 7-valine, and 7-leucine, respectively. The trend is such that the temperature dependence of water-to-bilayer peptide transfer becomes more pronounced as the nonpolar surface area of the peptide increases. Third, the nonlinearity of the ΔG_{tr} vs T plots in Figure 3 suggests a decrease in the heat capacity of the system upon transfer of the peptides from aqueous solution into lipid bilayers. Finally, the isothermal differences in ΔG_{tr} between the Cox IVp variants $[\Delta\Delta G_{tr} = \Delta G_{tr}(7\text{-X}) - \Delta G_{tr}(7\text{-glycine})]$ are the membrane affinities for the amino acid side chains substituted at the guest site. The membrane affinities of the alanine, valine, and leucine side chains at 21 °C are equal to the previously determined values within experimental uncertainty and correlate well with water−octanol solvent transfer studies of amino acid derivatives (Thorgeirsson *et al.*, 1996). The removal of nonpolar and amphipathic molecules from water

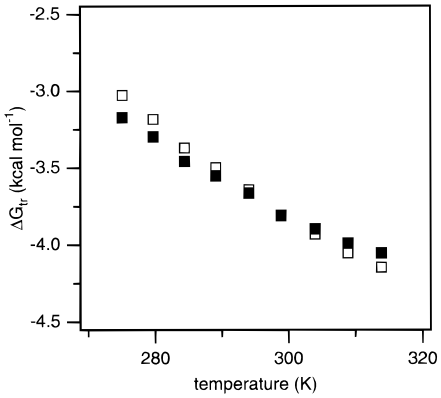

FIGURE 4: Temperature dependence of ΔG_{tr} for Cox IVp 7-alanine both uncorrected (□) and corrected (■) for estimated bilayer surface expansion effects. Surface expansion effects alone are not sufficient to account for the increased bilayer binding of the peptides with increasing temperature.

due to the hydrophobic effect is often characterized phenomenologically as an entropy-driven, enthalpy-neutral process at and around room temperature (Tanford, 1980) and can be more specifically identified by a reduction of heat capacity upon the removal of nonpolar surface from water (Baldwin, 1986). Since the water-to-bilayer transfer of the Cox IVp polypeptides is apparently driven by the change in entropy near room temperature, promoted by increasing nonpolar surface area, and accompanied by a reduction of heat capacity, the hydrophobic effect is clearly a major contributor to the bilayer incorporation of these polypeptides.

An increase in the bilayer incorportion of smaller polypeptides (containing 3−8 amino acid residues) with an increase in temperature has also been observed for tryptophan-containing peptides (Jain *et al.*, 1985), Ala-X-Ala-O-*tert*-butyl peptides (Jacobs & White, 1989), and the fusion-inhibiting peptide Z-D-Phe-L-Phe-Gly (Turner *et al.*, 1995). The increased bilayer incorporation of these peptides with increased temperature was mainly driven by the change in entropy and apparently due to the hydrophobic effect. A study on the water−bilayer partitioning of the cyclic peptide (+)-D-Phe-Cys-Phe-D-Trp-(+)-Lys-Thr-Cys-Thr(ol) also showed entropy-driven incorporation of the peptide into extruded lipid vesicles with a diameter ≥50 nm. However, transfer of the peptide from aqueous solution into smaller (diameter = 30 nm), sonicated lipid vesicles was driven instead by a favorable change in enthalpy (Beschiaschvili & Seelig, 1992). These and other results, showing that the bilayer incorporation of several small amphipathic molecules is enthalpy-driven under certain conditions (Huang & Charlton, 1972; Seelig & Ganz, 1991), have led to the proposal that the incorporation of amphipathic peptides and other amphipathic solutes into bilayers is driven by a "nonclassical" hydrophobic effect governed by solute−lipid van der Waals attractions and changes in the internal tension of bilayers (Beschiaschvili & Seelig, 1992).

