Synthetic Model Proteins: The Relative Contribution of Leucine Residues at the Nonequivalent Positions of the 3–4 Hydrophobic Repeat to the Stability of the Two-Stranded α-Helical Coiled-Coil†

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ABSTRACT: Our de novo designed coiled-coil model protein consists of two identical 35-residue polypeptide chains arranged in a parallel and in-register alignment via interchain hydrophobic interactions and a disulfide bridge at the position 2 between two helices. To quantitate the relative contribution of leucine residues at the nonequivalent position of the 3–4 hydrophobic repeat to the stability of the two-stranded α-helical coiled-coil, a single alanine was systematically substituted for a leucine in each chain at position "a" (9, 16, 23, or 30) or "d" (5, 12, 19, 26, or 33). The formation and stability of the coiled-coils were determined by circular dichroism studies in the absence and presence of guanidine hydrochloride. All the proteins with an alanine substituted at position a have a similar stability ($[Gdn{\cdot}HCl]_{1/2}$ ranges from 2.6 to 2.9 M), while all the proteins with an alanine substituted at position d have similar stability ($[Gdn{\cdot}HCl]_{1/2}$ ranges from 3.6 to 4.2 M), except for the proteins with an alanine substituted in the C-terminal heptad. The greater decrease in stability observed for a Leu → Ala mutation at position a (the average $\Delta\Delta G_u$ value is 3.3 kcal/mol) compared to those where the substitution was effected at position d (the average $\Delta\Delta G_u$ value is 2.0 kcal/mol) indicates that an Ala mutation at position a has a greater effect on the side-chain packing and hydrophobic interactions in the coiled-coil than an Ala mutation at position d. Analyses of the retention behaviors of these coiled-coils during reversed-phase chromatography and computer modeling also suggest that the isopropyl group (which is the structural difference between the side chain of Leu and Ala) of the leucine side chain is more buried at position a than at position d. The difference between the leucine residues at positions a and d in terms of their contribution to the coiled-coil stability is not dependent on the disulfide bridge location. Rather, the disulfide bridge locks the coiled-coil structure in a conformation which mimics the conformation observed in the X-ray structure of the GCN4 leucine zipper.

The two-stranded α-helical coiled-coil, which consists of two amphipathic α-helices associated together in parallel and in register, is an important structural and biologically abundant motif (Cohen & Parry, 1990, 1986). More than 200 proteins have been predicted to contain coiled-coil domains (Lupas et al., 1991). These coiled-coil segments are thought to play functionally important roles in a diverse group of proteins, such as muscle regulatory proteins (McLachlan & Karn, 1982; Phillips, 1986; Epstein & Fischman, 1991), DNA-binding proteins (Landschulz et al., 1988; O'Shea et al., 1989a, 1991; Gentz et al., 1989), cGMP-dependent protein kinase (Atkinson et al., 1991), tumor suppressor (Bourne, 1991), oncogene products (Martin-Zanca et al., 1986), and Epstein–Barr virus transactivator (Flemington & Speck, 1990). Both NMR[1] and X-ray crystallographic data support the view that the two α-helices in the coiled-coil have 2-fold symmetry with respect to the coiled-coil axis (O'Shea et al., 1991; Oas et al., 1990; Atkinson et al., 1991). The coiled-coil also has been identified as an ideal model for protein design (Talbot & Hodges, 1982; Hodges et al., 1981, 1988, 1990; Lau et al., 1984; O'Neil & Degrado, 1990; Engel et al., 1991; Zhou et al., 1992b) because it contains a defined secondary, tertiary, and quaternary structure and only one type of secondary structure, i.e., α-helix, which is the easiest type of secondary structure to monitor in aqueous solution at neutral pH using such techniques as circular dichroism and NMR spectroscopy. The main advantage of studying coiled-coils in preference to single-stranded α-helices is that coiled-coils are stabilized by both intrachain and interchain interactions. Model coiled-coils can be used to investigate all the noncovalent interactions involved in maintaining the three-dimensional structure of proteins.

The most striking feature of the coiled-coil structure is the hydrophobic 3–4 or 4–3 repeat where each heptad is designated by the letters a–g, in which hydrophobic residues at positions a and d fall on the same face of the helix, resulting in a hydrophobic interface between the two helices of the coiled-coil (NXXNXXXNXXNXXX... where N is the nonpolar residue at position a and d). Hodges and co-workers (Hodges et al., 1972; Sodek et al., 1972) sequenced the first two-stranded α-helical coiled-coil (tropomyosin), identified this repeat, and proved using synthetic peptides that the hydrophobic residues at positions a and d in the heptad repeat $(abcdefg)_n$ (Figure 1) were responsible for stabilizing the coiled-coil structure (Hodges et al., 1981). However, the relative contributions of hydrophobic residues at position a and d to the formation and stability of the coiled-coil still remain to be elucidated. A previous study (Hodges et al., 1990) from our laboratory has demonstrated that the large hydrophobic leucine residues at

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[1] Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Gdn·HCl, guanidine hydrochloride; RPC, reversed-phase chromatography.

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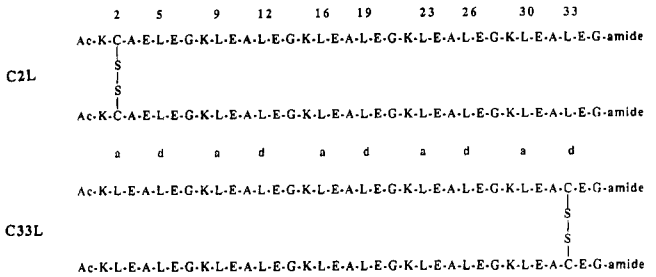

FIGURE 1: Amino acid sequence of the 70-residue synthetic two-stranded α-helical coiled-coil. The α-amino group of each chain is acetylated, and the C-terminal carboxyl group is amidated. The two series of hydrophobes (denoted as "a" and "d") which repeat at seven-residue intervals along the polypeptide chain (also referred to as a 3–4 or 4–3 hydrophobic repeat) are responsible for the formation and stabilization of the coiled-coil. A disulfide bridge is formed between two cysteines in chain 1 and in chain 2 at either position 2 (denoted as C2L) or position 33 (denoted as C33L). A leucine residue was systematically substituted in each chain at the same position by an alanine residue to create a series of coiled-coil analogues with a single Ala–Ala pair replacing one of nine Leu–Leu pairs at positions 5, 9, 12, 16, 19, 23, 26, 30, and 33. The coiled-coil analogues in which an alanine substitution is made at position 5 in chain 1 and chain 2 in protein C2L is denoted as C2A5d and similarly C2A9a, C2A12d, C2A16a, C2A19d, C2A23a, C2A26d, C2A30a, or C2A33d as well as C33A16 or C33A19d.

the a and d positions contribute more than other hydrophobic residues (Ile, Val, Phe, Tyr, and Ala) to the stability of the coiled-coil. In native coiled-coil proteins, not all positions a and d are occupied by hydrophobic leucine residues, and the distribution of leucine at a and d positions is different for coiled-coil proteins with different functions. For instance, in DNA-binding coiled-coils, leucine is usually found at position d, whereas in classical coiled-coils only one-third of the residues at position d are leucine and leucine is almost equivalent in its occupancy of the a and d positions (Cohen & Parry, 1990). Genetic analysis of the GCN4 leucine zipper has shown that the conserved leucines at positions d generally are less tolerant of amino acid substitutions than the alternate hydrophobic residues at positions a (Hu et al., 1990). Thus, a detailed understanding of the coiled-coil structure, especially the contributions of interface hydrophobic residues to the formation and stabilization of the coiled-coil structure, may delineate the reasons for a wide-range of hydrophobic residues in the hydrophobic interface and provide more insights on the relationship between the coiled-coil structure and biological function. The purpose of this study is to determine the relative contribution of interface leucine residues at positions a and d to the coiled-coil stability.

Materials and Methods

All peptides were synthesized by the solid-phase technique starting with copoly(styrene, 1% divinylbenzene)-benzhydrylamine hydrochloride resin using an Applied Biosystems peptide synthesizer model 430A as described previously (Hodges et al., 1988). The peptides were cleaved from the resin by reaction with hydrogen fluoride (20 mL/g of resin) containing 10% anisole and 2% 1,2-ethanedithiol for 1 h at −5 °C. The crude peptides were purified by reversed-phase HPLC on a Synchropak RP-P semipreparative C18 column (250 × 10 mm i.d., 6.5-μm particle size, 300-Å pore size) (Synchrom, Layfayette, IN), with a linear AB gradient (ranging from 0.2% to 1.0% B/min, depending on the peptide) at a flow-rate of 2 mL/min, where solvent A was 0.05% aqueous trifluoroacetic acid (TFA) and solvent B was 0.05% TFA in acetonitrile. Oxidized peptides (formation of a disulfide bond between two 35-residue monomers) were produced using air oxidization by stirring a solution of the reduced peptides in 100 mM NH_4HCO_3, pH 8.3, overnight at room temperature (Lee et al., 1991). The oxidized peptides were purified by reversed-phase HPLC on an analytical Aquapore RP-300 C8 column (220 × 4.6 mm i.d., 7-μm particle size, 300-Å pore size, Brownlee Labs, Santa Clara, CA) with a linear AB gradient of 1% B/min and a flow rate of 1 mL/min, where solvent A was 0.05% TFA in water and solvent B was 0.05% TFA in acetonitrile. The purified peptides were homogeneous as determined by analytical reversed-phase HPLC, amino acid analysis, and mass spectroscopy. For amino acid analysis, purified peptides were hydrolyzed in 6 N HCl containing 0.1% phenol at 110 °C for 24 h or 1 h at 160 °C in evacuated sealed tubes. Cysteine and cystine were determined following conversion to cysteic acid by incorporation of 2% dimethyl sulfoxide in the hydrolysis medium (Hodges et al., 1990). Amino acid analysis was performed on a Beckman model 6300 amino acid analyzer (Beckman Instruments Inc., Fullerton, CA). The correct primary ion molecular weights were confirmed by time of flight mass spectroscopy on a BIOION-20 Nordic (Uppsala, Sweden).

Circular dichroism (CD) spectra were recorded at 20 °C on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) attached to a Jasco DP-500N data processor and a Lauda (model RMS) water bath (Brinkman Instruments, Rexdale, Ontario, Canada) used to control the temperature of the cell. The instrument was routinely calibrated with an aqueous solution of recrystallized d_{10}-camphorsulfonic acid (the recommended Jasco standard), and the molecular ellipticity of a 0.06% solution at 290.5 nm was found to be +7910, in accordance with the literature (Takakuwa et al., 1985). CD spectra were the average of three scans obtained by collecting data at 0.25-nm intervals from 250 to 190 nm. Ellipticity is reported as mean residue ellipticity $[\theta]$, and the limits of error of measurements at 220 nm were ±500°. The guanidine hydrochloride (Gdn·HCl) denaturation studies were carried out by preparing mixtures of a stock solution of peptide in buffer (0.1 M KCl, 50 mM PO4, pH 7), buffer alone, and a solution of 6 M Gdn·HCl in buffer where the ratios of buffer and 6 M Gdn·HCl solutions varied to give the appropriate final Gdn·HCl concentrations for the CD measurements. Peptide concentrations of ~1 mM stock solutions were determined by amino acid analysis.

Energy minimization and molecular dynamics were carried out on a Silicon Graphics Personal Iris with the INSIGHT II and DISCOVER programs (Biosym Technologies Inc., San Diego, CA) as described by Zhou et al. (1992a). The molecular surface program (MS) (Connolly, 1983) in the UCSD Molecular Modeling System (MMS) was used to measure the molecular surface area of the coiled-coil from a computer modeling structure with probe sphere radius of 1.4 Å and surface density of 3.0 points/Å^2.