To determine the forces participating in the water-to-bilayer transfer of the amphipathic Cox IVp peptides, the thermodynamic parameters ΔC_p, $\Delta H°$, and $\Delta S°$ were calculated by fitting ΔG_{tr} vs temperature according to eq 6. The transfer of each of the Cox IVp peptides from solution into bilayers has a negative ΔC_p ranging from -117 to -208 cal K^{-1} mol^{-1}. Other studies report water-to-bilayer ΔC_p values of -32 to -90 cal K^{-1} mol^{-1} for amphipathic cations

and anions (Seelig & Ganz, 1991), −18 to −67 cal K^{-1} mol^{-1} for tryptophan side chain analogs (Wimley & White, 1993), −113 cal K^{-1} mol^{-1} for Z-D-Phe-L-Phe-Gly (Turner *et al.*, 1995), and greater than −100 cal K^{-1} mol^{-1} for *N*-acetyl-Trp-Leu-Trp-Leu-Leu-OH (Wimley & White, 1993). The relatively small reduction in heat capacity upon the removal of the 25 amino acid residue Cox IVp peptides from water is perhaps consistent with the negative ΔC_p due to nonpolar side chains being offset by a positive ΔC_p from polar side chains and the peptide backbone (Makhatadze & Privalov, 1990; Murphy & Freire, 1992), provided that the polar side chains and peptide backbone are buried in the bilayer (Thorgeirsson *et al.*, 1996). On the other hand, the differences in ΔC_p [$\Delta\Delta C_p = \Delta C_p$(7-X) − ΔC_p(7-glycine)] are −12, −22, and −91 cal K^{-1} mol^{-1} for the alanine, valine, and leucine sidechains, respectively. The $\Delta\Delta C_p$ is increasingly negative as the nonpolar surface area of the guest amino acid at position 7 is larger, consistent with the hydrophobic effect.[3]

To interpret the ΔH_{tr} and ΔS_{tr} values in terms of their origins from specific thermodynamic forces, the total change in the Gibbs free energy upon transfer of a solute from solution into bilayers (ΔG_{tot}) can be expressed as a sum of various free energy components (Ben-Tal *et al.*, 1996; Jacobs & White, 1989; Jähnig, 1983):

$$\Delta G_{tot} = \Delta G_{hyd} + \Delta G_{el} + \Delta G_{lip} + \Delta G_{imm} + \Delta G_{con} + \Delta G_{H-bond} + \Delta G_{vdw} \quad (8)$$

These free energy components account for the hydrophobic effect (ΔG_{hyd}), electrostatic interactions (ΔG_{el}), lipid perturbation upon solute binding (ΔG_{lip}), peptide immobilization in bilayers (ΔG_{imm}), peptide conformational changes (ΔG_{con}), hydrogen bonding (ΔG_{H-bond}), and lipid−lipid and lipid−solute van der Waals dispersion interactions (ΔG_{vdw}). At 298 K, ΔG_{hyd} is dominated by an increase in entropy ($\Delta G_{hyd} \cong -T\Delta S_{hyd} < 0$) (Dill, 1990; Tanford, 1980), whereas ΔG_{lip} and ΔG_{imm} are expected to have entropic origins disfavoring incorporation of the peptides into bilayers ($\Delta G_{lip} \cong -T\Delta S_{lip} > 0$, and $\Delta G_{imm} \cong -T\Delta S_{imm} > 0$) (Ben-Tal *et al.*, 1996). The ΔG_{H-bond} and ΔG_{vdw} values are due to changes in hydrogen bonding and van der Waals dispersion forces, respectively, which can have either an increase or a decrease in enthalpy. Examination of the water-to-bilayer transfer of the Cox IVp peptides at 298 K shows that the transfer is accompanied by positive ΔS_{tr} values ranging from 22.6 to 33.5 cal K^{-1} mol^{-1}. The ΔS_{tr} values are the sum of entropic components included in ΔS_{hyd}, ΔS_{lip}, and ΔS_{imm}; therefore, the ΔS_{hyd} contribution stabilizing the bilayer-incorporated-peptide state is expected to be even larger than ΔS_{tr} and is the main driving force behind the incorporation of the peptides into bilayers. On the other hand, each peptide has a ΔH_{tr} value of approximately 4 kcal mol^{-1}, showing that the incorporation of the peptides into bilayers is opposed by the change in enthalpy. The unfavorable ΔH_{tr} could be due to the loss of a hydrogen bond, lipid−lipid van der Waals interactions, and/or other unknown interactions. Even though it is not possible to further dissect the forces contributing to the positive ΔH_{tr}, it is still apparent that the enthalpic terms corresponding to a proposed "nonclassical" hydrophobic effect are not driving the amphipathic Cox IVp peptides into bilayers.