Results

The de novo designed protein consists of two identical 35-residue polypeptide chains, parallel and in register (Figure 1), arranged in a two-stranded α-helical coiled-coil structure. The selection of the amino acid sequence of the heptapeptide repeat, Lys-Leu-Glu-Ala-Leu-Glu-Gly, and the chain length of 35 residues, have been described in detailed (Hodges et al., 1981; Lau et al., 1984). This coiled-coil structure is predominantly stabilized by the hydrophobic interactions between nine leucine residues at positions a and d on both helices. The oppositely charged residues at positions g (Lys-1, -8, -15, -22, and -29) and e of adjacent heptads (Glu-6, -13, -20, -27, and -34) strongly favor the formation of interhelical ion pairs (e–g′ and

Table I: Circular Dichroism and Reversed-Phase Chromatography Results of the Synthetic Model Peptides

peptide[a]	$[\theta]_{220}$[b] (deg·cm²·dmol⁻¹)	$[\theta]_{220}/[\theta]_{208}$[c]	$[Gdn·HCl]_{1/2}$[d] (M)	$\Delta G_u^{H_2O}$[e] (kcal·mol⁻¹)	$\Delta\Delta G_u$[f] (kcal·mol⁻¹)	R_t[g] (min)	ΔR_t[h] (min)
C2L	−30 250	1.05	5.3	6.9		20.6	
C2A5d	−29 550	1.07	3.6	5.1	2.3	20.5	−0.1
C2A12d	−31 230	1.04	4.0	6.1	1.8	20.3	−0.3
C2A19d	−30 520	1.03	3.7	6.0	2.1	20.5	−0.1
C2A26d	−30 430	1.04	4.2	5.5	1.6	20.5	−0.1
C2A33d	−28 880	1.02	4.9	6.3	0.7	20.6	0.0
C2A9a	−29 840	1.05	2.9	4.5	3.2	20.9	0.3
C2A16a	−30 450	1.04	2.6	3.8	3.6	21.2	0.6
C2A23a	−30 620	1.03	2.9	4.0	3.2	21.3	0.7
C2A30a	−29 200	1.01	4.3	5.7	1.4	21.0	0.4

[a] The peptide sequences are shown in Figure 1. [b] The mean residue ellipticities at 220 nm were measured at 20 °C in 0.1 M KCl, 0.05 M PO_4 buffer, pH 7.0. [c] $[\theta]_{220}/[\theta]_{208}$ is the ratio of ellipticity at 220 nm to ellipticity at 208 nm. [d] $[Gdn·HCl]_{1/2}$ is the transition midpoint, the concentration of guanidine hydrochloride (M) required to give a 50% decrease in ellipticity at 220 nm. [e] $\Delta G_u^{H_2O}$ is the free energy of unfolding in the absence of guanidine hydrochloride and is estimated by extrapolating the free energy of unfolding at each individual concentration of guanidine hydrochloride (ΔG_u) to zero concentration assuming that they are linearly related (Pace, 1986; Shortle, 1989), $\Delta G_u = \Delta G_u^{H_2O} - m[Gdn·HCl]$. ΔG_u was calculated from the equation $\Delta G_u = -RT \ln [f_u/(1 - f_u)]$ (Pace, 1986; Shortle, 1989), where f_u is the molar fraction of denatured peptide as determined from the ellipticity at 220 nm. [f] $\Delta\Delta G_u$ is the free energy difference between the native protein (C2L) and the Ala mutant protein at 4.0 M Gdn·HCl. For example, $\Delta\Delta G_u$(C2A5d) = ΔG_u(C2L) − ΔG_u(C2A5d), where ΔG_u is the free energy of unfolding at 4 M Gdn·HCl. [g] R_t is peptide retention time (min) obtained on an analytical C18 column (Synchropak RP-P, 250 × 4.6 mm i.d., 300-Å pore size) using a linear AB gradient (2% B/min), where solvent A is 0.05% TFA in water and solvent B is 0.05% TFA in acetonitrile; flow rate, 1 mL/min. [h] ΔR_t is the retention time difference between the oxidized Ala mutant protein and the oxidized native protein (C2L) and represents the decrease or increase in retention time for the Ala analogues compared to the native Leu-protein. For example, ΔR_t(C2A5d) = R_t(C2A5d) − R_t(C2L) = 20.5 − 20.6 = −0.1 (min).

e′–g) that would be expected to stabilize a parallel and in-register arrangement of the coiled-coil (Stone et al., 1975; McLachlan & Stewart, 1975; Talbot & Hodges, 1982). In addition, a disulfide bridge was introduced between the two cysteines either at the N-terminus (position 2) (denoted as C2L) or at the C-terminus (position 33) (denoted as C33L) of the polypeptide to maintain the two helices in a parallel alignment. To evaluate the relative contribution of interface hydrophobic residues at positions a and d to coiled-coil stability, a leucine residue was systematically substituted in each chain at the same position by an alanine residue to create a series of coiled-coil analogues with a single Ala–Ala pair replacing one of nine Leu–Leu pairs at positions 5, 9, 12, 16, 19, 23, 26, 30, and 33. The coiled-coil analogue in which an alanine substitution is made at position 5 in chain 1 and chain 2 in protein C2L is denoted as C2A5d, where d refers an Ala substitution at position d, and similarly C2A9a, C2A12d, C2A16a, C2A19d, C2A23a, C2A26d, C2A30a, or C2A33d as well as C33A16a or C33A19d.

Characterization of the Parallel Coiled-Coils. Our previous study (Zhou et al., 1992a) has demonstrated that, in the reduced state, all Ala analogues used in this study form two-stranded α-helical coiled-coils under physiological conditions. The solution conformations of the oxidized Ala analogues were studied by circular dichroism. The CD spectra for all peptide analogues are very similar to each other and the native coiled-coil protein (C2L). All showed two mimima, one near 220 nm and another in 207 nm in benign conditions (0.1 M KCl, 50 mM PO_4, pH 7) with ~30 000 deg·cm²·dmol⁻¹ of mean residue ellipticity (Figure 2 and Table I), indicating that they are predominantly α-helical. In principle, isolated single-stranded helices are unstable and transient in aqueous solutions (Dyson et al., 1988; Saudek et al., 1991) and require the additional stabilization provided by the tertiary/quaternary structure or a less polar solvent, e.g., 1,1,1-trifluoroethanol (TFE) to induce α-helical structure. The peptides did not show any increase in helicity upon addition of the α-helix inducing solvent TFE, as measured by molar ellipticity at 220 nm (Figure 2). Thus the α-helical structures of these peptides are stabilized by the association of the two helices via interhelical hydrophobic interactions between the helices. This was confirmed by the concentration dependence of the ellipiticity at 220 nm on peptide concentration for the reduced peptide