Wimley and White (1993) have studied the temperature dependence of the partitioning of tryptophan side chain analogs between aqueous solution and lipid bilayers. The incorporation of the tryptophan side chain analogs into bilayers was also accompanied by a significant reduction of heat capacity; however, the process was driven more by the change in enthalpy than the change in entropy. Their analysis of ΔG_{tot} into changes in free energy due to the hydrophobic effect (ΔG_{hyd}) and the "bilayer effect" ($\Delta G_{bil} = \Delta G_{lip} + \Delta G_{vdw} + \Delta G_{el}$ for the tryptophan side chain analogs) shows that ΔG_{hyd} has a small, positive ΔH_{hyd} and a larger, negative $-T\Delta S_{hyd}$ at 298 K whereas ΔG_{bil} has a very large, negative ΔH_{bil}. In contrast to the tryptophan side chain analogs, the Cox IVp peptides have a positive ΔH_{tr} at 298 K and are incorporated into bilayers mainly by the positive ΔS_{tr} due to the hydrophobic effect. The present work suggests that once the enthalpic opposition to the bilayer incorporation of the 25-residue peptides is overcome, the water-to-bilayer transfer is driven mainly by the hydrophobic effect.

If the water-to-bilayer transfer of the Cox IVp peptides were purely due to the hydrophobic effect, one would expect that the ΔH_{tr} values would be approximately 0 kcal mol^{-1} at 298 K. Opposition to the bilayer incorporation of the peptides due to the change in enthalpy is most likely due to the transfer of the peptide backbone from water to bilayers since a previous study has shown that the transfer the amino acid side chains substituted at a guest site is exclusively accounted for by their hydrophobicities as assessed by solvent transfer studies (Thorgeirsson *et al.*, 1996). Experimental determination of the bilayer affinity, ΔH_{tr}, and ΔS_{tr} of the peptide backbone is perhaps necessary to more thoroughly dissect the forces involved in the incorporation of polypeptides into phospholipid bilayers.

A common feature of mitochondrial signal presequences is that they are positively charged potential amphipathic helix formers and that the balance of hydrophobic and electrostatic interactions is more important than the exact identity of the presequence (Roise & Schatz, 1988; Tamm, 1991). Schatz and co-workers have reported the successful import of yeast cytochrome *c* oxidase subunit IV into yeast mitochondria after replacing the naturally occurring signal sequence with various artificial signal sequences (Allison & Schatz, 1988; Baker & Schatz, 1989; Lemire *et al.*, 1985). Another study has shown that the secretion of alkaline phosphatase in *Escherichia coli* can be retained after replacing its signal sequence with synthetic signal sequences and that the physical and conformational properties of the synthetic sequences parallel their biological activity (Izard *et al.*, 1995). There is also evidence that signal presequences are exposed to lipid while the translocating portions of proteins are in a proteinaceous environment during the insertion and translocation of some proteins (Martoglio *et al.*, 1995). The thermodynamic analysis of the bilayer incorporation of the Cox IVp polypeptides studied here, along with the experimentally determined affinities of individual amino acid side chains for lipid bilayers (Thorgeirsson *et al.*, 1996), should provide useful information for the *de novo* design of signal

[3] Alternatively, direct calorimetric measurement of the ΔC_p values of the Cox IVp peptides (and hence $\Delta\Delta C_p$ values of the guest amino acid side chains) would provide a more precise relationship between the nonpolar surface area of each guest amino acid side chain and the corresponding change in heat capacity upon transfer.

sequences for the specific targeting of proteins and drugs to desired cellular membranes and membrane-bound organelles.

In conclusion, while a variety of forces (i.e., electrostatic interactions, peptide immobilization and conformational changes, "bilayer effects," and van der Waals dispersion forces) determine the precise value of ΔG_{tr} for the transfer of amphipathic polypeptides into bilayers, the main driving force behind the bilayer incorporation of the 25 amino acid residue peptides studied here is the hydrophobic effect. The major role of the hydrophobic effect in the water-to-bilayer transfer of the polypeptides is consistent not only with the classical viewpoint by Tanford (1980) but also with a recent study confirming the long-held view that the membrane affinities of amino acid side chains equal their hydrophobicities as assessed by solvent transfer studies (Thorgeirsson *et al.*, 1996).

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