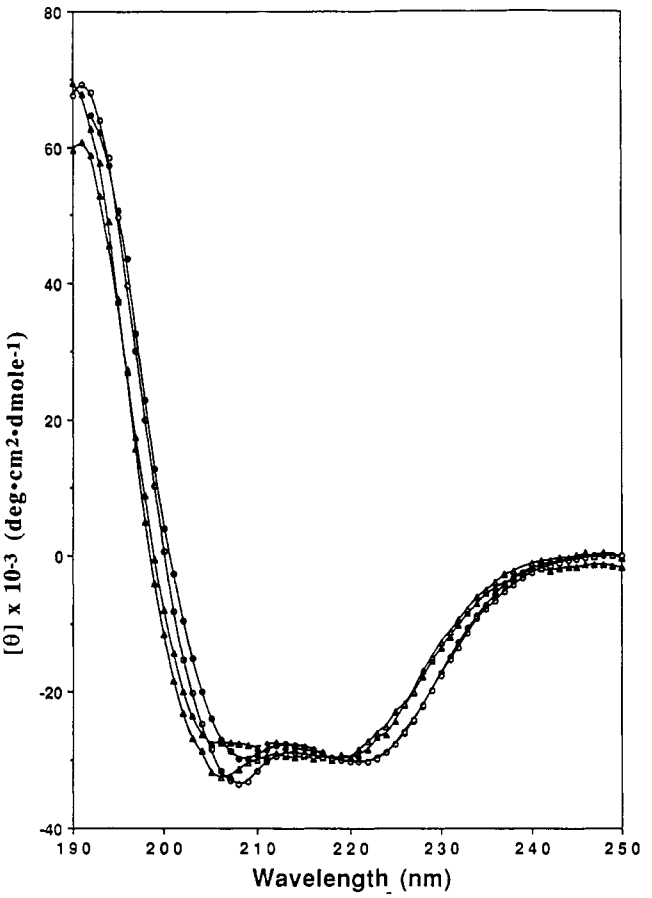

FIGURE 2: Circular dichroism spectra of the coiled-coil proteins in the presence (open symbols) and absence (closed symbols) of the α-helix inducing solvent, TFE. Circles denote the native protein C2L. Triangles denote an alanine analogue C2A5d. The nondenaturing (benign) buffer used was 0.05 M aqueous potassium phosphate containing 0.1 M KCl, pH 7. For samples containing TFE, the above buffer was diluted 1:1 (v/v) with TFE. The temperature was 20 °C for ellipticity measurements.

analogues (without the disulfide bridge between the two helices) and the dimeric molecular weight of the reduced peptide analogues in benign media as determined by size-exclusion chromatography and sedimentation equilibrium experiments (Lau et al., 1984; Zhou et al., 1992a). Our previous

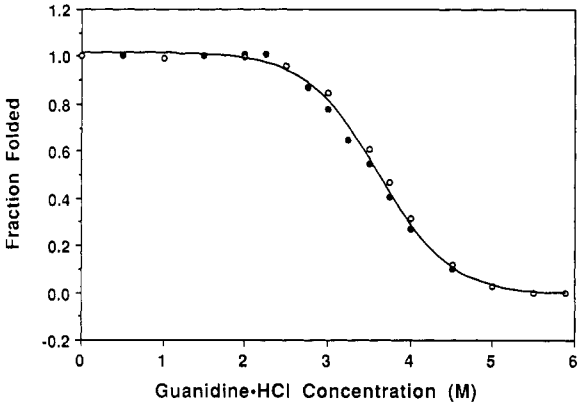

FIGURE 3: Guanidine hydrochloride (Gdn·HCl) denaturation profiles of coiled-coil analogue C2A5d at different peptide concentrations in 0.05 M PO_4, 0.1 M KCl buffer, pH 7 at 20 °C. The molar fraction of folded peptide (f_n) was calculated as $f_n = ([\theta] - [\theta]_u)/([\theta]_n - [\theta]_u)$, where $[\theta]$ is the observed mean residue ellipticity at 220 nm at any particular Gdn·HCl concentration and $[\theta]_n$ and $[\theta]_u$ are the mean residue ellipticities at 220 nm of the native (folded) and unfolded states, respectively. Concentrations of peptide C2A5d were 23 (○) and 113 μM (●).

studies on coiled-coil peptides (Lau et al., 1984; Hodges et al., 1988, 1990; Zhou et al., 1992a) suggested that the ratio of $[\theta]_{220}/[\theta]_{208}$ is about 0.86 for a single-stranded α-helix (in presence of TFE) and about 1.03 for a two-stranded α-helical coiled-coil in benign medium. The ratios of $[\theta]_{220}/[\theta]_{208}$ for these oxidized coiled-coils (1.04 ± 0.03, Table I) are consistent with our previous studies on coiled-coils.

The introduction of a disulfide bond at the N-terminal end of the coiled-coil is expected to ensure that the two α-helices are in a parallel and in-register alignment. If the coiled-coil is parallel, then the stability of the oxidized coiled-coil should be independent of the peptide concentration, because the disulfide bridge joins the two helices at the N-terminus in both chains. If the orientation is antiparallel, the disulfide-linked peptide chains should associate and show concentration-dependent stability (O'Shea et al., 1989b). The stability of the oxidized coiled-coils reported in this study is independent of peptide concentration (Figure 3), indicating that the coiled-coil is parallel. Sedimentation equilibrium results have previously shown that the oxidized and reduced coiled-coils have the same molecular weight corresponding to the 70-residue coiled-coil (Hodges et al., 1981, 1990; Lau et al., 1984; Zhou et al., 1992a).

Stability of Coiled-Coil Analogues. To quantitate the stability changes resulting from an Ala substitution for a Leu residue, Gdn·HCl denaturation of the coiled-coil proteins was monitored by measuring the ellipticities of the peptides at 220 nm as a function of Gdn·HCl concentration at 20 °C (Figures 3 and 4A). The ellipticity of of the peptide decreases with increasing Gdn·HCl concentration. The stability of the disulfide-bridged coiled-coil is independent of peptide concentration in the Gdn·HCl denaturation experiments. For instance, the Gdn·HCl unfolding profiles of peptide C2A5d at two different peptide concentrations (23 and 113 μM) show essentially the same denaturation curve (Figure 3) and the transition midpoints ($[\text{Gdn·HCl}]_{1/2}$ is the concentration of Gdn·HCl at which 50% of the peptide is unfolded) are 3.6 M for these two peptide concentrations within experimental error. The Gdn·HCl denaturation curves were analyzed by assuming that the folding/unfolding transition is two-state, in which case an equilibrium constant (K_u) can be obtained at each individual Gdn·HCl concentration by

$$K_u = (1 - f_n)/f_n$$

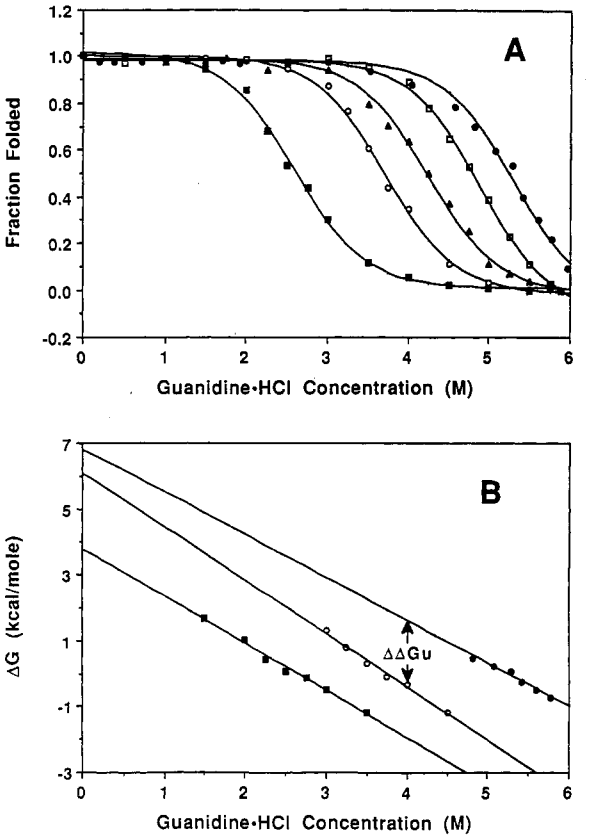

FIGURE 4: (A) Guanidine hydrochloride denaturation profiles of the native coiled-coil protein C2L (●) and Ala analogues C2A33d (□), C2A30a (▲), C2A19d (○), and C2A16a (■) in 0.1 M KCl, 0.05 M PO_4 buffer, pH 7. The fraction folded is defined in the legend to Figure 3. (B) Linear dependence of ΔG_u on the Gdn·HCl concentration allows simple determination of $\Delta G_u^{H_2O}$ and $\Delta\Delta G_u$. ΔG_u is the free energy of unfolding at each individual concentration of denaturant, and $\Delta G_u^{H_2O}$, the free energy of unfolding in the absence of Gdn·HCl, is estimated by extrapolating ΔG_u to zero assuming that they are linearly related. $\Delta\Delta G_u$ is the free energy difference between the native protein (C2L) and the Ala mutant at 4.0 M Gdn·HCl. The symbols are the same as in panel A. See text for the details.

where f_n is the fraction folded as determined from ellipticity at 220 nm, $f_n = ([\theta] - [\theta]_u)/([\theta]_n - [\theta]_u)$, where $[\theta]$ is the observed ellipticity at a particular Gdn·HCl concentration and $[\theta]_n$ and $[\theta]_u$ are the ellipticities of the native and unfolded states, respectively. The free energy of unfolding in the absence of Gdn·HCl ($\Delta G_u^{H_2O}$) was estimated by linear extrapolation of the free energy of unfolding at each individual concentration of Gdn·HCl ($\Delta G_u = -RT \ln K_u$) to zero Gdn·HCl concentration (Shortle, 1989; Pace, 1986) according to

$$\Delta G_u = \Delta G_u^{H_2O} - m[\text{Gdn·HCl}]$$

(see Figure 4B). The $\Delta G_u^{H_2O}$ values and the transition midpoints of the peptides are listed in Table I.

While the above procedure is adequate for estimating the approximate value of $\Delta G_u^{H_2O}$, the extrapolations are over too wide a range of Gdn·HCl concentrations to measure reliably small differences in the stability of the Ala analogues since the errors from the slope (m) are magnified in the values of $\Delta G_u^{H_2O}$. Therefore, an alternative method (Kellis et al., 1988, 1989) was used to measure the free energy difference ($\Delta\Delta G_u$) between the native protein and the Ala analogues. A concentration of denaturant intermediate between the values required for denaturation of the native protein and the Ala mutants was selected (Figure 4B). This assumes linearity in the dependence of ΔG_u on Gdn·HCl concentration only over

Table II: Effects of Ala Substitutions on Protein Stability Where the Disulfide Bond Is Located either at Position a (Position 2) or Position d (Position 33)

peptide[a]	$[\theta]_{220}$[b] (deg·cm²·dmol⁻¹)	$[Gdn·HCl]_{1/2}$[c] (M)	$\Delta[Gdn·HCl]_{1/2}$[d] (M)	peptide[a]	$[\theta]_{220}$[b] (deg·cm²·dmol⁻¹)	$[Gdn·HCl]_{1/2}$[c] (M)	$\Delta[Gdn·HCl]_{1/2}$[d] (M)
C2L	−30 250	5.3		C33L	−30 810	5.1	
C2A16a	−30 450	2.6	−2.7	C33A16a	−30 360	2.4	−2.7
C2A19d	−30 520	3.7	−1.6	C33A19d	−30 820	3.5	−1.6

[a] The peptide sequences are shown in Figure 1. [b] The mean residue ellipticities at 220 nm were measured at 20 °C in 0.1 M KCl, 0.05 M PO_4 buffer, pH 7.0. [c] $[Gdn·HCl]_{1/2}$ is the transition midpoint, the concentration of guanidine hydrochloride (M) required to give a 50% decrease in ellipticity at 220 nm. [d] $\Delta[Gdn·HCl]_{1/2}$ is the difference in the $[Gdn·HCl]_{1/2}$ between the Ala analogues and the native coiled-coil and represents the decrease in stability of the Ala analogues compared to the native Leu-protein. For example, $\Delta[Gdn·HCl]_{1/2}$(C33A16a) = $[Gdn·HCl]_{1/2}$(C33A16a) − $[Gdn·HCl]_{1/2}$(C33L) = 2.4 − 5.1 = −2.7.

a small range beyond the experimental data (Kellis et al., 1988, 1989). The values of $\Delta\Delta G_u$ at 4 M Gdn·HCl are listed in Table I.

The Relative Contribution of the Interface Leucine Residues to Coiled-Coil Stability. Figure 4A compares the Gdn·HCl denaturation profiles for the native protein (C2L) and the Ala-substituted analogues (C2A16a, C2A19d, C2A30a, and C2A33d). All the Ala-substituted coiled-coils are less stable than the native coiled-coil as shown by the transition midpoints and ΔG^{H_2O} values (Figure 4 and Table I). Ala destablizes the coiled-coil due to its decreased hydrophobicity and inability to provide the same intra- and interchain contacts compared to the Leu residue. These results are in agreement with our previous studies on the coiled-coils (Hodges et al., 1988, 1990; Zhou et al., 1992a). However, the relative contribution of the interface hydrophobic residues at different positions in the coiled-coil are not identical. It has been demonstrated (Zhou et al., 1992a) that the Leu–Leu hydrophobic interactions are less important at the ends of the coiled-coil and the Ala substitution for Leu residue at position 5 or 33 only slightly decreases the coiled-coil stability in the reduced coiled-coil. Figure 5 plots the Gdn·HCl transition midpoint vs the position of the Ala substitution for both the oxidized and reduced coiled-coils [the data reduced coiled-coils were taken from Zhou et al. (1992a)]. The difference in the stability between the peptides with an Ala substituted at position a and the peptides with an Ala substituted at position d was observed in the oxidized coiled-coils. All the peptides with an Ala substituted at position a have similar stability ($[Gdn·HCl]_{1/2}$ for peptide C2A9a, C2A16a, and C2A23a was 2.9, 2.6, and 2.9 M, respectively), and all the peptides with an Ala substituted at position d have similar stability ($[Gdn·HCl]^{1/2}$ for peptide C2A5d, C2A12d, C2A19d, and C2A26d was 3.6, 4.0, 3.7, and 4.2 M, respectively), except for the peptides C2A30a and C2A33d in which an Ala was substituted in the C-terminal heptad. The peptides with an Ala substituted at position d are more stable than those with Ala substituted at position a (Figure 5). The greater decrease in stability observed for the Leu → Ala substitution at position a suggests that the Ala mutation at position a destabilizes the native coiled-coil protein more than an Ala mutation at position d and the side chain of Leu residues at position a make a greater contribution to the coiled-coil stability than those at position d. The better way to compare the stability changes for the mutation of Leu → Ala at different positions is to compare the $\Delta\Delta G_u$ values in Table I. The differences in free energy of unfolding in the presence of 4.0 M Gdn·HCl between the native protein and the Ala mutants ($\Delta\Delta G_u$) are greater for the Ala mutants at position a compared to the Ala mutants at position d. The average $\Delta\Delta G_u$ value for C2A9a, C2A16a, and C2A23a is 3.3 kcal/mol (range from 3.2 to 3.6) versus 2.0 kcal/mol (range from 1.6 to 2.3) for C2A5d, C2A12d, C2A19d, and C2A26d.

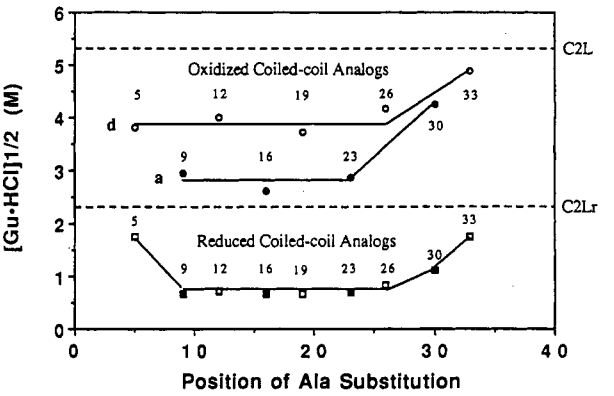

FIGURE 5: Plot of transition midpoint of the guanidine hydrochloride denaturation profiles (Table I) vs the position of the Ala substitution (5, 9, 12, 16, 19, 23, 26, 30, or 33). Dashed lines indicate the transition midpoint of the native coiled-coil proteins C2L and C2Lr (C2Lr denotes the native reduced coiled-coil where no disulfide bond is formed between two α-helical chains). Open symbols denote the coiled-coil analogue in which an Ala substitution is made at the d position. Closed symbols denote the coiled-coil analogue in which an Ala substitution is made at the a position. Circles denote the oxidized coiled-coils in which a disulfide bridge is formed at position 2 between the two α-helical chains and squares denote the reduced coiled-coils. The data for reduced coiled-coils was taken from Zhou et al. (1992a).

Effects of the Disulfide Bridge Location on the Relative Contribution of Interface Hydrophobic Residues to the Coiled-Coil Stability. By comparison of the oxidized and reduced coiled-coils (Figure 5), the contributions of interface hydrophobic residues to the stability are significantly different between position a and d in the oxidized coiled-coil, whereas no detectable difference between position a and d was observed along the majority of the reduced coiled-coil (Zhou et al., 1992a). This raises the following question: is the difference between position a and d due to the fact that the interhelical disulfide bridge at position a (position 2) controls the coiled-coil conformation, resulting in a greater contribution of the Leu residues at position a than those at position d? To address this question, the other three coiled-coil analogues (C33L, C33A16a, and C33A19d) in which an interhelical disulfide bridge is located at position d (position 33) were synthesized and the stabilities of these coiled-coils determined by Gdn·HCl denaturation studies. The peptides C2L and C33L (both peptides contain nine Leu and one Cys residues at 3–4 repeat hydrophobic positions and differ only in the disulfide bridge location, i.e., C2L has a disulfide bridge at the N-terminal a position, and C33L has a disulfide bridge at the C-terminal d position) have similar stability ($[Gdn·HCl_{1/2}$ of 5.3 and 5.1 M for C2L and C33L, respectively). If the formation of the disulfide bridge at position a causes a greater contribution of the Leu residue at position a to the coiled-coil stability compared to those at position d, one might expect to find that Leu residues at position d should make a greater contribution than those at position a in the coiled-coils with a disulfide bridge

at position d and C33A16a should be more stable than C33A19d. However, the oxidized coiled-coil C33A19d is still more stable than C33A16a (Table II), and the decreases in the stability for the Leu → Ala substitution at position a (position 16) are identical (Δ[Gdn·HCl]$_{1/2}$ values for C2A16a and C33A16a are 2.7 M) and greater than the substitution at position d (position 19) (Δ[Gdn·HCl]$_{1/2}$ values for C2A19d and C33A19d are 1.6 M). These results have demonstrated that the difference between the leucine residues at positions a and d in terms of their contribution to the coiled-coil stability is not dependent on the disulfide-bridge location.

Retention Behavior of the Coiled-Coil Analogues in Reversed-Phase Chromatography. It has been shown that there is a considerable similarity between the variables that direct intermolecular docking in biological macromolecules and those that determine the chromatographic behavior of the proteins (Regnier, 1987). The hydrophobic interaction between a peptide and the hydrophobic surface of the nonpolar stationary phase during RPC is likely to reflect similar interprotein interactions between nonpolar residues that stabilize the folded or three-dimensional structure of the native protein (Zhou et al., 1990; Mant et al., 1992). Analysis of peptide retention behavior in reversed-phase chromatography maybe very useful in understanding the peptide structure, and it may be possible to predict secondary structure properties of peptides relevant to biological systems (Zhou et al., 1990; Ostresh et al., 1991). The reversed-phase chromatographic behavior of these coiled-coil analogues provides some interesting results. The predicted retention time of the completely unfolded 70-residue disulfide-linked dimers would be greater than their corresponding monomers based upon the increase in hydrophobicity (Hodges et al., 1990). However, all oxidized 70-residue peptides are eluted prior to their corresponding reduced 35-residue peptides, suggesting the oxidized peptides retain a large portion of their coiled-coil structure during RPC. No significant decreases in the retention time for the substitution of one Leu residue with a less hydrophobic Ala residue in each chain of the coiled-coil was observed (Table I), which suggests that the side chains of Leu and Ala residues at positions a and d are buried in the coiled-coil structure. Only those residues at or near the exterior surface of the protein have a major impact on chromatographic behavior (Regnier, 1987). These results are in good agreement with the X-ray structure of the GCN4 leucine zipper, where the side chains of residues at positions a and d are 83% buried (O'Shea et al., 1991). By comparing the retention time of all Ala-substituted coiled-coils (Table I), it is clearly seen that all peptides with an Ala residue at position d have similar retention times (these range from 20.3 to 20.6 min) and are eluted prior to the peptides with an Ala at position a which have average retention time 21.1 ± 0.2 min (range from 20.9 to 21.3 min). The peptides with an Ala residue substituted for a Leu residue at position a are eluted after the native peptide (ΔR_t values of the peptides are all positive, Table I) while those with substitution at position d are eluted with retention times similar to or slightly prior to the native peptide (ΔR_t values of the peptides are negative, Table I). These findings suggest that the mutation at position a exposes the interior hydrophobic groups more than at position d and results in more hydrophobic groups being able to interact with the stationary phase during RPC. This result also implies that the coiled-coils with an Ala substitution at position a are more destabilizing of the coiled-coil structure than at position d, resulting in more unfolding of the coiled-coil during RPC.

Discussion

Previous studies by our laboratory (Hodges et al., 1988, 1990; Lau et al., 1984) have demonstrated that a large part of the stability of the coiled-coils is due to the presence of hydrophobes in the 3–4 repeat positions exactly as predicted. In the present study, the single alanine replacements of a leucine residue at hydrophobic positions a and/or d are all shown to destabilize the coiled-coil structure, implying that all of the hydrophobic interactions between these positions contribute to the coiled-coil stability. However, positions a and d respond differently to Ala substitutions in this Leu-stabilized coiled-coil, and the Leu at position a is less tolerant of an Ala mutation than those at position d. These results suggest that the Leu side chains at positions a contribute more to the Leu stabilized coiled-coils than those at positions d and the isopropyl groups (which is the structural difference between the side chain of Leu and Ala) are more buried at positions a than at positions d. The retention behavior of these coiled-coils during reversed-phase chromatography also supports this conclusion. This variation must be a consequence of the difference in the local structure between position a and d which can be explained by the X-ray structure of tropomyosin (Phillips et al., 1986) and the 1.8-Å resolution structure of the GCN4 coiled-coil (O'Shea et al., 1991). The positions a and d are structurally distinct in coiled-coil proteins: the distance between the α-carbon of one chain and the corresponding α-carbon of the other chain is 1.7 Å closer at positions a than those at positions d in tropomyosin. The β-carbons of symmetry-related leucines in GCN4 are almost 2 Å closer together in positions d compared to those in positions a, and the Leu side chains at positions a and d have different orientations: the Cα–Cβ vectors are pointed into the interface at positions d in GCN4 coiled-coil structure. In our computer modeling studies, the direction of the Cβ–Cγ bond points away from the hydrophobic interface at positions d, whereas they point into the hydrophobic interface at positions a. This results in a closer packing of the isopropyl group of the leucine side chains at position a compared to position d (Figure 6). The isopropyl groups of leucines at positions a are 80% buried from formation of the coiled-coil structure from single-stranded α-helices, while the isopropyl groups of leucines positions d are only 64% buried. The isopropyl groups of leucines at positions a have an average exposure area of 12 Å^2 compared to 19 Å^2 at positions d. Thus a mutation of Leu to Ala at

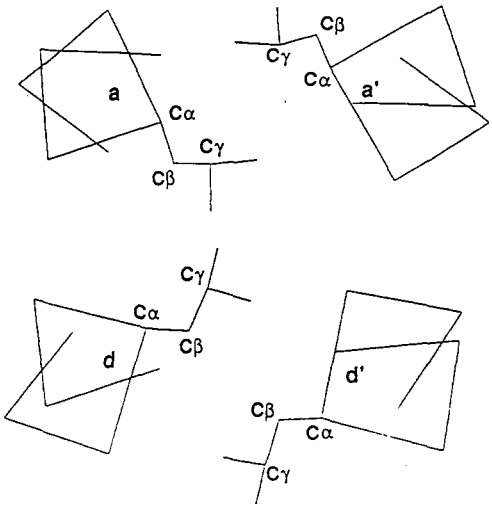

FIGURE 6: Computer modeling of the coiled-coil structure to show the different orientations of Leu side chains at positions a and d. Only side chain carbons of leucine at positions 16 (a and a′) and 19 (d and d′) as well as seven main chain α-carbons arounded this leucine residue are displayed. The view is along the coiled-coil axis from the N-terminus.

position a has a greater effect on the packing and hydrophobic interactions than an Ala mutation at position d and therefore results in a greater decrease in the stability of the coiled-coil. It has been shown that the contribution of an amino acid to protein stability considerably depends on its position within a protein, and the mutations using less hydrophobic residues in place of more hydrophobic ones are generally more deleterious for protein stability at more buried positions (Alber et al., 1987; Hughson & Baldwin 1989; Kellis et al., 1989; Ganter & Pluckthum 1990; Shortle et al., 1990; Sandberg & Terwilliger 1991). For example, stability studies with single Leu to Ala mutation in the hydrophobic core of staphylococcal nuclease showed variations in $\Delta\Delta G_u$ from 1.6 to 5.8 kcal/mol (Shortle et al., 1990). In the present study, where a Leu–Leu interaction was mutated to an Ala–Ala interaction in the central region of the coiled-coil, the $\Delta\Delta G_u$ values varied from 1.6 to 3.6 kcal/mol or 0.8 to 1.8 kcal/mol per single Leu to Ala mutation. It has been previously observed in heterodimeric coiled-coils where two Leu–Leu interactions were mutated to two Leu–Ala interactions, the $\Delta\Delta G_u$ values varied from 1.7 to 2.7 kcal/mol or 0.8 to 1.4 kcal/mol per single Leu to Ala mutation (Zhu et al., 1992).

It has been shown (Zhou et al., 1992a) that in the reduced coiled-coil the Leu–Leu hydrophobic interactions are less important at the ends of the coiled-coil and the ends of the coiled-coil are more flexible. Only the hydrophobes in the central region (residue 9–26) make a major contribution to the coiled-coil stability. In the oxidized coiled-coil, the greater decrease in the stability for the Leu → Ala mutation at position 5 than at position 33 compared to the similar decrease at both positions in the reduced coiled-coil (Figure 5) suggested that the disulfide bridge between two helical chains at position 2 increased the hydrophobic interactions around the Leu residue at position 5 and decreased the flexibility of the N-terminal region of the peptide chains, resulting in more stable coiled-coils and more fixed side chain packing in the hydrophobic core. Molecular dynamics simulations of the oxidized coiled-coil showed an increased flexibility only in the C-terminal region. By comparison, the reduced coiled-coil showed end effects at both the N- and C-terminal regions of the coiled-coil (Zhou et al., 1992a).

However, no detectable stability difference between position a and d was observed along the majority of the reduced coiled-coil. The reduced coiled-coil can tolerate the mutation more easily than the oxidized coiled-coil by adjusting the interchain packing around the mutation sites (Zhou et al., 1992b). Thus, it is possible to adjust the side chain packing more easily within the hydrophobic core in the less stable and short reduced coiled-coils. The presence of the disulfide bond is somehow restricting the way the side chains interact between the two helices. We suggest that the disulfide bridge locks the coiled-coil structure in a conformation where the packing of the residues at positions a and d are different. Interestingly, when β-branched amino acids (Ile or Val) are substituted for leucines at positions a or d, differences between positions a and d are observed even in the reduced coiled-coils (without disulfide bridge between two helices) (Zhu et al., unpublished results). These findings suggest that the disulfide bond in the oxidized coiled-coils or the β-branched amino acids can place packing constraints on the two interacting α-helices to mimic the conformation observed in the X-ray structure of the DNA-binding protein (GCN4 leucine zipper).

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Binding of a Mitochondrial Presequence to Natural and Artificial Membranes: Role of Surface Potential†

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ABSTRACT: The binding of a synthetic mitochondrial presequence to large, negatively charged, unilamellar vesicles and to unenergized yeast mitochondria has been measured. The presequence, which corresponds to the amino-terminal 25 residues of the yeast cytochrome oxidase subunit IV precursor, was labeled with a fluorescent probe and used to examine the importance of the surface potentials of membranes on the interactions with the presequence. Binding of the fluorescent presequence to the membranes was determined by measuring a decrease in the fluorescence emission of the bound presequence. Binding both to the vesicles and to the mitochondria could be described as a simple partitioning of the presequence between the aqueous and lipid phases. The partitioning was found to depend on the ionic strength of the medium, and the Gouy–Chapman theory could be used to describe the partitioning at various ionic strengths. Application of the theory allowed the determination of an apparent charge on the presequence (+2.31 ± 0.25), salt-independent apparent partition coefficients for vesicles (99 ± 84 M^{-1}) and for unenergized mitochondria (14.5 ± 3.6 L g^{-1}), and an estimated charge density for the mitochondrial outer membrane (−0.0124 ± 0.0016 C m^{-2}). This study shows that electrostatic effects are significant for the binding of a mitochondrial presequence both to lipid vesicles and to mitochondria, the natural target membrane of the presequence. The accumulation of positively charged presequences at the negative mitochondrial surface and the subsequent partitioning of the presequences directly into the mitochondrial outer membrane probably represent early steps in the translocation of precursor proteins into mitochondria.

The targeting of a protein from the cytoplasm to the mitochondria is typically mediated by an amino-terminal targeting sequence, the presequence. Experiments with gene fusions have demonstrated that mitochondrial presequences are necessary and can be sufficient for import of attached proteins into mitochondria (Verner & Schatz, 1988; Hartl et al., 1989). The physical properties of mitochondrial presequences have been studied with synthetic peptides that have sequences corresponding to various natural and artificial presequences (Roise & Schatz, 1988). The synthetic presequences are surface-active, have a strong affinity for membranes containing negatively charged lipids, and adopt secondary structure in hydrophobic environments. It was recently shown that synthetic presequences can be imported into mitochondria in vitro (Ono & Tuboi, 1988; Glaser & Cumsky, 1990; Pak & Weiner, 1990; Furuya et al., 1991; Roise, 1992).

The mechanism of interaction of surface-active peptides with membranes is thought to involve a combination of electrostatic and hydrophobic effects (Sargent & Schwyzer, 1986). Most studies on this process have used model systems, either phospholipid monolayers or bilayers, to mimic the natural membranes (Schoch & Sargent, 1980; Briggs et al., 1985; Roise et al., 1986; Schwarz et al., 1986; Tamm, 1986; Rizzo et al., 1987; Skerjanc et al., 1987; Kuchinka & Seelig, 1989; Seelig & Macdonald, 1989; Beschiaschvili & Seelig, 1990a,b; Frey & Tamm, 1990). While the models allow the experimental conditions to be altered as desired and are amenable to the measurement of binding by a variety of physical tech-

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